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Novel amphiphilic block copolymers and their self-assembled injectable hydrogels for gene delivery

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by

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To my parents and grand parents…

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ABSTRACT

This work describes the development and investigation of a family of novel "smart" copolymers as non-viral gene delivery vectors. The copolymers have five blocks, and thus named pentablock, with a central block of a hydrophobic polymer, surrounded by two blocks of a hydrophilic polymer, and capped at each terminal end with cationic polymer blocks, arranged in an architecture to provide temperature and pH sensitivity to the copolymers. They are derived from commercially available triblock Pluronic copolymers. The cationic copolymers can efficiently condense negatively charged plasmid DNA in nanostructures with efficient cellular uptake. The amphiphilic nature of copolymers causes them to exist as micelles in aqueous solutions that help them traverse cellular membranes with minimal cell membrane damage. Intra-cellular trafficking of copolymer/DNA complexes revealed that they are up-taken by the cells predominately via endocytosis and are able to deliver the ferried gene into the nuclei. The copolymers efficiently protect the condensed DNA against degradation by nucleases while their protonation capability at low pH assists them in escape from endosomal vesicles into the cytoplasm. The efficiency of the copolymers to deliver condensed DNA into the cells *in vitro* was comparable to the commercially available polymeric transfection vectors, and they were also found to be significantly less cytotoxic. Adding non-ionic Pluronic copolymers to the formulation of pentablock copolymer/DNA complexes sterically shielded their surface charge and protected them against aggregation with serum proteins. These stabilized formulations were able to retain their ability to transfect cells even in complete growth media supplemented with serum proteins, warranting efficient transfection efficiency in an *in vivo* application. The amphiphilic nature of copolymers further permits copolymer/ DNA complexes to form thermo-reversible hydrogels at physiological temperatures. At concentrations above 15 wt%, copolymer/DNA complexes existed as solutions at room temperature and formed elastic hydrogels at 37°C that dissolved over seven days in excess buffers to release colloidally stable polyplexes. The system thus permits an injectable aqueous pharmaceutical preparation at room temperature that can be injected subcutaneously in tissues/cavities to form a localized depot *in situ,* which provides a long-term sustained release of therapeutic genes well protected inside the copolymer/DNA complexes.

CHAPTER 1
GENERAL INTRODUCTION

Gene therapy- it's a medical art to deliver genetic information coded in nucleic acids to the targeted somatic cells of a patient for producing specific therapeutic proteins that can modulate the disease [1]. Providing a therapeutic gene may circumvent the limitations associated with direct administration of therapeutic proteins, like low bioavailability, systemic toxicity, in vivo instability, and high hepatic and renal clearance rates [2]. Originally designed for the treatment of hereditary genetic disorders [3], gene therapies are now being developed to treat cancer [4], heart diseases [5], AIDS [6], diabetes mellitus [7], and other treatments like tissue regeneration [8], wound healing [9] and immunization [10]. However, despite initial promise in many animal models, the translation of gene therapy to the clinical arena has been slow, and has been limited by the development of an efficient gene delivery system, not by the paucity of gene expression systems. Among all the gene carriers, viruses have most widely been investigated, as they have evolved to overcome human immune defenses and deliver their genetic payload efficiently into the host cells [11]. Approximately 70% of the 1260 approved gene therapy clinical trials have been conducted using engineered viruses as vectors to ferry therapeutic genes, Fig. 1 and 2 [12]. However, viral vectors have some practical limitations. They can carry only a limited amount of genetic information, and there is always a remote danger of recombination events that can produce a replicating virus. In addition, there is also possibility of the replicating virus to integrate into the host genome at undesired sites and permanently altering its genetic structure. Besides, mammalian immune systems have developed strategies to eliminate viral invaders as well. Repeated administration of these viral vectors (more than once or twice) can provoke an immune response, strong enough to result even in the death of the host. A big jolt came to this viral vector gene therapy on 17 September 1999, when Jesse Gelsinger, a teenage volunteer, died during a clinical trial at the University of Pennsylvania in Philadelphia [13]. An out-of-control immune response to the virus used in the therapy was cited as the reason for his death. Since that incident several other set backs involving secondary oncogenesis

[14], or transfection of untargeted germ cell lines [15] have been encountered in clinical trials of viral gene therapy. These incidents have boosted a renewed interest among scientists to develop biologically inactive non-viral methods of gene delivery [16], and researchers have been forced again to make a choice: domesticate viruses, or develop intelligent synthetic vectors?

The research on non-viral methods of gene delivery has been gathering steam over the last two decades- with the goal to design a vector that could 1) provide the transfection efficiency attained by viral vectors, 2) carry large amount of genetic information, 3) bypass the immune response, and 4) be safe. With safety and large scale manufacturing as their advantages, the clinical usefulness of these methods is limited by their low transfection efficiency and inability to confer long term transgene expression, important issues that need to be technologically improved. Novel cationic polymers and lipids have shown great promise as efficient non-viral vectors for gene and oligonucleotide delivery. Other strategies include particle bombardment [17, 18], electroporation [19, 20], nucleofection [21], jet injection [22], ultrasound [23] and direct injection of naked DNA [24, 25]. However, their applicability is restricted to specific circumstances, and can be only rarely applied with reasonable efficiency, as has been review recently [16].

Cationic polymers or liposomes that electrostatically condense negatively charged DNA molecules into nanoparticles have proven to be efficient gene delivery systems, giving transgene expression in targeted cells of several magnitudes higher than that achieved with naked plasmid delivery both in vitro and in vivo [26]. Polycations are ensembles of a certain repeating structural unit that are easy to manufacture and scale-up. Further, they are not architecturally constrained, and can be specifically tailored for the proposed application by choosing appropriate molecular weights/degree of branching, coupling of tissue specific targeting moieties, and/or performing other modifications that confer upon them specific physiochemical and physiological properties (temperature and pH sensitivity). Various off-the shelf polymers [27], like polyethylenimine (PEI), poly-L-lysine (PLL), methacrylates, dendrimers, and liposomes [28] like DOTAP (1,2-Dioleoyl-3-Trimethylammonium-Propane) and lipofectamine have been shown as efficient gene delivery vectors, but their use in clinical trials is held back by problems like cytotoxicity, in vivo colloidal stability, and low and

transient gene expression. New generations of block and graft copolymers, and liposomes designed specifically for gene delivery are now being investigated to address these issues [29, 30].

An efficient cationic non-viral gene delivery vector needs to overcome numerous obstacles at the systemic and cellular levels as discussed in detail elsewhere [31]. Briefly, on systemic level, cationic vectors should avoid non-specific interactions with erythrocytes, vessel endothelia, and plasma proteins like albumin, fibronectin, immuoglobulins, complement factors, and fibrinogen. These interactions can result in aggregation and accumulation of polymer/DNA complexes in the "first pass organs" such as lung (consequently causing pulmonary embolism), liver and spleen, and finally opsonization and clearance by the reticuloendothelial system (RES) [32, 33]. This leads to the very short plasma half-lives for these vectors, decreasing their circulation time and cellular uptake, and negatively influencing their biodistribution and gene expression patterns, making them of limited value for therapeutic applications. Second, the vectors should be targeted to specific cell types so that they deliver the therapeutic genes only the desired cells to produce expected physiological effect without any side effects. On cellular level, once these vectors reach the targeted cells, they should effectively traverse across the cell membrane (mediated mostly by endocytosis); protect the ferried gene from the low pH, nucleases rich environment of endosomes; escape the lysosomes; enter the nucleus and, finally unpack from the gene for it to be up-taken by nuclei machinery for transcription (Fig. 3). Lastly, non-viral gene delivery methods provide only transient protein expression because of the ultimate loss of the un-integrated plasmid DNA from the transfected nuclei. Besides, injecting gene delivery vectors formulated in large buffer volumes gives limited bioavailability of the bolus dose as most of the injected vector is lost, or is degraded rapidly in the tissue, or is removed from the tissue by lymphatic system. Efficient gene delivery systems that produce enough amount of therapeutic protein in the transfected tissue all along the duration of therapy to give an appreciable physiological response are needed.

In the work presented here, we have attempted to develop novel "smart" polymers designed specifically for gene delivery that encompass all the above mentioned qualities of an ideal gene delivery vector. These are cationic amphiphilic copolymers with five blocks of

three different polymers arranged in an architecture to confer temperature- and pH-sensitivity to them. The copolymers can condense negatively charged plasmid DNA molecules at physiological pH in nanoparticles of 100-200nm diameter for efficient uptake by targeted cells, while protecting the compacted plasmid against degradation by nucleases in extra-cellular matrix or inside the cells. The amphiphilic nature allows these copolymers to exist in micellar type of structures that helps them traverse across the amphiphilic lipid bilayer of cell membranes with minimum cytotoxicity. The pH sensitivity permits in the protonation of copolymers when entrapped in low pH environment of endosomal compartments which aids in the disruption of such vesicles and their final escape in the cytoplasm. The copolymers have reactive ends in their architecture to facilitate attachment of cell-specific ligands for target recognition, or nuclear localization signals (NLS) for improving nuclear translocation. Finally, the copolymers display an interesting thermo-reversible gelation at higher concentrations and physiological temperatures. While they exist as solutions at room temperatures, the polymeric network self-assembles at body temperature to form an elastic hydrogel. Thus, the copolymers can be mixed with the therapeutic gene in an aqueous phase at low temperatures (below 10°C) where they exist as sols and are injectable. On subcutaneous/intramuscular injection and subsequent heating to body temperatures, the copolymers self-assemble into a hydrogel in situ. The hydrogel act as a reservoir for the entrapped plasmid DNA and can dissolve over time in the tissue fluid to provide long-term sustained-release of the compacted gene. This would maintain the long term local bioavailability of DNA vectors to the surrounding tissues by continual replacement of the factors that get cleared or degraded, increasing the probability of cellular uptake, improving the optimal use of drug, and circumventing the need for repeated administration with increased patience compliance. Thus, these novel copolymers can be used for systemic delivery of genes in solution at low concentrations, and for localized sustained gene delivery to specific tissues/ cavities at higher concentrations.

Investigation and development of these novel copolymers as efficient non-viral gene delivery systems can facilitate improvement of the existing polymeric gene delivery technology, and help harness the great powers of gene therapy.

THESIS ORGANIZATION

The above stated overall objectives for developing and characterizing these novel copolymers as non-viral gene delivery vectors were addressed by completing a set of specific goals (SGs), and the progress towards completion of each goal has been documented in the accompanying chapters of this dissertation.

SG1

To characterize the physiochemical properties of pentablock copolymers pertaining to plasmid DNA compaction and protection against nucleases; and, hydrodynamic size, surface charge, and morphology of polymer/DNA complexes in aqueous solutions.

Chapters 3, 5 and 6 address this specific goal. Chapter 3 investigates the ability of pentablock copolymers to condense plasmid DNA, and protection they provide to DNA against degradation by nucleases. Morphology of the copolymer/DNA complexes is presented using Transmission Electron Microscopy (TEM). The apparent molecular mass and radius of gyration of copolymers and their polyplexes in aqueous solutions was investigated using Multi-Angle Laser Light Scattering (MALLS). Chapter 5 presents a detailed study on the DNA condensation by pentablock copolymers under different physiological conditions and polymer concentrations. Cryo-TEM was used to study the morphology of these micellar copolymers and their DNA condensates, as it enables direct real-space imaging of nanostructures in their native state in aqueous conditions by vitrifying the samples, avoiding staining and drying artifacts involved in conventional TEM. Chapter 6 provides a detailed investigation of the particle size and surface charge of these copolymer/DNA complexes, and presents strategies to shield their surface charge and prevent aggregation in serum supplemented buffers that will optimize their formulation for future in vivo applications.

SG2

To tailor the copolymer design and, improve the colloidal stability of their DNA complexes with optimized formulations for maximum gene transfection in cells with

minimum cytotoxicity, and investigate their intracellular trafficking pathway to identify steps that limit their transfection efficiency.

Chapters 3, 4, 5 and 6 collectively address SG2. In chapter 3, four different pentablock copolymers with different cationic content are screened for their cytotoxicity relative to cell membrane damage, and the transfection of efficiency of one of the copolymers is tested for the first time in a cancer cell line using two different reporter genes: one to account for the percentage of cells expressing the transfected reporter genes, and the other to provide the total amount of transgene expression in a population of the transfected cells. Chapter 4 examines in detail the biocompatibility of various pentablock copolymers on two different cancer cell lines using several cell-based assays to elucidate the mechanism of cell death induced by these copolymers, and compares it with that of another commercially available polycationic transfection reagent. The chapter illustrates how the cytotoxicity of the copolymers can be tuned by tailoring their molecular weight or cationic content. Chapter 5 investigates the pathway utilized by pentablock copolymer/DNA complexes to transfect a cell- from traversing across the cell membrane to the delivery of the DNA to the nucleus. Fluorescent labeling techniques and confocal microscopy were used. Chapter 6 provides a novel strategy to screen the cationic surface charge of the pentablock copolymer/DNA complexes, preventing their aggregation with serum proteins and significantly improving their transfection efficiency and biocompatibility in complete growth media. The results warrant good performance of this multi-component gene delivery system in systemic applications *in vivo*.

SG3

To develop injectable self-assembled in situ forming hydrogels of pentablock copolymer/DNA complexes for long-term sustained gene delivery, modulate their *in vitro* dissolution profile, and improve the formulations for maximum gene stability and transfection efficiency.

Chapter 7 describes the work done in the pursuit of SG3. It reports the mechanical properties of the hydrogels of copolymer/DNA complexes made with different formulations, and describe their sustained DNA release profile. The resuls present therin confirms the

release of condensed DNA, but not naked DNA, from the hydrogels and, examines the colloidal stability of released polyplexes. Finally the transfection efficiency of the polyplexes released from the hdyrogels is tested on cell lines *in vitro*, confirming that these injectable hydrogels display great potential as sustained gene delivery devices that have distinct advantages over other investigated systems.

Finally, Chapter 8 summarizes the conclusions obtained from this body work, and Chapter 9 offers some guidelines to test these copolymers and their injectable hydrogels in *in vivo* murine models, and discusses their future applications in gene therapy.

References

1. M.A. Kay, D. Liu, P.M. Hoogerbrugge, Gene Therapy, PNAS 94 (24) (1997) 12744-12746.
2. R. Langer, New methods of drug delivery, Science 249 (1992) 1527-1533.
3. R.W.A. Herzog, Valder R., Update on gene therapy for hereditary hematological disorders., Expert Review of Cardiovascular Therapy 5 (7) (2003) 576-587.
4. M.G. Castro, R. Cowen, I.K. Williamson, A. David, M.J. Jimenez-Dalmaroni, X. Yuan, A. Bigliari, J.C. Williams, J. Hu, P.R. Lowenstein, Current and future strategies for the treatment of malignant brain tumors, Pharmacology & Therapeutics 98 (1) (2003) 71-108.
5. J.N. Schroder, M.L. Williams, W.J. Koch, Gene delivery approaches to heart failure treatment., Expert Opinion on Biological Therapy 4 (9) (2004) 1413-1422.
6. R. Wolkowicz, G.P. Nolan, Gene therapy progress and prospects: Novel gene therapy approaches for AIDS, Gene Therapy 12 (6) (2005) 467-476.
7. V. Yechoor, L. Chan, Gene Therapy Progress and Prospects: Gene therapy for diabetes mellitus, Gene Therapy 12 (2) (2005) 101-107.
8. H. Storrie, D.J. Mooney, Sustained delivery of plasmid DNA from polymeric scaffolds for tissue engineering, Advanced Drug Delivery Reviews 58 (4) (2006) 500-514.
9. L.K. Branski, C.T. Pereira, D.N. Herndon, M.G. Jeschke, Gene therapy in wound healing: present status and future directions, Gene Therapy 14 (1) (2006) 1-10.
10. H.L. Robinson, C.A.T. Torres, DNA vaccines, Seminars in Immunology 9 (5) (1997) 271-283.
11. L.S. Young, P.F. Searle, D. Onion, V. Mautner, Viral gene therapy strategies: from basic science to clinical application, The Journal of Pathology 208 (2) (2006) 299-318.
12. M. Edeistein, *Vectors used in gene therapy clinical trials http://www.wiley.co.uk/genmed/clinical/*. 2006, John Wiley and Sons Ltd.
13. I.M. Verma, A Tumultuous Year for Gene Therapy, Molecular Therapy 2 (5) (2000) 415-416.
14. E. Check, Gene therapy: A tragic setback, Nature 420 (6912) (2002) 116-118.
15. N. Boyce, Trial halted after gene shows up in semen, Nature 414 (6865) (2001) 677.
16. G.D. Schmidt-Wolf, I.G.H. Schmidt-Wolf, Non-viral and hybrid vectors in human gene therapy: an update, Trends in Molecular Medicine 9 (2) (2003) 67-72.

17. N. Yang, J. Burkholder, B. Roberts, B. Martinell, D. McCabe, In vivo and in vitro Gene Transfer to Mammalian Somatic Cells by Particle Bombardment, PNAS 87 (24) (1990) 9568-9572.
18. N.-S. Yang, W.H. Sun, Gene gun and other non-viral approaches for cancer gene therapy, 1 (5) (1995) 481-483.
19. S. Somiari, J. Glasspool-Malone, J.J. Drabick, R.A. Gilbert, R. Heller, M.J. Jaroszeski, R.W. Malone, Theory and in Vivo Application of Electroporative Gene Delivery, Molecular Therapy 2 (3) (2000) 178-187.
20. P.J. Canatella, M.R. Prausnitz, Prediction and optimization of gene transfection and drug delivery by electroporation, Gene Therapy 8 (19) (2001) 1464-1469.
21. P. Buttgereit, I.G.H. Schmidt-Wolf, Gene therapy of lymphoma, Journal of Hematotherapy and Stem Cell Research 11 (3) (2002) 457-467.
22. D. Sawamura, S. Ina, K. Itai, X. Meng, A. Kon, K. Tamai, K. Hanada, I. Hashimoto, In vivo gene introduction into keratinocytes using jet injection, Gene Therapy 6 (10) (1999) 1785-1787.
23. M. Endoh, N. Koibuchi, M. Sato, R. Morishita, T. Kanzaki, Y. Murata, Y. Kaneda, Fetal Gene Transfer by Intrauterine Injection with Microbubble-Enhanced Ultrasound, Molecular Therapy 5 (5) (2002) 501-508.
24. M.J. Mann, G.H. Gibbons, H. Hutchinson, R.S. Poston, E.G. Hoyt, R.C. Robbins, V.J. Dzau, Pressure-mediated oligonucleotide transfection of rat and human cardiovascular tissues, PNAS 96 (11) (1999) 6411-6416.
25. G. Zhang, V. Budker, J.A. Wolff, High Levels of Foreign Gene Expression in Hepatocytes after Tail Vein Injections of Naked Plasmid DNA, Human Gene Therapy 10 (10) (1999) 1735-1737.
26. M. Thomas, A.M. Klibanov, Non-viral gene therapy: polycation-mediated DNA delivery, Applied Microbiology and Biotechnology 62 (1) (2003) 27-34.
27. D.W. Pack, A.S. Hoffman, S. Pun, P.S. Stayton, Design and development of polymers for gene delivery, Nat Rev Drug Discov 4 (7) (2005) 581-593.
28. S. Simoes, A. Filipe, H. Faneca, M. Mano, N. Penacho, N. Duzgunes, M. Pedroso de Lima, Cationic liposomes for gene delivery, Expert Opinion on Drug Delivery 2 (2) (2005) 237-254.
29. D. Putnam, Polymers for gene delivery across length scales, Nature Materials 5 (2006) 439–451.
30. P.P. Karmali, A. Chaudhuri, Cationic liposomes as non-viral carriers of gene medicines: Resolved issues, open questions, and future promises, Medicinal Research Reviews 9999 (9999) (2006) n/a.
31. T. Merdan, J. Kopecek, T. Kissel, Prospects of cationic polymers in gene and oligonucleotide therapy against Cancer, Adv. Drug Delivery Reviews 54 (2002) 715-758.
32. M. Ogris, E. Wagner, Targeting tumors with non-viral gene delivery systems, Drug Discovery Today 7 (8) (2002) 479-485.
33. D.B. Fenske, I. MacLachlan, P.R. Cullis, Long-circulating vectors for the systemic delivery of genes, Current Opinion in Molecular Therapeutics 3 (2) (2001) 153-158.

Figures
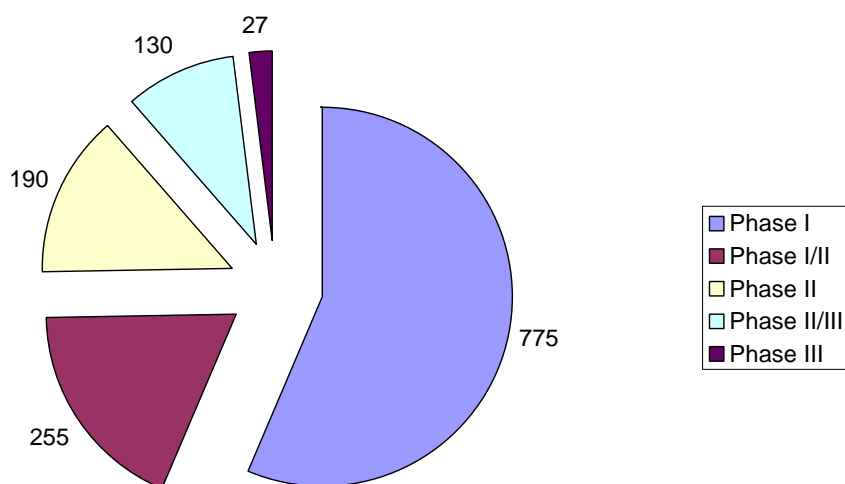
Fig. 1: Number of Gene Therapy clinical trails in different phases, updated January 2007 http://www.wiley.co.uk/genmed/clinical/.
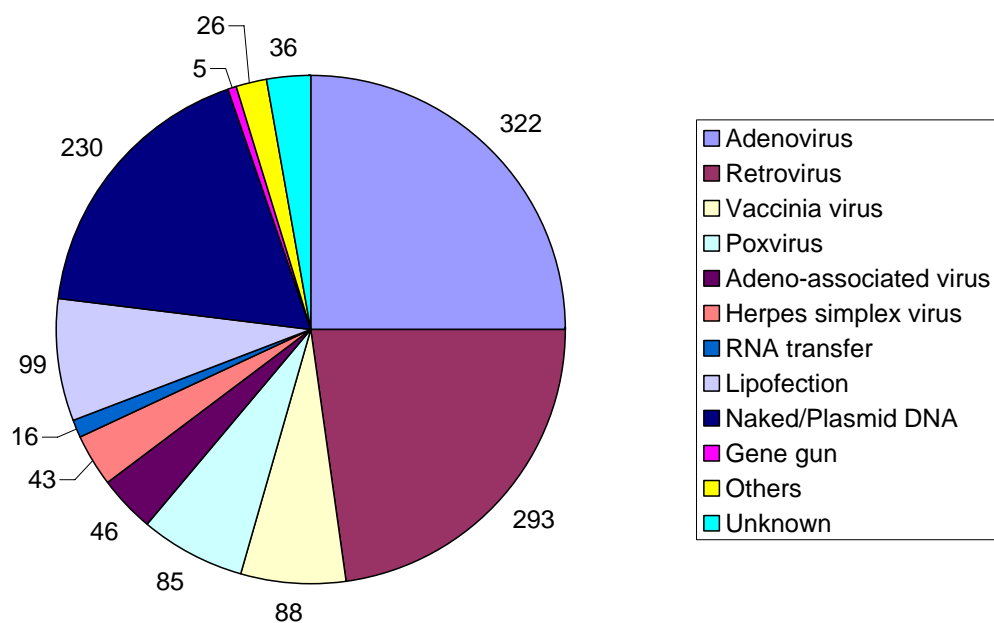
Fig. 2: Gene delivery vectors/methods used in Gene Therapy clinical trails, updated January 2007. http://www.wiley.co.uk/genmed/clinical/.
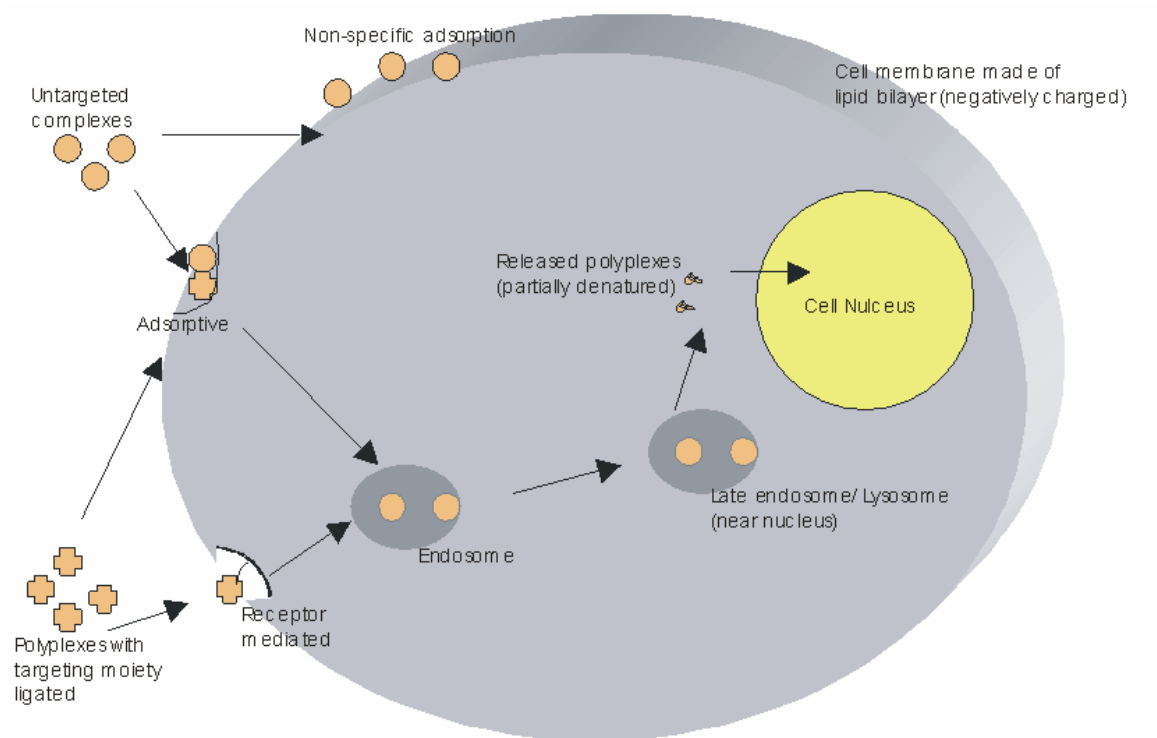
Fig. 3: Intracellular trafficking of a gene delivery vector.

CHAPTER 2

SYNTHETIC SUSTAINED GENE DELIVERY SYSTEMS

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Abstract

Gene therapy in clinical trials today is hampered by the need of a safe and efficient gene delivery system that can provide a sustained therapeutic effect without causing any cytotoxicity or invoking an unwanted immune response. Bolus gene delivery in solution using plasmid DNA or viral vectors results in the loss of the delivered factors via lymphatic system, and may cause undesired physiological responses by the escape of these bioactive molecules to distant sites. A controlled gene delivery system that can act as a localized depot of genes with sustained release profile would maintain the therapeutic level of expressed protein for extended period of times, circumventing repeated administrations and reducing the drug dosage. It would also protect the DNA in the nuclease rich extra-cellular or systemic environment, limiting its degradation. Several controlled release technologies developed for delivering therapeutic molecules have been adapted for gene delivery while more novel approaches are being investigated. DNA encapsulated in nanospheres and microspheres of degradable polymers can be administered systemically or orally to be up taken by the targeted tissues and provide long term release once internalized. Alternatively, DNA entrapped in hydrogels and polymer scaffolds can be injected or implanted at localized locations as platforms for sustained gene delivery. Here either the incorporated DNA is released by diffusion through the polymer matrix to be up taken by surrounding cells, or

DNA tethered to the matrix is internalized by the infiltrating cells as they invade through the matrix. The present review examines these different modalities used for sustained delivery of viral and non-viral vectors, and various synthetic and natural polymers used to synthesize them. Design parameters and release mechanisms for different delivery systems are presented along with their prospective applications, and opportunities for continuous development.

Outline

1.   Introduction

Gene therapy is an experimental technique of delivering genetic material to a patient's somatic cells in order to express therapeutic proteins to correct or modulate a targeted disease[1]. With the completion of the sequencing of the human genome, a lot of advances have been made in identifying target genes for the purposes of treating genetic and infectious diseases, but the bottle neck in the success of gene therapy has been in developing a safe and

efficient gene delivery system. Viruses are most efficient in delivering their genetic payload to the mammalian cells, and have been modified by the researchers to deliver the therapeutic genes instead[2]. However, the problems with immunogenicity, oncogenicity by insertional mutagenesis, toxicity, limitation of repeated administrations, targeting, and possibility of recombination events by replication, has hindered the successful use of viral vectors in gene therapy. Several set backs[3-5] in clinical trials using viral vectors have further questioned their safe usage, and researchers have become more interested in developing non-viral modes for gene delivery[6,7].

In non-viral gene delivery, the gene of interest is inserted into a cassette of expression plasmid that also contains other DNA sequences for controlling the effective translocation of the gene, and final transcription into targeting proteins. Injection of plasmid DNA generates systemic protein expression. However, expression is transient due to the eventual loss of unintegrated plasmid DNA from transfected cell nuclei, and thus decreases rapidly over a week or two. Specific or non-specific mechanisms may be involved in the loss of exogenous gene expression. Repeated administration of gene drug is required to maintain the therapeutic level of the expressed protein drug for an effective therapy. Development of sustained gene delivery devices that can maintain the long term local availability of DNA vectors to the surrounding tissues can achieve a sustained systemic protein production, circumventing the need for repeated administration. In fact, sustained and regulated gene expression is more effective than repetitive single dose administrations with high transient expression for the treatment of certain localized disease conditions, such as angiogenesis, bone regeneration, restenosis (a vasculoproliferative condition), inducing neovasculature in cardiac and limb ischemia[8-11].

The primary drawback of several unformulated plasmid and viral based formulations, accompanying the large buffer volume, is the limited bioavailability of the bolus dose. Most of the injected plasmid runs off, or is degraded rapidly in the tissue, or is removed from the tissue by lymphatic system. Sustained delivery systems can maintain the elevated levels of delivered therapeutics within the extracellular environment by continual replacement of the factors that get cleared or degraded. Besides, such a system can have a high DNA loading efficiency, can protect the DNA from endonculeases, and has the potential to deliver a

controlled, predictable, and sustained supply of DNA for prolonged expression. The controlled presence of the genetic medicine in the cell microenvironment within desired range can improve the effectiveness of drug by increasing patience compliance, reducing toxicity and requiring fewer administrations.

A lot of work has already been done on the development of polymeric controlled release systems for low-molecular weight drugs and proteins (growth factors, antibodies, hormones), and several of such systems have even been commercialized including Nutropin Depot, Gliadel wafer, Norplant, and Cypher Stent. Adapting these systems for the sustained delivery of DNA would be a great advantage in the practice of medicine, because delivering plasmid DNA to generate therapeutic proteins has many advantages over traditional protein based approaches. For one, gene therapy is not restricted to proteins that interact with cell-surface receptors. It can be used to express genes encoding intracellular proteins which could be used to control the fate of pluripotent cells. In this regard, gene therapy can target more cellular processes. The quantity and duration of protein production from gene delivery can be manipulated using inducible promoters, or can be restricted to a specific tissue through tissue specific promoters. With protein drugs, the delivery system should maintain the three dimensional conformation of the protein in order to maximize bioactivity. However, plasmid DNA, where the essential information is encoded in its linear sequence of bases, has a stable flexible chemistry that is compatible with established polymer-based drug delivery system. Because physical properties of DNA are similar regardless of its linear sequence, multiple plasmids can easily be incorporated into a single delivery system. A critical challenge in developing delivery systems for multiple proteins is developing processing conditions that maintain the bioactivity of all constituents. Besides, plasmid diffusion from the delivery site would not cause toxicity because of the high efficiency of DNA turnover in the bloodstream. From a commercial point of view, plasmid DNA is economical and relatively simple to manufacture compared to protein therapeutics and is non-toxic if manufactured properly.

This review examines the current development of polymeric sustained gene delivery systems, and discusses how continuous advances can impart momentum to the success of gene therapy. Different strategies for controlled gene delivery and, opportunities for continuous development have been discussed.

2. Design parameters for controlled release systems

Gene therapy can be controlled pharmaceutically at several levels. The gene delivery system itself can physically control the dose, location, and distribution of the administered gene. Designing of such delivery system should take into account a convenient and conventional administration route, and proper cellular targeting. On a second level, the gene expression systems (plasmids or viruses) can be designed to provide a controlled production and distribution of therapeutic proteins within the body, which may include tissue-specific promoters, transcript stabilizer, and which may persist in the cells according to their biochemical half-life. Though this second point is beyond the scope of this review, and has been discussed recently in detail elsewhere[12], it reinforces again the advantages of gene delivery over direct protein delivery, which is manifested in the wide therapeutic window gene delivery provides for long term protein production in targeted cells acting as bioreactors.

Controlled release of the DNA vectors eliminates the risks of under and over dosing, and provides an extended period of time to exploit the therapeutic potential of sustained protein expression. The key point is that an excess of delivered factors may produce undesirable side effects (eg. cytotoxicity) while lower levels of gene would produce insufficient protein to generate the desired therapeutic or physiological effect. The release profile of the delivered DNA vectors should therefore be designed to keep expressed protein levels within a therapeutic range, and can be based on their degradation and clearance rates within the local environment.

A controlled drug delivery device can be pre-designed to provide either constant or cyclic release of the drug over a long period of time, and can be triggered by environment or external events. Primary release mechanisms of gene delivery vectors from such devices can involve diffusion of DNA vectors through polymer matrix, degradation of polymer matrix, or swelling of matrix followed by diffusion of water. One or all of these mechanisms can occur in a single delivery system. In systems where entrapped DNA is transported by diffusion, concentration gradients can be established by appropriately manipulating the release kinetics. The diffusion of vectors can occur on a macroscopic scale, as through the pores in the

polymer matrix, or on a molecular level, by passing between the polymer chains (like in micellar packing). Strategies like controlling the rate of degradation of polymer matrix by adjusting density of biodegradable or hydrolysable linkers, or controlling the dissolution rate and pore size of polymer matrix by adjusting polymer weight concentration, can be used to tune the release profile. In swelling-based systems, mostly based on hydrogels, the swelling can be triggered by a change in environment surrounding the delivery system, such as pH, temperature, or ionic strength.

The non-ionic polymeric delivery devices can be cationized to promote DNA binding and loading efficiency. The binding of DNA to the polymer matrix hinders its diffusion, thereby prolonging its release. Such systems would also release compacted DNA upon degradation/dissolution of the polymeric matrix, further aiding in transfecting the cells. Some polymers used in the sustained delivery devices that contain functional groups (like carboxylic acids, amines) in their backbone can be readily modified to manipulate the interactions between polymer and DNA. However this may sometime limit the uptake of DNA by cells due to strong interactions between the DNA and polymer. Alternatively, viral vectors, or complexes of polycation and DNA pre-formed in solution can be loaded or immobilized on the polymeric delivery device. Specific binding (such as antigen-antibody, or biotin-avidin) or non-specific interactions (like molecular interactions with lipids, proteins, or polymer) can be used to immobilize the vectors on the polymeric devices. The number of binding sites in the matrix, affinity of DNA vectors for these sites, and the degradation rate of polymer matrix can determine the amount of vectors that can be loaded, as well as their release profiles. The vectors bound to these polymer matrices may either be released by degradation of linkage between vector and the material, or can directly be internalized by infiltrating cells.

Exploiting the therapeutic potential of genetic medicine requires the most efficacious mode of delivery, and arguably must be tailored specifically for different applications (tissues, disease conditions), with a tunable release, high bioavailability, device fabrication techniques that maintain bioactivity of encapsulated factors, and patient compliant injectability.

3.   Delivered factors

One of the first and most successful approaches in gene therapy involved direct injection of naked plasmid DNA in the interstitial space of the tissue, especially the skeletal muscles[13]. The application has been demonstrated to induce physiological effects using genes encoding systematically secreted proteins, such as erythroprotein (EPO)[14] and interluikin-5[15] and, locally acting proteins, such as basic fibroblast growth factor[16], vascular endothelial growth factor[17] and dystrophin[18].   However, the level of transfection is often variable and inefficient[13]. The plasmid DNA injected in excess buffer gets rapidly cleared or is internalized by phagocytic cells (macrophages). It is very susceptible to degradation by nucleases in the extra-cellular matrix (ECM) or inside the cells. Further, plasmids are large molecules (10^3-10^4 base pairs, >100nm hydrodynamic diameter) with high negative charge density that can limit their transport through tissues and can prevent their diffusion across biological barriers such as an intact endothelium, plasma membrane or nuclear membranes[19]. Clearance and degradation of plasmid DNA can also be attributed to its sequence-specific recognition by the immune system. Bacterially derived methylation pattern of CpG sequences on the plasmid backbone increase the immune response to the encoded transgene by promoting the production of immunostimulatory cytokines[20,21]. Such immune responses can eliminate the transgene expressing cells, and in effect reduce the duration of transgene expression[22]. Encapsulating naked DNA in sustained gene delivery systems can reduce this immune response by shielding CpG sequences[23], increase its residence time in tissues, and protect it against degradation.

Cationic polymers or liposomes that electrostatically condense negatively charged DNA molecules into nanoparticles have proven to be efficient gene delivery systems, giving transgene expression in targeted cells of several magnitudes higher than that achieved with naked plasmid delivery both *in vitro* and *in vivo*[24]. The approach is to neutralize the anionic surface charge of DNA, and reduce its molecular size. The polymers/liopsomes protect the encapsulated DNA from degradation by nucleases in ECM, blood stream, and endosomes inside the cells, and aid in the targeting to desired cells, transport across the cellular membrane, intracellular trafficking, and nucleus uptake. Various off-the shelf polymers[25],

like polyethylenimine (PEI), poly-L-lysine (PLL), methacrylates, dendrimers, and liposomes[26] like DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) and lipofectamine have been shown to be efficient gene delivery vectors, while new generations block and graft copolymers and liposomes designed specifically for gene delivery are being investigated to improve their serum stability, toxicity and transfection efficiency[27,28]. However, the shorter duration and lower level of gene expression than achieved by viral vectors are important issues that need to be technologically improved. One possible way to tackle the issue is long-term release of these vectors using a sustained gene delivery system that would also improve their colloidal stability, decrease their cytotoxicity, and increase bioavailability.

Viral vectors are biological systems derived from naturally evolved viruses capable of transmitting their genetic materials into the host cells. Many viruses including retrovirus, adenovirus, herpes simplex virus (HSV), adeno-associated virus (AAV) and pox virus have been modified to eliminate their pathogenicity (cytopathic effects) and maintain their high gene transfer capability[2]. However, the limitations associated with the use of viral vectors in terms of their safety, and in terms of their limited payload of cDNA, have encouraged researchers to increasingly focus on non-viral vectors as an alternative for gene delivery[6,29]. Further improvements are required to make these viral vectors less toxic and immunogenic[1,29]. Encapsulating viral vectors in controlled release systems can provide several advantages, including stability against degradation- as they have short half lives on the order of half hours at 37°C; reduced immonogenecity by avoiding escape to distant sites and making them available to only targeted cells at localized site; and reduced recognition by immune system by entrapping them inside the polymeric systems, limiting the binding of neutralized antibodies on their surface[30-32]. Sustained delivery may also minimize the amount of viral vector necessary to get the desired physiological response.

4 Different modalities of sustained delivery devices

A detailed examination of the advantages and limitations of different sustained gene delivery modalities, along with their delivery mechanism, and examples of successful applications is presented in this section. Both synthetic and natural polymers have been

employed to produce these devices. Advantages of different polymers along with various techniques used to encapsulate DNA vectors into the devices of these polymers are also provided.

4.1   Nanospheres

Nanoparticles are sub-micron sized (50 to 700nm) polymeric particles in which a drug molecule can be encapsulated or absorbed onto the polymeric matrix or conjugated to the surface[33]. Large surface area to volume ratio enables these nanoparticles to encapsulate large molecules of plasmid DNA efficiently without condensing it (electrostatic plasmid condensation involved in use of cationic polymers). Nanospheres loaded with plasmid DNA are internalized by the cells, and DNA diffuses out from the pores over time, as opposed to decomplexing from a cationic polymer or lipid. The sub-cellular and sub-micron size of nanoparticles has distinct advantages over microparticles. They can penetrate deep into the tissues through fine capillaries, cross the fenestration present in epithelial lining (e.g. liver), and have generally higher intracellular uptake compared to microparticles[34,35]. Though the transfection levels achieved with such nanoparticles *in vitro* are significantly lower than with cationic polymers and lipids, a substantial increase in expression has been observed through one week of culture, indicating DNA is released in a sustained manner intracellularly[36-38]. Intramuscular delivery of such nanospheres produced one to two order of magnitudes higher expression of plasmid DNA after seven days compared to lipofectamine, and the expression also sustained for longer period of times (up to 28 days) than liposomal plasmid DNA[37,39,40].

Both synthetic and natural polymers have been utilized in formulating bio-degradable nanoparticles. Synthetic polymers, like polylactide-polyglycolide copolymers, have the advantage of releasing the encapsulated DNA over a period of days to weeks, compared to shorter duration with natural polymers like gelatin and collagen. However, synthetic nanoparticles are limited by the use of organic solvents and relatively harsher formulation conditions. Polylactides (PLA) and poly(D,L- lactide-co-glycolide) (PLGA) nanospheres have been studied most extensively for sustained drug delivery because they form biologically compatible and metabolizable moeties (lactic acid and glycolic acid) after hydrolysis[37,38,41]. Nanosphere formulations of these polyesters are advantageous over

microspheres because they prevent DNA damage caused by the acidic environment of polymer degraded materials; the large surface area to volume ratio of nanoparticles facilitates fast diffusion of acidic degradation products from the particles into the tissue fluid. DNA loaded PLGA nanoparticles have been formulated mainly using a double emulsion solvent evaporation technique. Though the DNA loading efficiency is low, these nanoparticles can be delivered in higher doses to deliver required amounts of DNA without worrying about polymer associated toxicity because of their demonstrated long-term biocompatibility *in vitro*[42] and *in vivo*[43]. Plasmid loaded PLGA nanoparticles have been shown to rapidly escape endosomes, within 10 minutes of their incubation with cells[44]. The mechanism of rapid escape is by selective reversal of their surface charge (from anionic to cationic) in the acidic endo-lysosomal vesicles which causes the nanoparticles to interact with the endo-lysosomal membrane and escape into the cytosol. *In vitro* studies have indicated that smaller particle size and uniform size-distribution are important to enhance nanoparticles-mediated gene expression[45]. In a rat bone osteotomy model, sustained expression of reporter genes released from PLGA nanoparticles was observed in tissues retrieved from the gap five weeks after the surgery[41]. This suggests that such a strategy can be used to facilitate bone healing using therapeutic genes encoding bone morphogenic protein. Gene-coated sutures using an emulsion of PLGA nanoparticles, used to close an incision in rat skeletal muscles, demonstrated gene expression in the tissue at the site two weeks after surgery[46]. Such gene coated sutures encoding for growth factors like vascular endothelial growth factor could facilitate wound healing. *In vitro* studies have indicated that smaller particle size and uniform size-distribution are important to enhance nanoparticles mediated gene expression[45]. Nanoparticle-mediated *wt*-p53 gene delivery displayed sustained and greater anti-proliferative activity compared to lipofectamine in a breast cancer cell line *in vitro*[47]. However, efficiency of these nanoparticles to generate physiological responses *in vivo* using therapeutic genes still needs to be demonstrated.

Nanospheres of biodegradable natural polymers like gelatin and chitosan have also been shown to provide transfection comparable to that of lipofectamine[40]. DNA-gelatin nanospheres formed by salt-induced complex coacervation of gelatin and plasmid DNA, and stabilized by crosslinking gelatin matrix with crosslinking agent, produced greater and more

prolonged reporter gene expression after intra-muscular injections in mice[40]. Chitosan is a biodegradable natural polysaccharide derived from chitin that possesses both bioadhesion and mucus absorption enhancing capacities[48], making it a unique adjuvant for nasal, oral or rectal delivery of drugs. DNA loaded chitosan nanospheres have been used for the delivery of mucosal gene expression vaccine. In a mouse model of respiratory syncytial virus (RSV) infection, a single intranasal administration of chitosan-DNA nanospheres (25 μg/mouse), containing a mixture of plasmid DNAs encoding RSV antigens, resulted in a significant reduction of viral titers and viral antigen load after acute RSV infection of these mice[49].

The surface of the nanoparticles can be modified with ligands like poly(ethylene glycol) for tumor targeting and prolong blood circulation during systemic administration. DNA encapsulated in PEG-modified gelatin nanoparticles using a water-ethanol solvent displacement method have been shown to provide sustained gene expression in solid tumors after both i.v. and i.t. injections[50], and have longer tumor and plasma half-lives than unmodified gelatin nanoparticles with preferential localization in the tumor mass[51].

Nanospheres of polymers with cationic functional groups in their backbone can electrostatically bind DNA providing higher loading efficiency, more sustained release profile, and enhanced transfection efficiency. Nanospheres of water soluble and biodegradable polyphosphoesters, like poly(2-aminoethyl propylene phosphate) (PPE-EA), condensed plasmid DNA and provided sustained delivery as the high molecular weight polymer degraded through the cleavage of the backbone phosphate bonds up to 12 days *in vitro*[52]. The nanoparticles gave enhanced reporter gene expression after intramuscular injections into mice as compared to naked DNA[52]. Alternatively, plasmid DNA precondensed in nanostructures by cationic copolymers can be encapsulated into nanospheres. Plasmid DNA compelxed with two polylysine-based dendrons had greater encapsulation efficiency than naked plasmid DNA into PLGA nanospheres (less than 1μm in size), and displayed decreased release rate[53].

4.2   Microspheres

Molecules of plasmid DNA encapsulated into the microspheres of degradable polymers can provide sustained gene delivery in remote parts of the body after subcutaneous or intra-peritoneal injections using conventional syringes[54,55] or oral delivery[56-58]. These microspheres are not readily internalized by the cells, but are retained in the tissue providing prolonged DNA release. The released DNA can transfect the cells at the delivery site with the protein product acting locally or distributed systematically. Because the microspheres are too large to enter the cells by endocytosis, they can be preferentially up-taken by phagocytic cells such macrophages by size exclusion. Thus, microspheres provide ideal DNA carriers for vaccination or induction of cytotoxic T cell response. Microspheres of polymers that display bioadhesive properties are further suitable for mucosal immunization and can be delivered orally[56,57,59]. Mucosal immunization through gastrointestinal, nasal, or vaginal routes are most desirable because most pathogens enter through these routes[60], and induction of mucosal immunity offers the most effective line of defense at the port of entry[61]. DNA loaded microspheres of bio-adhesive polymers have been shown to be absorbed by the mucus and traverse through the mucosal barriers while protecting the DNA against nucleases[57,59]. Further, it is difficult for microspheres to diffuse out of the injected tissue to other sites because of their size and thus selective gene expression at the site of injection (e.g. localized tumors) can be obtained preventing distribution to distant sites No accumulation of radioactive-labeled DNA was detected in liver, kidney, or thyroid gland after subcutaneous injection of gelatin microspheres containing plasmid DNA into the backs of mice[54].

Fast degrading hydrophobic polymer microspheres containing surface carboxylic acid groups display biological adhesive properties and can be absorbed by gastro-intestinal mucus and cellular linings. This is aids in delaying the passage of such DNA loaded microspheres through gastrointestinal tract after oral administration, increasing the DNA delivery to the circulation[56,62]. Bioerodible and biologically adhesive microspheres (0.1-1 μm diameter) of polyanydride copolymers of fumaric and sebacic acid, poly (FA:SA), have been shown to provide improved DNA uptake into cells lining the small intestine and into hepatocytes after oral administration[56]. These DNA loaded microspheres were made using phase invertion nanoencapsulation (PIN). They maintained contact with intestinal epithelium for extended

periods of time and penetrated it, through and between the cells, increasing the absorption of administered plasmid DNA into the circulation.

Biocompatible and biodegradable polymers of FDA approved poly(D,L,-lactic-co-glycolic acid) (PLGA) are among the most commonly used material for microencapsulation of therapeutics. The PLGA microspheres have been studied extensively for the controlled delivery of proteins therapeutics and antigens to macrophages for mucosal immunization, and have recently been adapted to encapsulate DNA and oligonucleotides. Plasmid DNA loaded PLGA microspheres have been shown to elicit systemic and mucosal antibody responses after oral administration[57] and, induce cytotoxic T cell responses[59]. They have also been used to provide sustained delivery of gene silencing nucleotides (e.g. siRNAs) with expression persisting for longer periods than free oligonucleotides after subcutaneous injections into mice[63].

Although the preparation methods for PLGA microspheres have been well established[64,65], encapsulation of highly hydrophilic therapeutic agents with large molecular masses like plasmid DNA is challenging. The extremely hydrophilic character of DNA could lead to low entrapment levels and high initial release rates. The acidic degradation products from PLGA might also degrade the encapsulated DNA. Different strategies have been explored to improve the loading efficiency and stability of DNA into PLGA-type microspheres. One of the most common techniques is the double-emulsion solvent evaporation method[66,67]. However, there is partial degradation of the plasmid DNA due to the shear stress[68] induced during homogenization process and buffer salt crystallization during freeze-drying which deleteriously convert plasmid from the supercoiled form to the nicked or linear form[69,70]. Also this process gives low encapsulation efficiency (~20-40%) in the hydrophobic core of PLGA[66,71]. Cryopreparation is another technique for microencapsulation, where aqueous phase of the primary emulsion containing the plasmid DNA is frozen, resulting in a solid particulate suspension[70,72]. Because shear stress is zero within a solid, minimum damage is caused to the frozen plasmid DNA during homogenization to form secondary emulsion[68]. This process also gives higher encapsulation efficiency (~85%) because diffusion of frozen DNA out of microspheres is prevented during homogenization. Inclusion of saccharides in the primary emulsion have been shown to

disrupt the formation of DNA-nicking crystals during homogenization and lyophilization, preserving its super-coiled topology[70]. Spray-drying method is another DNA microencapsulation method, where aqueous DNA solution is dispersed in organic polymer solution (ethyl formate, or methylene chloride) by sonication, and resulting water/oil dispersions are spray dried[73,74]. Different variables like amount of DNA, solvent for polymer, addition of NaHCO$_3$ to aqueous DNA solution, sonication time, etc need to be optimized for highest encapsulation efficiency and retaining DNA integrity[73,74]. The process gives encapsulation efficiency between 30 to 100%, with higher ones giving higher burst release.

Composition and molecular weight (MW) of the PLGA polymers affects the release profile of encapsulated DNA[71,75], and affects the hydrophobicity of the microspheres, influencing their interaction with cells and mucosal membranes. Microspheres made with high MW polymers or with higher concentration of polymer can retain DNA for longer times due to denser polymer network or increased particle wall thickness. However, *in vivo*, the microspheres made with low MW polymers gave significantly prolonged gene expression that sustained up to 172 days as compared to high MW polymers where expression decreased in only 14 days[72]. High MW PLG microspheres were found to aggregate to a greater extent than the low MW PLG. Polymer degradation generally decreases the hydrophobicity[76] and thus the faster degrading polymers would be less likely to aggregate. Thus, it was suggested that faster degrading low MW PLG microspheres had higher surface hydrophilicities that reduced their aggregation providing a larger surface area for gradual release of DNA in the cellular microenvironment[72]. Hydrophilic PLGA polymers give higher encapsulation efficiency and faster release of intact DNA compared to hydrophobic ones[66,73,75]. They also show higher rates of phagocyotosis by macrophages and dendritic cells without affecting their viability[73].

Biodegradable microspheres of cationized gelatin present another interesting system for sustained gene delivery. Gelatin is prepared form collagen, and can be readily cationized by introducing amine residues on to its carboxyl groups[77]. The micro-encapsulated DNA is electrostatically immobilized to the polymer matrix and is released as the microspheres are degraded by proteases that make the cross-linked gelatin soluble in water. This release mechanism is different from that based on plasmid DNA diffusion from the release carriers,

as observed with PLGA microspheres. Because released DNA can be bound to the degraded fragments of cationized gelatin, this further aids in the DNA protection against degradation and its cellular uptake. The kinetics of release can be controlled by the extent of crosslinking in gelatin. However, the duration of DNA release is limited by the enzymatic degradation of gelatin, observed up to ~3-4 weeks *in vivo*[54,78]. Microspheres of cationized gelatin have been shown to provide sustained and enhanced gene expression *in vivo* (up to 28 days) suppressing tumor metastasis and arresting the progress of disseminated pancreatic cancer cells[54,55]. Sustained release of matrix metalloproteinase gene from gelatin microspheres was shown to prevent the onset of renal sclerosis in streptozotocin-induced diabetic mice[78]. Microspheres containing siVEGF (a DNA vector based on a small interfering (si) RNA system that targets Vascular endothelial growth factor (VEGF)) were used to normalize tumor vasculature and have been shown to inhibit tumor growth in a NRS-1 squamous cell carcinoma xenograft model[79]. The gelatin microspheres were found around the tumor up to 10 days after injection while free siVEGF had vanished by that time.

Plasmid DNA loaded chitosan microspheres have been used shown to provide long term gene delivery *in vivo*[80,81]. Cationic chitosan electrostatically binds DNA to form homogeneous and stable microparticles, providing a non-immunogenic and non-toxic system for mucosal delivery of plasmid DNA[82]. Plasmid loaded chitosan microspheres (1.45-2 μm) prepared by complex coacervation process using a precipitation technique[83,84] gave high plasmid DNA encapsulation efficiency (82-92%). Sonication and organic solvents are not used for the preparation of chitosan microspheres, inflicting minimum damage to the DNA integrity. Encapsulated interleukin-2 (IL-2) expression plasmid DNA was continuously released from chitosan microspheres for as long as 140 days *in vitro* that provided similar IL-2 expression as obtained with lipofectin, suggesting useful strategy for long term gene based immunotherapy[85]. Molecular weight and concentration of chitosan, along with amount of plasmid DNA affected the *in vitro* release profile from the microspheres[80,85]. Intramuscular injection of microspheres loaded with two plasmids encoding beta-galactosidase and luciferase productions gave high protein expression for both genes in mice that was sustained up to 12 weeks post-injection[81].

Yun et al[86] prepared hyaluronan (HA) microspheres loaded with DNA by crosslinking native HA using an adipic dihydrazide crosslinking chemistry at room temperatures, avoiding use of any organic solvents, and showed an extended release of intact DNA from these microspheres up to 2 months. The release rates could be controlled by adjusting the extent of crosslinking in the microspheres. Hyaluronan is a naturally occurring glycosaminoglycan distributed throughout the ECM, connective tissues, and organs of all higher animals, and is therefore a suitable polymer for delivery devices demanding long-term biocompatibility.

The microspheres can be coated with antibodies or ligands to selectively adhere to cells expressing those receptors. HA-DNA microspheres coated with a humanized mAb to E- and P-selectin (by conjugating with HuEP) showed more than 40 fold increase in the adhesion of HUVECs activated by IL-β relative to unactivated HUVECs, and a six-fold increase in adhesion to CHO-P (Chinese hamster ovary cells stably expressing P-selectin) relative to CHO cells[86].

Microspheres have also been used for sustained delivery of viral vectors in gene therapy to reduce their immunogenicity and increase their half-life in tissues[30]. Adenoviral vectors were microencapsulated in biodegradable chitosan microspheres by ionotropic coacervation of chitosan with encapsulation efficiency higher than 84%[87]. *In vitro*, the release of viral vector in aqueous media was negligible but, when in contact with monolayers of the cells, an effective release of bioactive adenovirus was obtained. Thus, encapsulation in microparticles not only protect the adenovirus from the external medium, but can also delay their release that is fully dependent on cell contact, an advantage for mucosal vaccination purposes. The formulations developed were able to maintain AdV infectivity and permit a delayed release of the bioactives that is promoted by digestion *in situ* of the microparticles by the cell monolayers. In another study, it was demonstrated that encapsulation of recombinant adenovirus in biodegradable alginate microparticles effectively circumvented the vector-specific immune response[31]. Reporter gene expression was significantly higher in immunized mice (containing virus-specific antibodies) when microencapsulated viral vectors were used compared to non-encapsulated ones. Recently, it was demonstrated that injection of degradable starch microspheres loaded with adenovirus vector (lacZ) through hepatic artery (tumor feeding) produced enhanced and cancer-selective gene expression in hepatocellular

carcinoma in rats[88]. Starch microspheres could be trapped within tumor to locally release gene vector that provided sustained and selective gene transfer than vector alone.

Molding microspheres in 3-D constructs supplements their drug delivery capacity with the structural support afforded by a scaffold. Microspheres with encapsulated proteins/DNA can be embedded within hydrogel or matrices, resulting in prolonged release profile of vectors. These points are discussed in the implantable hydrogels and scaffolds section below.

4.3   Hydrogels

Hydrogels are very attractive delivery systems for hydrophilic macromolecules like DNA as the entrapped vectors are soluble in the hydrated gel, enabling high loading efficiency. They provide a protective environment for DNA, and allow easy control of encapsulated gene transport by adjusting cross-linking densities and modulating network structure. Hydrogels are formed by chemical or physical crosslinking of special class of polymers that imbibe a considerable amount of water while maintaining their shape. They are composed of hydrophilic materials that can either be synthetic or natural, or a combination of two. Hydrogels of naturally occurring biopolymers like chitosan, alginate, gelatin, collagen, and hyaluronic acid have high DNA encapsulation efficiency and are much less damaging to DNA than synthetic polymers and their degradation products. DNA is released from these hydrogels by ionic exchange or degradation of the biopolymer by cell-secreted enzymes in the tissue matrix. However, this gives less control over the DNA release profiles from these hydrogels and can reduce the ability to sustain release for longer period of times. Synthetic biomaterials like polyanhydrides, functionalized PEG, and amphiphilic block copolymers can be used to make self-assembled or cross-linked hydrogels. The mechanical properties and degradation rate of such hydrogels can be manipulated by varying the extent of cross-linking or concentration of polymer networks. They can be bioerodible, or incorporate hydrolysable crosslinkers or matrix metalloproteinase sites (MMP, targets of invading macrophages). The hydrolysable blocks degrade over time, leading to a decrease in the gel cross linking density, mass loss, and ultimate DNA release. Different monomer chemistries and molecular weights can be used to control gel dissolution rates. Synthetic hydrogels offer broader control over

the release characteristics than natural polymers but, the gelation conditions and the chemical environment must be carefully selected to limit damage of DNA integrity.

4.3.1   In-situ forming Hydrogels

Polymers whose aqueous solutions can be subcutaneously injected by needle in the desired tissue, organ, or body cavity followed by instant *in situ* hydrogel formation that maintains its integrity for extended period of time form a special class of controlled delivery systems with distinct advantages over matrices that need to be surgically implanted. The flowing nature of these hydrogels during injection further enables a good fit when injected into a body cavity or defect.   The *in situ* gelation can occur either by chemical crosslinking[23,89] or by self-assembly of polymer network in response to physiological environmental stimuli like temperature[90] or pH[91]. The simplicity of preparing pharmaceutical formulation in aqueous solution, convenient administration, and *in situ* gel formation without any organic solvents / copolymerization agents/ or external stimulation makes this a convenient non-invasive controlled drug delivery system. Under *in vivo* conditions, the ingress of tissue fluid into the hydrogel results in dissolution or degradation of the hydrogel matrix facilitating a sustained release of encapsulated DNA. Typically, these hydrogels dissolve or degrade into non-toxic components that can be excreted through the renal clearance in the body precluding invasive removal procedures. Though many studies have been reported on the use of *in situ* gelling hydrogels for delivering protein therapeutics[92-94] and hydrophobic drugs[95-97], their use for gene delivery has not been extensively explored.

Genetically produced silk and elastin like polymers (SELP) that undergo irreversible sol to gel transition when transferred from room to body temperature have been investigated as injectable sustained gene delivery systems. SELPs consist of alternating silk-like (Gly-Ala-Gly-Ala-Gly-Ser) and elastin-like (Gly-Val-Gly-Val-Pro) blocks that can be produced by synthetic gene-directed biological production methods. SELPs with appropriate sequence and composition self-assemble at physiological temperatures to form hydrogels through crystallization of the silk-like blocks of the polymer chains, an irreversible, kinetic process[98]. Once localized *in situ* following injection, the entrapped DNA can be released from hydrogels by an ion-exchange mechanism while the polymer matrix degrades into relatively

nontoxic amino acids[99]. Release rates can be controlled by manipulating polymer concentration, cure time (time incubated at 37°C), and adding excipients that prevent or promote hydrogen-bond mediated chain crystallization. Sustained release of entrapped DNA from these hydrogels up to 30 days has been shown *in vitro*. The ability to precisely customize the structure and physicochemical properties of these protein polymers using recombinant techniques renders this class of polymers an interesting candidate for further evaluation in controlled gene delivery.

Amphiphilic multi-block copolymers, containing hydrophobic and hydrophilic polymer blocks within their molecular architecture, display thermo-reversible gelation and have been well investigated for sustained protein and gene delivery[100,101]. Above the lower critical solution temperature (LCST) of the hydrophobic block, where it dehydrates, these copolymers self-assemble to form micellar like structures with a hydrophobic core stabilized by a hydrophilic corona. Above a critical gelation temperature (CGC), these micellar solutions form a lyotropic liquid crystalline phase that results in a transparent hydrogel. The solution to hydrogel transition is driven by an increase in volume fraction of copolymer micelles (as in PEG-PPO-PEG), or an increase in the size of micelles (increase in aggregation number, as in PEG-PLGA-PEG) due to hydrophobic interactions between collapsed hydrophobic blocks resulting in ordered packing of the micelles into a crystalline lattice[100]. As the water diffuses into the gel matrix, solvating a boundary layer of gel and decreasing the polymer concentration below CGC, the gel boundary dissolves, allowing the entrapped plasmid DNA to be released along with polymer molecules. They rely on diffusion, and disruption of weak interactions between hydrophilic and hydrophobic components to release DNA. Using simple, free volume-based theories, mean field theory and percolation theory the diffusion in heterogeneous polymer networks can be described[102]. Thermoresponsive Pluronic (PEG-PPO-PEG) gels have been used for localization and sustained delivery of plasmid DNA and viral vectors. Stereotaxic delivery of lentiviral vector in 15% Pluronic F127 to the rat brain resulted in transduction of cells, predominantly astrocytes, close to the injection site[103]. Using a localized Pluronic gel based depot of viral vector release in the central nervous system (CNS) would have application in brain injury or ischemia and spinal cord trauma with the area of tissue damage capturing the semi-solid gel.

Delivery of adenoviral vector in poloxamer 407 gel via an endoluminal route to the vasculature of balloon-injured rat carotid arteries has been shown to increase local arterial transfection efficiency[104]. Recently, Pluronic F127 gel containing adenoviral vector has been applied to the perivascular surface of the common carotid artery of the rat[105]. *In vivo* gene transfer to the adventitia resulted in sustained transgene expression capable of labeling migrating adventitial cells within the media and neointima of injured vessels.

However, self-assembled Pluronic hydrogels have low mechanical strength, and a loosely cross-linked network structure, which results in rapid release of entrapped DNA molecules through diffusion during the early incubation stage[106]. Chemical modifications to Pluronics have been made which alter the gelation characteristics of the gel. The common systems used to modify Pluronic copolymers are polyacrylic acids, polybases, and biodegradable polyesters[107]. Addition of polyethylene glycol[108] and cellulose derivatives[109] to Pluronic F127 have been used to reduce the dissolution rate of drug from the gel. The addition of polyacrylic acid[110] or polycarbophil has increased the muco-adhesiveness of Pluronics for improved nasal delivery of plasmid DNA[111].

Pluronics were also modified at both ends with functional groups to improve the mechanical properties of hydrogels. A family of novel pentablock self-assembling copolymers has been developed by adding PDEAEM to the sides of Pluronic block copolymers using an ATRP reaction scheme by our group[112]. While the copolymers show reversible thermo-reversible gelation properties like Pluronics[113,114], the cationic PDEAEM groups condense the negatively charged DNA and show pH buffering capacity at low pH[115]. The polyplexes of the copolymers are biocompatible and give DNA transfection efficiency comparable to that of commercially available linear PEI ExGen 500®[116]. Copolymers condense DNA in solution at room-temperature that instantly form an elastic gel *in situ* after injection into the body (illustrated in Fig. 1). The gels dissolve in tissue fluid to release condensed DNA. While gels act as a DNA depot, the released DNA is also protected inside polyplex nanoparticles in both ECM and inside cells. The amphiphilic copolymers aid in the intracellular localization of polymer/DNA complexes by endocytosis and their release from endosomes after entrapment. Because plasmid DNA is electrostatically bound to copolymers, its release is controlled only by the dissolution profile of the hydrogels. The plasmid cannot

freely diffuse out of the polymeric network, preventing initial burst observed in gels/martices of non-ionic polymers where DNA release is governed by diffusion through pores. The 100 µl gels of copolymer at 15 wt% concentration have been shown to release condensed DNA up to 7 days *in vitro*, compared to complete naked DNA release in less than a day using only Pluronic gels (unpublished data). The release profile can be easily modulated by tailoring the cationic content in the copolymers, and by adjusting concentration of copolymers in the formulation. These gels also a have higher storage modulus as compared to Pluronic gels, and have been shown to be easily injectable into subcutaneous tumors and skeletal muscles by syringes equipped with 27 gauge needles. Such biocompatible cationic self-assembling copolymers display great potential as sustained gene delivery devices, and have distinct advantages over systems that release naked DNA.

Another example of such thermosensitive polymers is PEG-PLGA-PEG. When polymer solution containing a plasmid DNA encoding TGF-ß1 was administered to the excisional wounds at the back of diabetic mice, it formed an adhesive film *in situ*. Significantly accelerated re-epithelializaion, increased cell proliferation, and organized collagen were observed in the wound bed treated with thermosensitive hydrogel containing plasmid TGF-ß1 compared to controls[117].

Polymers containing hydrolytically labile linkages that form chemically crosslinked hydrogels on reacting with other polymers, and are in injectable solution form for a few minutes after reconstitution of polymer components have also been shown as injectable controlled delivery systems. Functionalized branched PEG polymers, with one of them containing hydrolytically liable ester linkages, formed crosslinked gels via amide linkages that could encapsulate plasmid DNA into the polymer network without any degradation[23]. The formulation was injectable within 15 minutes after reconstitution of polymer components and formed crosslinked matrix *in situ*. The reacting PEG polymers do not interact with DNA but entrap it in the matrix. The gel biodegraded in tissue fluid over time (up to 28 days) giving a prolonged release of plasmid DNA, and significantly increased the duration of gene expression in immunocompetent mice up to 92 days compared to only 30 days with naked DNA injections.

4.3.2   **Implantable hydrogels**

4.3.2.1   **Chemically crosslinked hydrogels**

Chemically crosslinked hydrogels have the flexibility to tune the degradation kinetics of the gels to produce tailored release profiles. Agarose, a natural polysaccharide derived from red algae, forms thermoreversible hydrogels, and have been used provide sustained release of DNA *in vivo*. Poly-L-lysine compacted DNA encapsulated in agarose gels provided sustained gene expression up to 35 days in skin tissues after intra-dermal injections in mice, compared to 5-7 days obtained with injections in solution, and invoked a wound-healing response through day 14[118]. The agarose hydrogel containing DNA was first gelled in 0.3ml syringe, and then injected intradermally using a 28.5 gauge needle. The encapsulated DNA is released by diffusion from the hydrogel. The DNA release profile can be controlled by changing the agarose concentration. Recently, controlled release of a DNA vaccine from intradermally implanted agarose hydrogels was shown to provide a sustained bovine herpesvirus 1-specific immune response similar to that obtained with two discrete administration of the vaccine 4 weeks apart in a bovine model suggesting a novel slow-release vaccination tool in cattle where repeated administrations are frequently necessary[119].

Biodegradable hydrogels of gelatin and cationized gelatin offer physiochemical and electrostatic immobilization of plasmid DNA in the polymer matrix. Aqueous solutions of gelatin can be cast into various molds and made into hydrogel sheets at 4°C, which can be cut into small discs (5 x5 x 51 mm^3) for implanting into a localized tissue in body[120]. Such a gelatin hydrogel had a sponge with pore-size of 500 μm[120]. Plasmid DNA can be impregnated into the freeze-dried hydrogel sheets by swelling process, dropping it in solution on the sheets and incubating them at 4°C overnight. Encapsulated DNA is released as the hydrogel degrades to generate water-soluble gelatin fragments. Gelatin doesn't degrade by simple hydrolysis, but is degraded by proteolysis, making it suitable for prolonged drug release. Gelatin hydrogels can be chemically crosslinked using reagents like glutaraldehyde for making denser (less water content) network, and increase the duration of biodegradation.

The *in vivo* degradation period of these hydrogels can thus be controlled from 7 to 21 ways depending on degree of crosslinking induced[120,121]. It has been observed that only gelatin hydrogels with higher degree of cationization (41.6 mol% or more) provided significant gene expression *in vivo* perhaps because they release DNA condensed with cationized gelatin fragments that improve cell adhesion and gene transfection[121]. Cationized gelatin-based hydrogels have been shown to maintain significantly higher gene expression than naked DNA injections for more than 21 days in the femoral muscles of mice[120,121]. Another advantage of these hydrogels is that their profile of controlled release is not influenced by the shape of their mold.

Biodegradable synthetic hydrogels based on the water-soluble polymer oligo(poly(ethylene glycol) fumarate) (OPF) have been shown to encapsulate DNA, retain its activity, and provide prolonged release up to 62 days, depending of degree of crosslinking[122]. The hydrogels can be crosslinked under physiological conditions to physically entrap plasmid DNA, and can be molded into sheets. The sheets can be cut into discs of approximately 6 mm diameter and 1 mm thickness, and implanted into the body at the site of therapy. Composites of plasmid DNA-loaded cationized gelatin microspheres in an oligo(poly(ethylene glycol)fumarate) (OPF) hydrogel were found to prolong the bioavailability of plasmid DNA upto 42 days relative to the injected plasmid DNA solution control and non-embedded cationized gelatin microspheres (7 to 21 days) in an *in vivo* murine model[123]. While the plasmid release form gelatin microspheres was limited by enzymatic degradation, the duration of release can be modulated by embedding the microspheres in OPF gels and controlling the release by modulating the crosslinking in the OPF gels. The sustained release of plasmid DNA from the composite group could be explained by the observed slower degradation of gelatin microspheres within the OPF, and the prolonged retention of degraded gelatin/ DNA fragments in the hydrogel.

4.3.2.2   **Photo-crosslinked hydrogels**

Gels crosslinked through photo-polymerization of the monomer and DNA solutions enable spatial and temporal control of gel formation (and gel dissolution) under physiological conditions[106,124]. For example, by spatially modulating the degree of crosslinking, the

encapsulated DNA can be localized on the surface of the gels for easier uptake by infiltrating cell in a tissue engineering application. Degree of polymerization can be easily modulated by controlling the UV irradiation time[106]. Gels are formed by exposing a solution of monomer and DNA, containing a photoinitiator for polymerization, to light (eg UV at 365nm, 5-11 mW/cm^2) for 10-30min[125]. It is important in this process to identify methods for protecting DNA from detrimental effects of photoinitiator and free radicals[126]. Addition of transfection agents and/or antioxidants can greatly reduce DNA damage by radicals[126]. Hydrogels formed from photo-polymerization of multifunctional PEG monomers were shown to encapsulate DNA with minimal damage, and release biologically active plasmid DNA for periods of 6-100 days depending upon the degree of photopolymerization[125]. Photo-polymerized di-methacrylated oligo(lactide)-*b*-poly(ethylene glycol)-*b*-oligo(lactide) macromer was used as a bioerodible hydrogel platform for delivering plasmid DNA. Gold stents coated with photo-polymerized styrenated gelatin have been shown to provide sustained release and expression of encapsulated DNA (adenoviral vector) up to 3 weeks in carotid arteries of rabbit[127]. The study confirmed that released DNA retained its activity after photo-encapsulation in gelatin hydrogels.

Multifunctional anhydride monomers were photocrosslinked to produce hydrophobic, highly crosslinked polymer networks that degrade by surface erosion. Surface-eroding polymers can deliver molecules of a wide range of sizes at sustained, steady rates, which is advantageous for DNA delivery where the high molecular weight may complicate control of the release profiles. However, when plasmid DNA was released from photocrosslinked polyanhydride matrices, DNA recovery was low ($\sim$25%)[128]. To reduce the damaging effects of polymer degradation and photoencapsulation, DNA was pre-encapsulated in alginate microparticles, which served as temporary coating that quickly dissolved upon microparticle release from the polyanhydride matrix, and increased the DNA recovery to 90%. Such hydrogels can provide both, a structural support and, a controlled release profile to the encapsulated DNA.

Recently, photopolymerized biodegradable hydrogels of Pluronic and HA were shown as potential controlled gene delivery platforms[106]. Pluronic F127 was di-acrylated to form a macromer and chemically cross-linked in a micellar gel state by UV irradiation to form a

hydrogel in the presence or absence of vinyl group-modified hyaluronic acid (HA). Mechanical strength of photo-cross-linked Pluronic hydrogels was much higher than that of physical Pluronic hydrogels produced by simply increasing the temperature. UV irradiation time and the presence of the vinyl group-modified HA affected the mechanical property of Pluronic hydrogels to a great extent, giving less swelling ratios and slower degradation profile. They showed much reduced burst releases and more sustained DNA release patterns. Functionally active DNA was slowly released from photo-cross-linked hydrogels over 10 days *in vitro* and its profile could be controlled by the degree of cross-linking.

While photo-polymerized hydrogels could be a potential candidate for temporal and spatially controlled sustained gene delivery, UV irradiation process should be carefully designed to achieve a desirable DNA release kinetic rate with minimal DNA structural damage. For instance, the UV curing time could be shortened by using more potent photo-sensitizers and/or using other vinyl monomers and macromers[106].

4.4 Implantable polymeric scaffolds

Encapsulating DNA therapeutics in a polymeric matrix that can be implanted at the site of injury or disease integrates the provision of controlled gene delivery with the structural support afforded by a scaffold. The three dimensional scaffold can provide support for cell adhesion and migration, and a template for tissue formation, while also creating and maintaining space for it. The scaffold provides a unique opportunity to control both the sustained delivery of genes and the cellular environment in which the gene transfer occurs, while the matrix retains the DNA at the site of implantation in a protective environment. The matrix can be loaded with a higher amount of DNA providing prolonged therapeutic benefits as compared to particulate gene delivery vehicles where the amount of DNA encapsulated depends on the charge ratio of polymer to DNA. Besides, such matrices would distribute DNA throughout the 3D space of the therapy site which may be more effective than injection of bolus doses in aqueous solutions. The basic properties of the scaffold can also be augmented to create a microenvironment that exploits synergy between multiple growth and transcription factors. Different combinations of genes and proteins can be combined in the

matrix, and various adhesion molecules, peptides and ECM matrix proteins can be immobilized to the biomaterials to regulate cellular interactions with the matrix. Specific cellular cues can be incorporated in order to target the attraction of the cell types to be transfected. Thus, while the matrix controls the release kinetics of the entrapped genes, it can also provide a more complex conducive and inductive environment for migration of targeted cells. Another advantage of such polymeric implants is the possibility of their removal by a small surgical procedure if adverse events necessitate discontinuation of therapy. The basic requirements for a gene delivery scaffold is being biocompatible, have sufficient mechanical integrity, large surface area, and an ideal surface morphology. If used for a tissue regeneration application, the scaffold should also be biodegradable as it cannot be removed surgically after neotissue growth. Various synthetic and natural polymers have been investigated to form such implantable controlled gene delivery devices.

4.4.1   Biodegradable matrices

Scaffolds fabricated with natural polymers, such as collagen and hyaluronan (HA), degrade by the cell secreted enzymes and allow the cells to migrate by specific cellular interactions within the matrix. They have the advantage of having the intrinsic property of the environmental responsiveness via degradation and remodeling by cell secreted enzymes. They are generally non-toxic even at high concentrations as they degrade into components that are similar to ECMs compostion and can, therefore, be readily incorporated into bolus matrix delivery systems.

Sponges made from such natural biopolymers impregnated with DNA, termed as "Gene activated matrix" (GAM)[129], have been shown to be therapeutically effective sustained gene delivery systems *in vivo*. Plasmid DNA can be encapsulated by absorption from aqueous solution onto pre-formed collagen sponges to form GAM capable of gene delivery. These three-dimensional, moldable, porous GAMs act as an acellular scaffold that provide a platform for gene delivery, while acting as bioreactors for seeding cells to secrete plasmid-encoded proteins that enhance cartilage natural healing process. The encapsulation content of DNA can be controlled by varying the polymer concentration and the conditions of the incorporation process. These biocompatible, naturally derived polymer matrices permit cell

infiltration for DNA uptake. Plasmid release as the matrix degrades provides a secondary mechanism for gene transfer to surrounding cells. Collagen based GAMs for sustained gene delivery have been implanted at different body sites for localized therapy and tissue engineering, including bone[9,130], cartilage[131], central nervous system[132], wounds[133,134], and cardiovascular tissues[135,136]. This flexibility obviates the need for prior graft colonization in cell culture. Bovine-derived collagen-I based GAM have been implanted into an adult rat femur[130] and a canine bone defect model[9]. Scaffolds loaded with 1mg of DNA in the rat model (with 5-mm gap defect) were capable of transfecting the migrating cells, and maintaining prolonged protein expression for up to 3 weeks that resulted in a significant increase in bone regeneration compared to localized plasmid or systemic hormone delivery. In the canine model (with 1cm bone gap) collagen matrix with 100mg of DNA were implanted, and the union of the gap was achieved after 8 weeks of treatment. The local retention and expression of plasmid DNA by granulation tissue was demonstrated for 6 weeks after implantation.

Collagen sponges loaded with DNA precondensed with cationic polymer or liposomes are superior in mediating sustained gene delivery *in vitro* and *in vivo* as compared to naked DNA-loaded sponges, in terms of both level and duration of gene expression[137]. Protective copolymers are particularly advantageous in promoting the transfection capacity of polyplex-loaded sponges upon subcutaneous implantation, likely due to their stabilizing and opsonization-inhibiting properties. The release of DNA complexes is significantly slower than that of naked DNA because of the differences in physical properties, providing prolonged therapeutic benefits.

Other natural proteic polymers, such as atecollagen[138] and fibrin[136], or polysaccharidic materials, such as chitosan-gelatin[139], glycosaminoglycan[140], and hyaluronic acid[141,142], have also been used to substitute collagen in GAMs. Gelatin sponge matrix loaded with canarypox virus ALVAC recombinants encoding the murine tumor necrosis factor-α, produced statistically significant growth inhibition of established tumor nodules after intratumoral inoculation[143]. Hybrid collagen-gelatin, and collagen-glycosylaminoglycan scaffold have demonstrated enhanced activity over bare collagen GAMs. These all natural cationic matrices can encapsulate more plasmid DNA, and provide greater control over the fabrication of

scaffolds with appropriate porousness and mechanical property. For example, chitosan increase the rate of gel formation, and the strength of the resulting gels, and gelatin changes the brittleness of chitosan reversely[144].

Cylindrical minipellets (0.6mm diameter, 10mm length) of atecollagen loaded with plasmid DNA have been shown to provide controlled release of the gene, maintaining high platelet count and sustained protein expression levels in serum up to 60 days after single intramuscular injection[138]. Intact gene was detected in peripheral blood up to 40 days while it was barely detectable after 21 days following naked DNA injections. Atecollagen is a very biocompatible material, and is prepared by eliminating antigenic telopeptides from the ends of the collagen molecule by pepsin treatment[145,146]. Adding 30 wt% glucose to the formulation made the collagen matrix structure made coarser, allowing substantial controlled release of DNA. The atecollagen implant remains as a solid mass that can be handled easily, and can be removed surgically with the vectors to regulate the duration of gene expression[138].

4.4.2 Synthetic scaffolds

Scaffolds synthesized with synthetic polymers like PLG are typically highly porous, which can allow efficient nutrient transport and cellular infiltration. In a tissue engineering application, cell infiltration from surrounding tissue is important for integration of the engineered tissue with the host and for the development of a vascular network through out the tissue to supply necessary metabolites once the tissue has developed. Synthetic polymers are more versatile than natural polymers for synthesizing gene delivery matrices as they provide greater control over matrix macrostructure, mechanical properties and degradation time. Hydrophilic polymers such as PEG can be crosslinked and funcationlized[147]. Most commonly used synthetic polymers for gene delivery matrices are made of polylactic acid (PLA) which degrades within the human body to form lactic acid, a naturally occurring chemical which is easily removed from the body. Other similarily used materials include polyglycolic acid (PGA) and polycaprolactone (PCL)- their degradation mechanism is similar to that of PLA, however, they exhibit a faster and a slower rate of degradation compared to PLA, respectively. Copolymers of these materials, like FDA approved PLG, can be designed to degrade over times ranging from weeks to more than a year. Scaffolds can be

formed as a mesh of fibers wound together or the polymer can be processed into a highly porous structure[147-150].

Mixtures of DNA and PLGA have been electrospun to form a non-woven nanofibrous and nanocomposite scaffold. The entrapped DNA was released from such scaffolds by controlled degradation of the biodegradable PLGA, transfecting cells adhered to the matrix[151]. Such scaffolds capitalize on the molecular interaction of block copolymers and plasmid DNA in solution, giving rise to novel structures and additional functionality. Three-dimensional scaffolds of DNA loaded PLG microspheres can be fabricated by the assembly and subsequent fusion of microspheres using a gas foaming/particulate leaching process[148,152]. The scaffolds had an interconnected open pore structure with high porosity, and exhibited sustained release of active DNA *in vitro* for 21 days, with minimal burst during the initial phase of release. Control over the release rates could be obtained through manipulating the properties of the polymer, microspheres diameter, and the foaming process. Such PLG matrices have been shown to be effective for sustained gene delivery in various applications of tissue engineering *in vivo*, facilitating enhanced matrix deposition and blood vessel formation in the developing tissue after implantation[153].

Biocompatible FDA approved Evac poly(ethylene-co-vinyl acetate) matrices loaded efficiently with up to 100mg of DNA have been shown to provide both short term and long term controlled release of DNA, up to 1 month, maintaining the integrity and activity of released plasmids[67]. DNA release from these Evac matrices was controlled by diffusion, and depended only on the size of the entrapped DNA, not on the size and geometry of the matrices. DNA release profiles were bi-phasic, with an initial burst followed by a slow but continuous release. Evac matrices have been shown as efficient and convenient vehicles for DNA vaccination via the murine vaginal tract that provided long-term immunity for as long as 56 days[154]. This immunization regimen avoided the need for multiple immunizations and invasive surgery required with other investigated methods of DNA vaccination in vaginal tracts[155,156]. The matrices were easily implanted by a simple surgical procedure[157] into the vaginal tract of mice, and provided sustained DNA release to the vaginal mucosal surface overcoming the barriers caused by the estrus cycle and physical environment of the vaginal tract. The matrices can be removed by a simple surgery after the treatment.

4.4.3   Substrate immobilization

Substrate immobilization is an alternative technique where DNA is actually immobilized to a surface or biomaterial that supports cell adhesion. The targeted cells migrate to the scaffold and engulf the DNA tethered to the polymeric matrix. This puts the DNA directly into the cellular microenvironment, increasing its local concentration and avoiding problems of mass transfer limitations or complex aggregation encountered in release systems. Immobilization maintains the DNA locally in the matrix, limiting any potential undesirable diffusion to distant sites. This also prevents any systemic loss of the drug, as it is taken up only the infiltrating cells. Cells cultured on the substrate can internalize the DNA either directly from the surface, or after release of the DNA from the surface. Immobilization techniques are a great tool to regulate the distribution of DNA across the scaffold and create gradients.

DNA or non-viral vectors complexed with DNA can be immobilized on the polymeric substrate through specific or non-specific interactions for delivery from the surface. Specific interactions can be introduced through complementary functional groups on the vector and surface, such as antigen/antibody or biotin/avidin. DNA complexed with PAMAM dendrimers were immobilized by drying onto bioerodible PLG and collagen based membranes and shown to give effective transfection both *in vitro* and *in vivo* in skin cells of mice[158]. Dendrimer/DNA complexes could mediate transfection after dissociation from these membranes and/or when retained on the surface of the membranes. DNA co-precipitated with calcium phosphate was adsorbed onto two and three dimensional PLG matrices, and SaOS-2 cells cultured on these 3D matrices were shown to efficiently internalize the immobilized DNA[159]. Biotinylated HA-DNA complexes have been immobilized to neutravidin modified substrates like HA-collagen hydrogel[160]. Cells cultured on the hydrogel were transfected while those adjacent to hydrogel did not. It was further shown in the same study that surface patterning these hydrogels with ridges and grooves could provide oriented cell growth. DNA complexed with other cationic polymers like PLL and PEI, which are functionalized with biotin, have also been immobilized to such neutravidin substrates[161,162],

and were shown to give 100-fold increased transgene expression in cells cultured on these DNA surfaces compared to bolus gene delivery of DNA complexes[162]. Degree of DNA immobilization and transgene expression were found to be dependent on the biotin content in the complexes. Though higher biotin contents increased immobilization, it gave decreased transfection. Since transfection was observed only at locations on matrices where DNA was immobilized, it suggested spatially controlled gene delivery is possible by immobilizing of DNA complexes on the substrate in a spatially controlled manner[161]. This can be useful in creating complex tissue architechtures.

Viral vectors have also been immobilized by both non specific or specific antigen/antibody interactions on collagen constructs coated with anti-viral antibodies, preventing escape of virus to distant locations[163,164]. Viral functional groups can either be modified with antibodies or biotin residues[165], or functional groups in viruses can be engineered enabling binding without chemical modification which could otherwise inactivate the virus[166,167]. Collagen-coated polyurethane was thiol activated and covalently bound to anti-adenovirus antibodies for subsequent binding to adenovirus[168]. Virus nonspecifically bound to polystyrene beads or microspheres have shown increased transduction efficiency and localized and targeted gene expression adjacent to the beads in contrast to free viral vector delivery both *in vitro* and *in vivo*[169,170].

5 Applications

Sustained gene delivery of viral or non-viral vectors using polymeric devices can be employed to promote gene transfer in the cells adjacent to the implant or, in organs deep into the body using systemic or oral administration. An efficient gene delivery systems design should consider both the specific application, and the requirements for efficacy. For example systems developed for nerve regeneration in spinal cord injury would be very different from the one used to promote angiogenesis in ischemic cardiac tissue. The polymeric gene delivery, depending on the gene product, can be designed to produce therapeutic proteins locally, like in suicide gene therapy, or wound healing treatment, or distributed systemically by transfected cells, as in treatment of hemophilia. Table 1 lists the *in vivo* studies using

polymeric sustained gene delivery systems that demonstrated physiological responses, and some of the most prominent applications of sustained gene delivery are summarized in this section.

5.1   Cancer Therapy

Various target therapeutic genes have been identified for gene therapy against cancer employing strategies like immunization, blocking molecular pathways for uncontrolled growth or angiogenesis, and suicide gene therapy. However, current human clinical trials against cancer using viral vectors are limited by adverse effects of virus itself. On the other hand, applications using non-viral vectors are limited by short duration and low level of gene expression. Because cancer cells have an invasive and aggressive growth profile, it is important to maintain the therapeutic level of the drug for complete eradication of the tumor in order to prevent any dissemination and metastasis during or after treatment. A controlled gene delivery method can prolong the maintenance of expressed protein drug in the system much longer than bolus drug delivery or controlled protein release systems, and, thus, provide one of the most efficient ways for eradicating neoplastic cells. Local delivery of cancer chemotherapeutics also reduces systemic side effects while maintaining sustained drug levels at the site of action.

Injection of biodegradable gelatin microspheres incorporating plasmid DNA encoding for NK4 protein, an antagonist for hepatocyte growth factor (HGF), have been shown to suppress the progress of disseminated pancreatic cancer cells in peritoneal cavity of mice by inhibiting growth of nascent blood vessels (angiogenesis) and increasing apoptosis in tumor tissue[55]. The controlled gene delivery enhanced and prolonged the NK4 protein level in the blood circulation, resulting in significantly greater suppression in tumor number and increased survival time of mice as compared to bolus plasmid delivery. Similar enhancement of tumor suppression effects of therapeutic plasmids inhibiting angiogenesis by controlled release gelatin microspheres were shown on mice bearing Lewis lung carcinoma tumor[54], and in a squamous cell carcinoma xenograft model[79]. A marked reduction in vascularity accompanied the inhibition of transfected tumor.

5.2   Vaccination

The advantage of a DNA-based approach is that the vaccines can be manufactured very rapidly and in large quantities, while yielding an efficacious immune response at low doses. DNA vaccines can encode multiple immunogenic epitopes at the same time, and can evoke both humoral and cell mediated immune responses. DNA vaccination shows promise in a number of areas including infectious diseases, allergy, and cancer immunotherapy. While not all safety concerns have been completely addressed, human trials in HIV patients are encouraging[171].

Long term maintenance of immunity requires repeated administration of DNA vaccine, which might involve side affects due to over or under dosing, and systemic loss of drug. Controlled gene delivery devices can circumvent this by acting as depot for DNA vectors, and providing sustained release. Release profiles from most of the polymeric controlled drug delivery modalities include an initial burst of the drug followed by slow and steady release for extended periods. The initial burst of gene drug may provide enough antigen to sensitize immune cells and initiate immune response to a new foreign antigen. The continuous relapse of lesser amounts of DNA may provide long-term antigen to repeatedly boost the immune system and maintain the pool of antibody producing cells or memory cells. By providing active antigen over an extended period, reliable immunization can be achieved that can be sustained for longer period of time. Controlled release of a DNA vaccine from intradermally implanted agarose hydrogels have shown to provide a sustained anti-bovine herpesvirus 1-specific immune response similar to that obtained with two discrete administration of the vaccine 4 weeks apart, in calves where repeated administrations are frequently necessary[119].

Though naked DNA vaccination strategy had been limited to intramuscular, cutaneous, and intradermal routes of immunization, controlled gene delivery devices can be effective in mucosal immunization through nasal, gastrointestinal, or vaginal routes, the port of entry for most pathogens[61]. Because naked DNA is ineffective in crossing mucosal barriers, and is rapidly degraded by nucleases, delivery systems that protect DNA and target it to antigen-presenting cells are essential for the success of DNA-based mucosal vaccines. DNA loaded microspheres and nanospheres can be absorbed by the mucus and traverse through mucosal

barriers, while protecting the encapsulated plasmid and releasing DNA for prolonged periods to maintain long-term immunity. Microspheres, because of their size, are not readily endocytosed, and are, therefore, preferentially up taken by the professional APCs and phagocytic cells. Biodegradable microspheres of PLGA containing plasmid DNA were shown to retain its activity and provide sustained delivery to induce cytotoxic T cell responses after oral administration[57,73]. Polymer microspheres containing surface carboxylic acid groups that display biological adhesive properties can be absorbed by gastrointestinal mucus and epithelial cells, delaying their passage through gastrointestinal system, and increasing the plasmid delivery to the circulation after oral administration[56,62]. EVac matrices loaded with DNA have been shown to provide long term local mucosal immunization after implantation in vaginal tracts of mice[154]. The matrices provided sustained release of DNA to the vaginal mucosal surface that was functionally active and capable of transfecting vaginal tissues.

5.3   Tissue engineering

Inductive tissue engineering involves delivering growth factors and cytokines to the progenitor cells in the surrounding tissue that can direct cell differentiation and induce tissue formation. Delivering the pharmacological doses of these short half-life factors and maintaining an environment with appropriate combination of signals that induce proper cell function and regenerate clinically useful amounts of new tissue *in vivo* have been critical challenges. Localized delivery of genes encoding these factors using polymeric scaffolds is a versatile technique which puts genes directly into the path of the infiltrating cells, directing specific cell processes. While sustained gene delivery maintains prolonged expression of encoded factors, the polymeric scaffolds create and maintain space and provide a conductive physical support that allows tissue regeneration. The matrix must mimic the numerous functions of the natural extracellular matrix (ECM). Gene delivering scaffolds synthesized from natural or biodegradable synthetic polymers, as discussed in sections above, have shown marked success in various tissue engineering models, including bone regeneration[9,129,130,150,172,173], nervous system (nerve regeneration)[132,174], angiogenesis[173,175,176], and cartilage formation[131,139,140].

Though several fundamental scaffold design requirements have been identified[177], guidelines for controlled delivery of genes from such scaffolds to provide maximal tissue formation are still poorly understood. Because the matrix provides support for cell adhesion and migration, and organizes cells into structures, improving the mechanical properties of the scaffold and mechanical stimulation of the tissue can influence tissue formation[172,178]. Scaffolds must create and maintain a space for tissue formation and should be resorbed or degraded at a rate that is comparable with new tissue formation. For any tissue regeneration application, different types of cells migrate and infiltrate the site of injury. Targeting a specific cell population or cell type for gene delivery can provide greater benefits and may be desirable for inducing differentiation toward specific fate or function. For example, in a nerve regeneration application, infiltrating fibroblasts cannot form functional relays or myelinate regenerating axons, and, therefore, gene delivery targeted to Shwann cells and olfactory ensheathing cells will be more effecive[174]. The type of gene delivered may also influence the maximal tissue regeneration. Though most studies have investigated delivering genes encoding a growth factor, a gene encoding the transcription factor that induces the production of growth factor in the cell machinery presents a viable alternative. The latter ensures the expression of all natural splice variants, and may regulate multiple separate genes[176]. Extent of transgene expression and number of cells expressing transgene by maintaining the microenvironmental concentration of genes can determine normal or aberrant tissue formation[175]. Gene delivery strategies with spatial (μm to mm) and temporal (days to months) control on transgene expression that promote an appropriate concentration of tissue inductive factors must be developed to recreate environmental complexities present during tissue formation[179]. Porous scaffolds fabricated from synthetic polymers, such as PLG, provide opportunity to simultaneously or sequentially deliver plasmid DNA, cells and inductive proteins to create a temporal cascade of signaling that accelerates and enhances the extent of tissue formation[150,180]. Excellent reviews exist that address these guidelines in detail for tissue engineering with DNA releasing scaffolds[180-182]. A more thorough understanding of biological requirements for tissue regeneration would serve to identify better strategies for sustained gene delivery that can best enhance the regenerative process.

6 Conclusions

Delivering DNA vectors in a controlled fashion from polymeric devices provides a continuous supply of vectors to the targeted cells over a period of time, maintaining an elevated DNA concentration in the cellular microenvironment, increasing the transfection probability, and thus generating prolonged gene expression. These devices reduce the amount of genetic material needed for therapy by preventing its rapid loss from the tissue reducing the dose and number of repeated administration of the vectors. A localized injection/implantation of these controlled delivery systems in target tissues can further avoid escape of delivered vectors to distant sites which could otherwise lead to toxicity to untargeted cells and unwanted immune responses. Both synthetic and naturally occurring polymers have been used for synthesizing such devices. Though natural polymers allow encapsulation of DNA vectors under mild conditions and degrade into biocompatible components of extra-cellular matrix, they provide limited range of physical and chemical properties for modulating the release profile of the vectors. In contrast, synthetic polymers, like polyesters, allow selective manipulation of many of the device properties. Specific or non-specific interactions can be introduced between the polymers and entrapped vectors to enhance loading efficiency, and to either control the release rate of vectors to be taken up by cells surrounding the device, or to immobilize the on polymeric surface for preferential uptake by the infiltrating cells. While prolonged physiological responses from these sustained delivery devices have been demonstrated in a number of gene therapy applications, variables important to the efficacy of these systems are not well understood and are under intense investigation. Advancement of biomaterials with specific mechanical and surface properties that can be easily modulated to tailor the release profile of vectors is needed for improvement of controlled gene delivery technology. Studies that correlate properties of these sustained gene devices to the distribution, duration and amount of total protein expressed by the delivered genes in the cells will lead to a more rational molecular-scale design of such delivery systems. Development of an efficient gene delivery system that can keep the level of expressed protein within therapeutic range for extended periods of time will be instrumental in the realization of clinical gene therapy.

Table 1: *In vivo* studies that demonstrated physiological response after delivery of therapeutically relevant genes using polymeric sustained delivery systems

Delivery system	Polymer	Vector	Species/ location	Gene	Result
Nano-spheres	Gelatin	Plasmid	Mice/ Kidney	TGF-βR siRNA	Enhanced anti-fibrotic activity[183]
	PLGA	Plasmid	Rat/ femoral fracture	pSEAP	Sustained gene expression after 5 weeks[41]
	Chitosan	Plasmid	Mice/intranasal	RSV antigen	Significant reduction of viral titers[49]
Micro-spheres	PLGA	Plasmid	Oral	Insect protein luciferase	Systemic and mucosal antibody responses[57]
	Gelatin	Plasmid	Mice/ peritoneal cavity	NK4	Suppress tumor growth by inhibiting angiogenesis[54,55]
	Gelatin	Plasmid	Mice/ renal subcapsule	MMP-1	Low level of blood urea nitrogen (diabetic model)[78]
	Alginate	HAd5	Mice/ intranasally or i.p.	AdCA36lacZ	Circumvented vector-specific immune response[31]
Hydrogels	Agarose	Plasmid	Calves/ neck skin	pCIgD-BoHV-1 glycoprotein D	Bovine herpesvirus 1-specific immune response[119]
	PEG-PLGA-PEG	Plasmid	Diabetic mice/ dermal wound	TGF-β1	Wound healing, accelerated re-epithelialization[117]
Scaffolds	Evac	Plasmid	Mice/ vagina	LDH-C$_4$	Immunization[154]
	Collagen-gelatin	Plasmid	Rabbit/ knee	TGF-β1	Cartilage regeneration[139]
	Gelatin	Canarypox virus ALVAC	Mice/ intratumoral	IL-2, IL-12, TNF-α	Grwoth inhibition of tumor nodules[143]
	Collagen	Plasmid	Dog/ beagle tibia	pMat-1	Bone regeneration[9]
	Collagen	AdV	Rats/ dermal wound	PDGF-B	Granulation and vascularization[134]
	Collagen	PLL / plasmid	Optic nerve	FGF2, BDNF, NT3	Survival of axotomized RGCs[132]
	Collagen	Plasmid	Rabbit/ears (ischemic dermal ulcers)	PDGF	Granulation tissue, wound closure[133]

Abbreviations: HAd5- Human adenovirus type 5; PDGF- platelet-derived growth factor; RGC- retinal ganglion cells; FGF2- fibroblast growth factor; BDNF- brain-derived neurotrophic factor; NT3- neurotrophin-3; IL-2- murine cytokines interleukin 2, TNF-α- tumor necrosis factor-α

References

1. Kay MA, Liu D, Hoogerbrugge PM. Gene therapy. PNAS 1997;94(24):12744-12746.
2. Young LS, Searle PF, Onion D, Mautner V. Viral gene therapy strategies: from basic science to clinical application. The Journal of Pathology 2006;208(2):299-318.
3. Verma IM. A Tumultuous Year for Gene Therapy. Molecular Therapy 2000;2(5):415-416.
4. Boyce N. Trial halted after gene shows up in semen. Nature 2001;414(6865):677.
5. Check E. Gene therapy: A tragic setback. Nature 2002;420(6912):116-118.
6. El-Aneed A. An overview of current delivery systems in cancer gene therapy. Journal of Controlled Release 2004;94(1):1-14.
7. Henry CM. Gene delivery--without viruses. Chemical & Engineering News 2001;79(48):35-41.
8. Richardson TP, Peters MC, Ennett AB, Mooney DJ. Polymeric system for dual growth factor delivery. Nature Biotechnology 2001;19(11):1029-1034.
9. Bonadio J, Smiley E, Patil P, Goldstein S. Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. Nature Medicine 1999;5(7):753-759.
10. Ohno T, Gordon D, San H, Pompili VJ, Imperiale MJ, Nabel GJ, Nabel EG. Gene therapy for vascular smooth muscle cell proliferation after arterial injury. Science 1994;265(5173):781-784.
11. Lee WS, Jain MK, Arkonac BM, Zhang D, Shaw SY, Kashiki S, Maemura K, Lee SL, Hollenberg NK, Lee et a. Thy-1, a novel marker for angiogenesis upregulated by inflammatory cytokines. Circulation Research 1998;82(8):845-851.
12. Glover DJ, Lipps HJ, Jans DA. Towards safe, non-viral therapeutic gene expression in humans. Nature Reviews Genetics 2005;6(4):299-310.
13. Herweijer H, Wolff JA. Progress and prospects: Naked DNA gene transfer and therapy. Gene Therapy 2003;10(6):453-458.
14. Tripathy SK, Svensson EC, Black HB, Goldwasser E, Margalith M, Hobart PM, Leiden JM. Long-term expression of erythropoietin in the systemic circulation of mice after intramuscular injection of a plasmid DNA vector. Proceedings of the National Academy of Sciences of the United States of America 1996;93(20):10876-10880.
15. Tokui M, Takei I, Tashiro F, Shimada A, Kasuga A, Ishii M, Ishii T, Takatsu K, Saruta T, Miyazaki J-I. Intramuscular injection of expression plasmid DNA is an effective means of long-term systemic delivery of interleukin-5. Biochemical and Biophysical Research Communications 1997;233(2):527-531.
16. Laham RJ, Chronos NA, Pike M, Leimbach ME, Udelson JE, Pearlman JD, Pettigrew RI, Whitehouse MJ, Yoshizawa C, Simons M. Intracoronary basic fibroblast growth factor (FGF-2) in patients with severe ischemic heart disease: Results of a Phase I open-label dose escalation study. Journal of the American College of Cardiology 2000;36(7):2132-2139.
17. Lathi KG, Vale PR, Losordo DW, Cespedes RM, Symes JF, Esakof DD, Maysky M, Isner JM. Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease: Anesthetic management and results. Anesthesia and Analgesia 2001;92(1):19-25.
18. Braun S, Thioudellet C, Rodriguez P, Ali-Hadji D, Perraud F, Accart N, Balloul J-M, Halluard C, Acres B, Cavallini B and others. Immune rejection of human dystrophin following intramuscular injections of naked DNA in mdx mice. Gene Therapy 2000;7(17):1447-1457.
19. Ledley FD. Pharmaceutical Approach to Somatic Gene Therapy. Pharmaceutical Research 1996;V13(11):1595-1614.

20.   Klinman DM, Yamshchikov G, Ishigatsubo Y. Contribution of CpG motifs to the immunogenicity of DNA vaccines. Journal Of Immunology (Baltimore, Md.: 1950) 1997;158(8):3635-3639.

21.   Yew NS, Zhao H, Wu I-H, Song A, Tousignant JD, Przybylska M, Cheng SH. Reduced Inflammatory Response to Plasmid DNA Vectors by Elimination and Inhibition of Immunostimulatory CpG Motifs. Molecular Therapy 2000;1(3):255-262.

22.   Yew NS, Zhao H, Przybylska M, Wu I-H, Tousignant JD, Scheule RK, Cheng SH. CpG-Depleted Plasmid DNA Vectors with Enhanced Safety and Long-Term Gene Expression in Vivo. Molecular Therapy 2002;5(6):731-738.

23.   Roy K, Wang D, Hedley ML, Barman SP. Gene delivery with in-situ crosslinking polymer networks generates long-term systemic protein expression. Molecular Therapy 2003;7(3):401-408.

24.   Thomas M, Klibanov AM. Non-viral gene therapy: polycation-mediated DNA delivery. Applied Microbiology and Biotechnology 2003;62(1):27-34.

25.   Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nat Rev Drug Discov 2005;4(7):581-593.

26.   Simoes S, Filipe A, Faneca H, Mano M, Penacho N, Duzgunes N, Pedroso de Lima M. Cationic liposomes for gene delivery. Expert Opinion on Drug Delivery 2005;2(2):237-254.

27.   Putnam D. Polymers for gene delivery across length scales. Nature Materials 2006;5:439–451.

28.   Karmali PP, Chaudhuri A. Cationic liposomes as non-viral carriers of gene medicines: Resolved issues, open questions, and future promises. Medicinal Research Reviews 2006;9999(9999):n/a.

29.   Boulaiz H, Marchal JA, Prados J, Melguizo C, Ara?nega A. Non-viral and viral vectors for gene therapy. Cellular and Molecular Biology 2005;51(1):3-22.

30.   Beer SJ, Matthews CB, Stein CS, Ross BD, Hilfinger JM, Davidson BL. Poly (lactic-glycolic) acid copolymer encapsulation of recombinant adenovirus reduces immunogenicity in vivo. Gene Therapy 1998;5(6):740-746.

31.   Sailaja G, HogenEsch H, North A, Hays J, Mittal SK. Encapsulation of recombinant adenovirus into alginate microspheres circumvents vector-specific immune response. Gene Therapy 2002;9(24):1722-1729.

32.   Matthews CB, Jenkins G, Hilfinger JM, Davidson BL. Poly-L-lysine improves gene transfer with adenovirus formulated in PLGA microspheres. Gene Therapy 1999;6(9):1558-1564.

33.   Panyam J, Labhasetwar V. Biodegradable nanoparticles for drug and gene delivery to cells and tissue. Advanced Drug Delivery Reviews 2003;55(3):329-347.

34.   Desai MP, Labhasetwar V, Walter E, Levy RJ, Amidon GL. The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent. Pharmaceutical Research 1997;14(11):1568-1573.

35.   Desai MP, Labhasetwar V, Amidon GL, Levy RJ. Gastrointestinal uptake of biodegradable microparticles: Effect of particle size. Pharmaceutical Research 1996;13(12):1838-1845.

36.   Sandor M, Mehta S, Harris J, Thanos C, Weston P, Marshall J, Mathiowitz E. Transfection of HEK cells via DNA-loaded PLGA and P(FASA) nanospheres. Journal of Drug Targeting 2002;10(6):497-506.

37.   Cohen H, Levy RJ, Gao J, Fishbein I, Kousaev V, Sosnowski S, Slomkowski S, Golomb G. Sustained delivery and expression of DNA encapsulated in polymeric nanoparticles. Gene Therapy 2000;7(22):1896-1905.

38.   Prabha S, Labhasetwar V. Nanoparticle-mediated wild-type p53 gene delivery results in sustained antiproliferative activity in breast cancer cells. Molecular Pharmaceutics 2004;1(3):211-219.

39.   Truong-Le VL, August JT, Leong KW. Controlled gene delivery by DNA-gelatin nanospheres. Human Gene Therapy 1998;9(12):1709-1717.

40.   Leong KW, Mao H-Q, Truong-Le VL, Roy K, Walsh SM, August JT. DNA-polycation nanospheres as non-viral gene delivery vehicles. Journal of Controlled Release 1998;53(1-3):183-193.

41.   Labhasetwar V, Bonadio J, Goldstein SA, Levy RJ. Gene transfection using biodegradable nanospheres: results in tissue culture and a rat osteotomy model. Colloids and Surfaces B: Biointerfaces 1999;16(1-4):281-290.

42.   Davda J, Labhasetwar V. Characterization of nanoparticle uptake by endothelial cells. International Journal of Pharmaceutics 2002;233(1-2):51-59.

43.   Guzman LA, Labhasetwar V, Song C, Jang Y, Lincoff AM, Levy R, Topol EJ. Local intraluminal infusion of biodegradable polymeric nanoparticles. A novel approach for prolonged drug delivery after balloon angioplasty. Circulation 1996;94(6):1441-1448.

44.   Panyam J, Zhou W-Z, Prabha S, Sahoo SK, Labhasetwar V. Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: Implications for drug and gene delivery. FASEB Journal 2002;16(10):1217-1226.

45.   Prabha S, Zhou W-Z, Panyam J, Labhasetwar V. Size-dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles. International Journal of Pharmaceutics 2002;244(1-2):105-115.

46.   Labhasetwar V, Bonadio J, Goldstein S, Chen W, Levy RJ. A DNA controlled-release coating for gene transfer: Transfection in skeletal and cardiac muscle. Journal of Pharmaceutical Sciences 1998;87(11):1347-1350.

47.   Prabha S, Labhasetwar V. Nanoparticle-Mediated Wild-Type p53 Gene Delivery Results in Sustained Antiproliferative Activity in Breast Cancer Cells. Mol. Pharmaceutics 2004;1(3):211-219.

48.   Thanou M, Verhoef JC, Junginger HE. Chitosan and its derivatives as intestinal absorption enhancers. Advanced Drug Delivery Reviews 2001;50(Supplement 1):S91-S101.

49.   Mohapatra SS. Mucosal gene expression vaccine: a novel vaccine strategy for respiratory syncytial virus. Pediatric Infectious Disease Journal 2003;22(2):Supplement:S100-S104.

50.   Kaul G, Amiji M. Tumor-Targeted Gene Delivery Using Poly(Ethylene Glycol)-Modified Gelatin Nanoparticles: In Vitro and in Vivo Studies. Pharmaceutical Research 2005;V22(6):951-961.

51.   Kommareddy S, Amiji M. Biodistribution and pharmacokinetic analysis of long-circulating thiolated gelatin nanoparticles following systemic administration in breast cancer-bearing mice. Journal of Pharmaceutical Sciences 2006;9999(9999):n/a.

52.   Wang J, Zhang P-C, Mao H-Q, Leong KW. Enhanced gene expression in mouse muscle by sustained release of plasmid DNA using PPE-EA as a carrier. Gene Therapy 2002;9(18):1254-1261.

53.   Ribeiro S, Hussain N, Florence AT. Release of DNA from dendriplexes encapsulated in PLGA nanoparticles. International Journal of Pharmaceutics 2005;298(2):354-360.

54.   Kushibiki T, Matsumoto K, Nakamura T, Tabata Y. Suppression of tumor metastasis by NK4 plasmid DNA released from cationized gelatin. Gene Therapy 2004;11(15):1205-1214.

55.   Kushibiki T, Matsumoto K, Nakamura T, Tabata Y. Suppression of the Progress of Disseminated Pancreatic Cancer Cells by NK4 Plasmid DNA Released from Cationized Gelatin Microspheres. Pharmaceutical Research 2004;V21(7):1109-1118.

56.   Mathiowitz E, Jacob JS, Jong YS, Carino GP, Chickering DE, Chaturvedi P, Santos CA, Vijayaraghavan K, Montgomery S, Bassett M and others. Biologically erodable microspheres as potential oral drug delivery systems. Nature 1997;386(6623):410-414.

57.   Jones DH, Corris S, McDonald S, Clegg JCS, Farrar GH. Poly(-lactide-co-glycolide)-encapsulated plasmid DNA elicits systemic and mucosal antibody responses to encoded protein after oral administration. Vaccine 1997;15(8):814-817.

58.   Aggarwal N, HogenEsch H, Guo P, North A, Suckow M, Mittal SK. Biodegradable alginate microspheres as a delivery system for naked DNA. Canadian Journal Of Veterinary Research 1999;63(2):148-152.

59.   Hedley ML, Curley J, Urban R. Microspheres containing plasmid-encoded antigens elicit cytotoxic T-cell responses. Nature Medicine 1998;4(3):365-368.

60.   Ruedl C, Wolf H. Features of oral immunization. International Archives of Allergy and Immunology 1995;108(4):334-339.

61.   Elson CO. In defense of mucosal surfaces: Regulation and manipulation of the mucosal immune system. Advances in Experimental Medicine and Biology 1997;412:373-385.

62.   Chickering DE, Mathiowitz E. Bioadhesive microspheres: I. A novel electrobalance-based method to study adhesive interactions between individual microspheres and intestinal mucosa. Journal of Controlled Release 1995;34(3):251-262.

63.   Khan A, Benboubetra M, Sayyed PZ, Ng KW, Fox S, Beck G, Benter IF, Akhtar S. Sustained Polymeric Delivery of Gene Silencing Antisense ODNs, siRNA, DNAzymes and Ribozymes: In Vitro and In Vivo Studies. 2004;12(6):393 - 404.

64.   Thomasin C, Corradin G, Men Y, Merkle HP, Gander B. Tetanus toxoid and synthetic malaria antigen containing poly(lactide)/poly(lactide-co-glycolide) microspheres: importance of polymer degradation and antigen release for immune response. Journal of Controlled Release 1996;41(1-2):131-145.

65.   Gander B, Wehrli E, Alder R, Merkle HP. Quality improvement of spray-dried, protein-loaded D,L-PLA microspheres by appropriate polymer solvent selection. Journal Of Microencapsulation 1995;12(1):83-97.

66.   Tinsley-Bown AM, Fretwell R, Dowsett AB, Davis SL, G.H. Farrar. Formulation of poly(-lactic-co-glycolic acid) microparticles for rapid plasmid DNA delivery. Journal of Controlled Release 2000;66(2-3):229-241.

67.   Luo D, Woodrow-Mumford K, Belcheva N, Saltzman WM. Controlled DNA Delivery Systems. Pharmaceutical Research 1999;V16(8):1300-1308.

68.   C. S. Lengsfeld TJA. Shear-induced degradation of plasmid DNA. Journal of Pharmaceutical Sciences 2002;91(7):1581-1589.

69.   Jones DH, Partidos CD, Steward MW, Farrar GH. Oral delivery of poly(lactide-co-glycolide) encapsulated vaccines. Behring Institute Mitteilungen 1997(98):220-228.

70.   Shuichi Ando DP, Daniel W. Pack, Robert Langer,. PLGA microspheres containing plasmid DNA: Preservation of supercoiled DNA via cryopreparation and carbohydrate stabilization. Journal of Pharmaceutical Sciences 1999;88(1):126-130.

71.   Wang D, Robinson DR, Kwon GS, Samuel J. Encapsulation of plasmid DNA in biodegradable poly(,-lactic-co-glycolic acid) microspheres as a novel approach for immunogene delivery. Journal of Controlled Release 1999;57(1):9-18.

72.   Jang J-H, Shea LD. Intramuscular delivery of DNA releasing microspheres: Microsphere properties and transgene expression. Journal of Controlled Release 2006;112(1):120-128.

73.   Walter E, Dreher D, Kok M, Thiele L, Kiama SG, Gehr P, Merkle HP. Hydrophilic poly(-lactide-co-glycolide) microspheres for the delivery of DNA to human-derived macrophages and dendritic cells. Journal of Controlled Release 2001;76(1-2):149-168.

74.   Walter E, Moelling K, Pavlovic J, Merkle HP. Microencapsulation of DNA using poly(-lactide-co-glycolide): stability issues and release characteristics. Journal of Controlled Release 1999;61(3):361-374.

75. Diez S, Tros de Ilarduya C. Versatility of biodegradable poly(d,l-lactic-co-glycolic acid) microspheres for plasmid DNA delivery. European Journal of Pharmaceutics and Biopharmaceutics 2006;63(2):188-197.

76. Butler SM, Tracy MA, Tilton RD. Adsorption of serum albumin to thin films of poly(lactide-co-glycolide). Journal of Controlled Release 1999;58(3):335-347.

77. Young S, Wong M, Tabata Y, Mikos AG. Gelatin as a delivery vehicle for the controlled release of bioactive molecules. Journal of Controlled Release 2005;109(1-3):256-274.

78. Aoyama T, Yamamoto S, Kanematsu A, Ogawa O, Tabata Y. Local delivery of matrix metalloproteinase gene prevents the onset of renal sclerosis in streptozotocin-induced diabetic mice. Tissue Engineering 2003;9(16):1289-99.

79. Matsumoto G, Kushibiki T, Kinoshita Y, Lee U, Omi Y, Kubota E, Tabata Y. Cationized gelatin delivery of a plasmid DNA expressing small interference RNA for VEGF inhibits murine squamous cell carcinoma. Cancer Science 2006;97(4):313-321.

80. Akbuga J, Aral C, Ozbas-Turan S, Kabasakal L, Keyer-Uysal M. Transfection efficiency of chitosan microspheres: Effect of DNA topology. S.T.P. Pharma Sciences 2003;13(2):99-103.

81. Ozbas-Turan S, Aral C, Kabasakal L, Keyer-Uysal M, Akbuga J. Co-encapsulation of two plasmids in chitosan microspheres as a non-viral gene delivery vehicle. J Pharm Pharm Sci 2003;6(1):27-32.

82. Janes KA, Calvo P, Alonso MJ. Polysaccharide colloidal particles as delivery systems for macromolecules. Advanced Drug Delivery Reviews 2001;47(1):83-97.

83. Aral C, Ozbas-Turan S, Kabasakal L, Keyer-Uysal M, Akbuga J. Studies of effective factors of plasmid DNA-loaded chitosan microspheres I. Plasmid size, chitosan concentration and plasmid addition techniques. S.T.P. Pharma Sciences 2000;10(1):83-88.

84. Berthold A, Cremer K, Kreuter J. Preparation and characterization of chitosan microspheres as drug carrier for prednisolone sodium phosphate as model for anti-inflammatory drugs. Journal of Controlled Release 1996;39(1):17-25.

85. Akbuga J, Ozbas-Turan S, Erdogan N. Plasmid-DNA loaded chitosan microspheres for in vitro IL-2 expression. European Journal of Pharmaceutics and Biopharmaceutics 2004;58(3):501-507.

86. Yun YH, Goetz DJ, Yellen P, Chen W. Hyaluronan microspheres for sustained gene delivery and site-specific targeting. Biomaterials 2004;25(1):147-157.

87. Lameiro MH, Malpique R, Silva AC, Alves PM, Melo E. Encapsulation of adenoviral vectors into chitosan-bile salt microparticles for mucosal vaccination. Journal of Biotechnology 2006;126(2):152-162.

88. Shiba H, Okamoto T, Futagawa Y, Misawa T, Yanaga K, Ohashi T, Eto Y. Adenovirus Vector-Mediated Gene Transfer Using Degradable Starch Microspheres for Hepatocellular Carcinoma in Rats. Journal of Surgical Research 2006;133(2):193-196.

89. Ismail FA, Napaporn J, Hughes JA, Brazeau GA. In situ gel formulations for delivery: Release and myotoxicity studies. Pharmaceutical Development and Technology 2000;5(3):391-397.

90. Ruel-Gariepy E, Leroux J-C. In situ-forming hydrogels--review of temperature-sensitive systems. European Journal of Pharmaceutics and Biopharmaceutics 2004;58(2):409-426.

91. Srividya B, Cardoza RM, Amin PD. Sustained ophthalmic delivery of ofloxacin from a pH triggered in situ gelling system. Journal of Controlled Release 2001;73(2-3):205-211.

92. Seong J-Y, Jun YJ, Kim BM, Park YM, Sohn YS. Synthesis and characterization of biocompatible poly(organophosphazenes) aiming for local delivery of protein drugs. International Journal of Pharmaceutics 2006;314(1):90-96.

93.   Determan MD, Cox JP, Mallapragada SK. Drug release from pH-responsive thermogelling pentablock copolymers. Journal of Biomedical Materials Research Part A 2006;9999(9999):NA.
94.   Lewis P. Stratton AD, Mark C. Manning, John F. Carpenter,. Drug delivery matrix containing native protein precipitates suspended in a poloxamer gel. Journal of Pharmaceutical Sciences 1997;86(9):1006-1010.
95.   Lin H-R, Sung KC, Vong W-J. In Situ Gelling of Alginate/Pluronic Solutions for Ophthalmic Delivery of Pilocarpine. Biomacromolecules 2004;5(6):2358-2365.
96.   Kang GD, Cheon SH, Song S-C. Controlled release of doxorubicin from thermosensitive poly(organophosphazene) hydrogels. International Journal of Pharmaceutics 2006;319(1-2):29-36.
97.   Jeong B, Bae YH, Kim SW. Drug release from biodegradable injectable thermosensitive hydrogel of PEG-PLGA-PEG triblock copolymers. Journal of Controlled Release 2000;63(1-2):155-163.
98.   Cappello J, Crissman JW, Crissman M, Ferrari FA, Textor G, Wallis O, Whitledge JR, Zhou X, Burman D, Aukerman L and others. In-situ self-assembling protein polymer gel systems for administration, delivery, and release of drugs. Journal of Controlled Release 1998;53(1-3):105-117.
99.   Megeed Z, Cappello J, Ghandehari H. Controlled Release of Plasmid DNA from a Genetically Engineered Silk-Elastinlike Hydrogel. Pharmaceutical Research 2002;V19(7):954-959.
100.   Jeong B, Kim SW, Bae YH. Thermosensitive sol-gel reversible hydrogels. Advanced Drug Delivery Reviews 2002;54(1):37-51.
101.   Kissel T, Li Y, Unger F. ABA-triblock copolymers from biodegradable polyester A-blocks and hydrophilic poly(ethylene oxide) B-blocks as a candidate for in situ forming hydrogel delivery systems for proteins. Advanced Drug Delivery Reviews 2002;54(1):99-134.
102.   Harland RS, Peppas NA. Drug transport in and release from controlled delivery devices of hydrophilic/hydrophobic block and graft copolymers. European Journal of Pharmaceutics and Biopharmaceutics 1993;39(6):229-233.
103.   Strappe PM, Hampton DW, Cachon-Gonzalez B, Fawcett JW, Lever A. Delivery of a lentiviral vector in a Pluronic F127 gel to cells of the central nervous system. European Journal of Pharmaceutics and Biopharmaceutics 2005;61(3):126-133.
104.   Feldman LJ, Pastore CJ, Aubailly N, Kearney M, Chen D, Perricaudet M, Steg PG, Isner JM. Improved efficiency of arterial gene transfer by use of poloxamer 407 as a vehicle for adenoviral vectors. Gene Therapy 1997;4(3):189-198.
105.   Siow RCM, Mallawaarachchi CM, Weissberg PL. Migration of adventitial myofibroblasts following vascular balloon injury: insights from in vivo gene transfer to rat carotid arteries. Cardiovascular Research 2003;59(1):212-221.
106.   Chun KW, Lee JB, Kim SH, Park TG. Controlled release of plasmid DNA from photo-cross-linked pluronic hydrogels. Biomaterials 2005;26(16):3319-3326.
107.   Xiong XY, Tam KC, Gan LH. Polymeric Nanostructures for Drug Delivery Applications Based on Pluronic Copolymer Systems. J Nanosci Nanotechnol 2006;113(2):164-72.
108.   Gilbert JC, Richardson JL, Davies MC, Palin KJ, Hadgraft J. The effect of solutes and polymers on the gelation properties of pluronic F-127 solutions for controlled drug delivery. Journal of Controlled Release 1987;5(2):113-118.
109.   Suketu D. Desai JB. In vitro evaluation of pluronic F127-based controlled-release ocular delivery systems for pilocarpine. Journal of Pharmaceutical Sciences 1998;87(2):226-230.

110. Choi H-G, Jung J-H, Ryu J-M, Yoon S-J, Oh Y-K, Kim C-K. Development of in situ-gelling and mucoadhesive acetaminophen liquid suppository. International Journal of Pharmaceutics 1998;165(1):33-44.
111. Jeong-Sook Park Y-KO, Ho Yoon, Jung Mogg Kim, Chong-Kook Kim,. In situ gelling and mucoadhesive polymer vehicles for controlled intranasal delivery of plasmid DNA. Journal of Biomedical Materials Research 2002;59(1):144-151.
112. Determan MD, Cox JP, Seifert S, Thiyagarajan P, Mallapragada SK. Synthesis and characterization of temperature and pH responsive pentablock copolymers. Polymer 2005;46(18):6933-6946.
113. Determan MD, Chieh-Tsung L, Thiyagarajan P, Mallapragada SK. Self-assembly of Temperature and pH-Responsive Pentablock Copolymers. Polymeric Materials:Science and Engineering 2005;23:808.
114. Determan MD, Guo L, Thiyagarajan P, Mallapragada SK. Supramolecular Self-Assembly of Multiblock Copolymers in Aqueous Solution. Langmuir 2006;22(4):1469-1473.
115. Agarwal A, Unfer R, Mallapragada SK. Novel cationic pentablock copolymers as non-viral vectors for gene therapy. Journal of Controlled Release 2005;103(1):245-258.
116. Agarwal A, Unfer R, Mallapragada SK. Investigation of in vitro biocompatibility of novel pentablock copolymers for gene delivery. Journal of Biomedical Materials Research Part A 2006;9999(9999):NA.
117. Lee P-Y, Li Z, Huang L. Thermosensitive Hydrogel as a Tgf-ß1 Gene Delivery Vehicle Enhances Diabetic Wound Healing. Pharmaceutical Research 2003;20(12):1995-2000.
118. Meilander-Lin NJ, Cheung PJ, Wilson DL, Bellamkonda RV. Sustained in Vivo Gene Delivery from Agarose Hydrogel Prolongs Nonviral Gene Expression in Skin. Tissue Engineering 2005;11(3-4):546-555.
119. Toussaint JF, Dubois A, Dispas M, Paquet D, Letellier C, Kerkhofs P. Delivery of DNA vaccines by agarose hydrogel implants facilitates genetic immunization in cattle. Vaccine;In Press, Corrected Proof.
120. Fukunaka Y, Iwanaga K, Morimoto K, Kakemi M, Tabata Y. Controlled release of plasmid DNA from cationized gelatin hydrogels based on hydrogel degradation. Journal of Controlled Release 2002;80(3):333-343.
121. Kushibiki T, Tomoshige R, Fukunaka Y, Kakemi M, Tabata Y. In vivo release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents. Journal of Controlled Release 2003;90(2):207-216.
122. Kasper FK, Seidlits SK, Tang A, Crowther RS, Carney DH, Barry MA, Mikos AG. In vitro release of plasmid DNA from oligo(poly(ethylene glycol) fumarate) hydrogels. Journal of Controlled Release 2005;104(3):521-539.
123. Kasper FK, Kushibiki T, Kimura Y, Mikos AG, Tabata Y. In vivo release of plasmid DNA from composites of oligo(poly(ethylene glycol)fumarate) and cationized gelatin microspheres. Journal of Controlled Release 2005;107(3):547-561.
124. Krsko P, Saaem I, Clancy R, Geller H, Soteropoulos P, Libera M. E-beam-patterned hydrogels to control nanoscale surface bioactivity. 2005 2005///.
125. Quick DJ, Anseth KS. DNA delivery from photocrosslinked PEG hydrogels: encapsulation efficiency, release profiles, and DNA quality. Journal of Controlled Release 2004;96(2):341-351.
126. Quick DJ, Anseth KS. Gene Delivery in Tissue Engineering: A Photopolymer Platform to Coencapsulate Cells and Plasmid DNA. Pharmaceutical Research 2003;V20(11):1730-1737.
127. Yasuhide Nakayama KJ-Y, Shogo Nishi, Hikaru Ueno, Takehisa Matsuda,. Development of high-performance stent: Gelatinous photogel-coated stent that permits drug delivery and gene transfer. Journal of Biomedical Materials Research 2001;57(4):559-566.

128. Quick DJ, Macdonald KK, Anseth KS. Delivering DNA from photocrosslinked, surface eroding polyanhydrides. Journal of Controlled Release 2004;97(2):333-343.

129. Fang J, Zhu Y-Y, Smiley E, Bonadio J, Rouleau JP, Goldstein SA, McCauley LK, Davidson BL, Roessler BJ. Stimulation of new bone formation by direct transfer of osteogenic plasmid genes. PNAS 1996;93(12):5753-5758.

130. Chen H, Frankenburg EP, Goldstein SA, McCauley LK. Combination of local and systemic parathyroid hormone enhances bone regeneration. Clinical Orthopaedics And Related Research 2003(416):291-302.

131. Pascher A, Palmer GD, Steinert A, Oligino T, Gouze E, Gouze J-N, Betz O, Spector M, Robbins PD, Evans CH and others. Gene delivery to cartilage defects using coagulated bone marrow aspirate. Gene Therapy 2004;11(2):133-141.

132. Berry M, Gonzalez AM, Clarke W, Greenlees L, Barrett L, Tsang W, Seymour L, Bonadio J, Logan A, Baird A. Sustained effects of gene-activated matrices after CNS injury. Molecular and Cellular Neuroscience 2001;17(4):706-716.

133. Tyrone JW, Mogford JE, Chandler LA, Ma C, Xia Y, Pierce GF, Mustoe TA. Collagen-embedded platelet-derived growth factor DNA plasmid promotes wound healing in a dermal ulcer model. Journal of Surgical Research 2000;93(2):230-236.

134. Chandler LA, Ma C, Gonzalez AM, Doukas J, Nguyen T, Pierce GF, Phillips ML. Matrix-enabled gene transfer for cutaneous wound repair. Wound Repair and Regeneration 2000;8(6):473-479.

135. Pakkanen TM, Laitinen M, Hippeläinen M, Hiltunen MO, Alhava E, Ylä-Herttuala S. Periadventitial lacZ gene transfer to pig carotid arteries using a biodegradable collagen collar or a wrap of collagen sheet with adenoviruses and plasmid-liposome complexes. The Journal of Gene Medicine 2000;2(1):52-60.

136. Christman KL, Fang Q, Yee MS, Johnson KR, Sievers RE, Lee RJ. Enhanced neovasculature formation in ischemic myocardium following delivery of pleiotrophin plasmid in a biopolymer. Biomaterials 2005;26(10):1139-1144.

137. Scherer F, Schillinger U, Putz U, Stemberger A, Plank C. Nonviral vector loaded collagen sponges for sustained gene delivery in vitro and in vivo. The Journal of Gene Medicine 2002;4(6):634-643.

138. Ochiya T, Takahama Y, Nagahara S, Sumita Y, Hisada A, Itoh H, Nagai Y, Terada M. New delivery system for plasmid DNA in vivo using atelocollagen as a carrier material: the Minipellet. Nat Med 1999;5(6):707-710.

139. Guo T, Zhao J, Chang J, Ding Z, Hong H, Chen J, Zhang J. Porous chitosan-gelatin scaffold containing plasmid DNA encoding transforming growth factor-[beta]1 for chondrocytes proliferation. Biomaterials 2006;27(7):1095-1103.

140. Samuel RE, Lee CR, Ghivizzani SC, Evans CH, Yannas IV, Olsen BR, Spector M. Delivery of plasmid DNA to articular chondrocytes via novel collagen-glycosaminoglycan matrices. Human Gene Therapy 2002;13(7):791-802.

141. Kim A, Checkla DM, Dehazya P, Chen W. Characterization of DNA-hyaluronan matrix for sustained gene transfer. Journal of Controlled Release 2003;90(1):81-95.

142. Kim AP, Yellen P, Yun YH, Azeloglu E, Chen W. Delivery of a vector encoding mouse hyaluronan synthase 2 via a crosslinked hyaluronan film. Biomaterials 2005;26(13):1585-1593.

143. Siemens DR, Austin JC, Hedican SP, Tartaglia J, Ratliff TL. Viral Vector Delivery in Solid-State Vehicles: Gene Expression in a Murine Prostate Cancer Model. J. Natl. Cancer Inst. 2000;92(5):403-412.

144. Chen T, Embree HD, Brown EM, Taylor MM, Payne GF. Enzyme-catalyzed gel formation of gelatin and chitosan: potential for in situ applications. Biomaterials 2003;24(17):2831-2841.

145.   Rubin AL, Stenzel KH, Miyata T, White MJ, Dunn M. Collagen as a Vehicle for Drug
        Delivery: Preliminary Report. J Clin Pharmacol New Drugs 1973;13(8):309-312.
146.   Sano A, Maeda M, Nagahara S, Ochiya T, Honma K, Itoh H, Miyata T, Fujioka K.
        Atelocollagen for protein and gene delivery. Advanced Drug Delivery Reviews
        2003;55(12):1651-1677.
147.   Luu YK, Kim K, Hsiao BS, Chu B, Hadjiargyrou M. Development of a nanostructured DNA
        delivery scaffold via electrospinning of PLGA and PLA-PEG block copolymers. Journal of
        Controlled Release 2003;89(2):341-353.
148.   Jang J-H, Shea LD. Controllable delivery of non-viral DNA from porous scaffolds. Journal of
        Controlled Release 2003;86(1):157-168.
149.   Huang Y-C, Riddle K, Rice KG, Mooney DJ. Long-Term In Vivo Gene Expression via
        Delivery of PEI DNA Condensates from Porous Polymer Scaffolds. Human Gene Therapy
        2005;16(5):609-617.
150.   Huang Y-C, Kaigler D, Rice KG, Krebsbach PH, Mooney DJ. Combined Angiogenic and
        Osteogenic Factor Delivery Enhances Bone Marrow Stromal Cell-Driven Bone Regeneration.
        Journal of Bone and Mineral Research 2005;20(5):848-857.
151.   Liang D, Luu YK, Kim K, Hsiao BS, Hadjiargyrou M, Chu B. In vitro non-viral gene
        delivery with nanofibrous scaffolds. Nucl. Acids Res. 2005;33(19):e170-.
152.   Moriah Nof LDS. Drug-releasing scaffolds fabricated from drug-loaded microspheres.
        Journal of Biomedical Materials Research 2002;59(2):349-356.
153.   Shea L, Smiley E, Bonadio J, Mooney DJ. DNA delivery from polymer matrices for tissue
        engineering. Nature Biotechnology 1999;17(6):551-554.
154.   Shen H, Goldberg E, Saltzman WM. Gene expression and mucosal immune responses after
        vaginal DNA immunization in mice using a controlled delivery matrix. Journal of Controlled
        Release 2003;86(2-3):339-348.
155.   Livingston JB, Lu S, Robinson HL, Anderson DJ. The induction of mucosal immunity in the
        female genital tract using gene-gun technology. Part 1: Antigen expression. Annals of the
        New York Academy of Sciences 1995;772:265-267.
156.   Livingston JB, Lu S, Robinson H, Anderson DJ. Immunization of the female genital tract
        with a DNA-based vaccine. Infection And Immunity 1998;66(1):322-329.
157.   Radomsky ML, Whaley KJ, Cone RA, Saltzman WM. Controlled vaginal delivery of
        antibodies in the mouse. BIOL. REPROD. 1992;47(1):133-140.
158.   Bielinska AU, Yen A, Wu HL, Zahos KM, Sun R, Weiner ND, Baker Jr. JR, Roessler BJ.
        Application of membrane-based dendrimer/DNA complexes for solid phase transfection in
        vitro and in vivo. Biomaterials 2000;21(9):877-887.
159.   Kofron MD, Laurencin CT. Development of a calcium phosphate co-precipitate/poly(lactide-
        co- glycolide) DNA delivery system: Release kinetics and cellular transfection studies.
        Biomaterials 2004;25(13):2637-2643.
160.   Segura T, Anderson BC, Chung PH, Webber RE, Shull KR, Shea LD. Crosslinked hyaluronic
        acid hydrogels: a strategy to functionalize and pattern. Biomaterials 2005;26(4):359-371.
161.   Segura T, Volk MJ, Shea LD. Substrate-mediated DNA delivery: Role of the cationic
        polymer structure and extent of modification. Journal of Controlled Release 2003;93(1):69-
        84.
162.   Segura T, Shea LD. Surface-tethered DNA complexes for enhanced gene delivery.
        Bioconjugate Chemistry 2002;13(3):621-629.
163.   Abrahams JM, Song C, DeFelice S, Grady MS, Diamond SL, Levy RJ. Endovascular
        Microcoil Gene Delivery Using Immobilized Anti-adenovirus Antibody for Vector Tethering.
        Stroke 2002;33(5):1376-1382.

164.   Klugherz BD, Song C, Defelice S, Cui X, Lu Z, Connolly J, Travis Hinson J, Wilensky RL, Levy RJ. Gene delivery to pig coronary arteries from stents carrying antibody-tethered adenovirus. Human Gene Therapy 2002;13(3):443-454.

165.   Pandori MW, Hobson DA, Sano T. Adenovirus-Microbead Conjugates Possess Enhanced Infectivity: A New Strategy for Localized Gene Delivery. Virology 2002;299(2):204-212.

166.   Parrott MB, Barry MA. Metabolic Biotinylation of Secreted and Cell Surface Proteins from Mammalian Cells. Biochemical and Biophysical Research Communications 2001;281(4):993-1000.

167.   Parrott MB, Barry MA. Metabolic Biotinylation of Recombinant Proteins in Mammalian Cells and in Mice. Molecular Therapy 2000;1(1):96-104.

168.   Stachelek SJ, Song C, Alferiev I, Defelice S, Cui X, Connolly JM, Bianco RW, Levy RJ. Localized gene delivery using antibody tethered adenovirus from polyurethane heart valve cusps and intra-aortic implants. Gene Therapy 2004;11(1):15-24.

169.   Cavanagh HMA, Dingwall D, Steel J, Benson J, Burton M. Cell contact dependent extended release of adenovirus by microparticles in vitro. Journal of Virological Methods 2001;95(1-2):57-64.

170.   Mah C, Fraites J, Thomas J., Zolotukhin I, Song S, Flotte TR, Dobson J, Batich C, Byrne BJ. Improved Method of Recombinant AAV2 Delivery for Systemic Targeted Gene Therapy. Molecular Therapy 2002;6(1):106-112.

171.   Dropulic B, June CH. Gene-Based Immunotherapy for Human Immunodeficiency Virus Infection and Acquired Immunodeficiency Syndrome
doi:10.1089/hum.2006.17.577. Human Gene Therapy 2006;17(6):577-588.

172.   Mason DJ, Genevar PG, Itaka K, Alini M. Glutamate signalling and its potential application to tissue engineering of bone. European Cells and Materials 2004;7:12-26.

173.   Geiger F, Bertram H, Berger I, Lorenz H, Wall O, Eckhardt C, Simank H-G, Richter W. Vascular Endothelial Growth Factor Gene-Activated Matrix (VEGF165-GAM) Enhances Osteogenesis and Angiogenesis in Large Segmental Bone Defects. Journal of Bone and Mineral Research 2005;20(11):2028-2035.

174.   Murray M, Fischer I. Transplantation and gene therapy: Combined approaches for repair of spinal cord injury. Neuroscientist 2001;7(1):28-41.

175.   Ozawa CR, Banfi A, Glazer NL, Thurston G, Springer ML, Kraft PE, McDonald DM, Blau HM. Microenvironmental VEGF concentration, not total dose, determines a threshold between normal and aberrant angiogenesis. Journal of Clinical Investigation 2004;113(4):516-527.

176.   Rebar EJ, Huang Y, Hickey R, Nath AK, Meoli D, Nath S, Chen B, Xu L, Liang Y, Jamieson AC and others. Induction of angiogenesis in a mouse model using engineered transcription factors. Nature Medicine 2002;8(12):1427-1432.

177.   Murphy WL, Mooney DJ. Controlled delivery of inductive proteins, plasmid DNA and cells from tissue engineering matrices. Journal of Periodontal Research 1999;34(7):413-419.

178.   Kong HJ, Liu J, Riddle K, Matsumoto T, Leach K, Mooney DJ. Non-viral gene delivery regulated by stiffness of cell adhesion substrates. Nature Materials 2005;4(6):460-464.

179.   Saltzman WM, Olbricht WL. Building drug delivery into tissue engineering. Nature Reviews Drug Discovery 2002;1(3):177-186.

180.   Salvay DM, Shea LD. Inductive tissue engineering with protein and DNA-releasing scaffolds. Molecular BioSystems 2006;2(1):36-48.

181.   Storrie H, Mooney DJ. Sustained delivery of plasmid DNA from polymeric scaffolds for tissue engineering. Advanced Drug Delivery Reviews 2006;58(4):500-514.

182.   Jang J-H, Houchin TL, Shea LD. Gene delivery from polymer scaffolds for tissue engineering. Expert Review of Medical Devices 2004;1(1):127-138.

183.   Kushibiki T, Nagata-Nakajima N, Sugai M, Shimizu A, Tabata Y. Enhanced anti-fibrotic activity of plasmid DNA expressing small interference RNA for TGF-[beta] type II receptor for a mouse model of obstructive nephropathy by cationized gelatin prepared from different amine compounds. Journal of Controlled Release 2006;110(3):610-617.
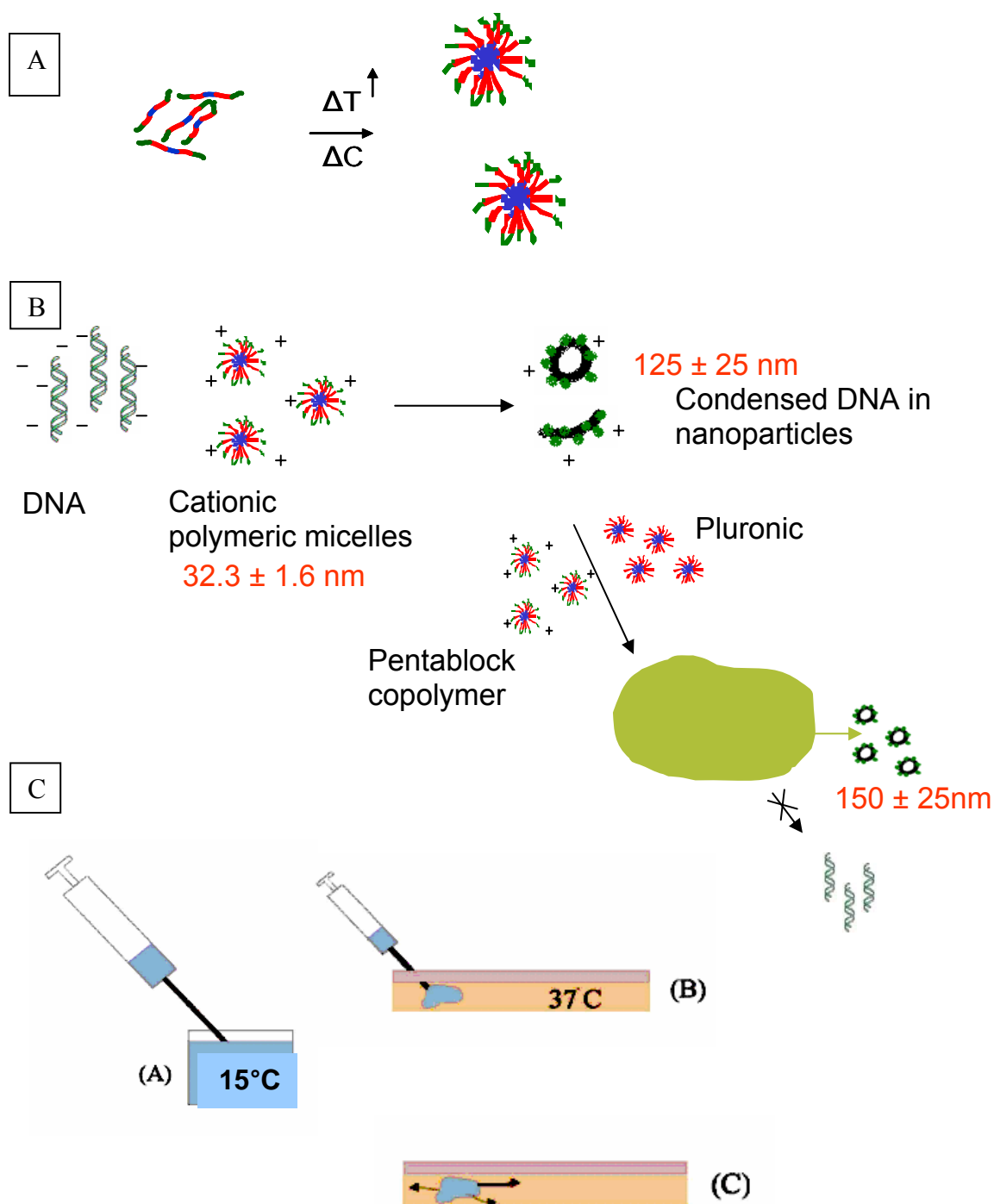
Fig. 1: (A) Pentablock copolymers exist as micelles in aqueous solutions, with a hydrophobic core and a hydrophilic corona. (B) The cationic polymers condense DNA in solution into nanoplexes that are easily taken up by the cells via endocytosis. Polyplex solutions

containing more than 15 wt% of copolymer, free flowing at 15°C or below, self-assemble to form reversible strong elastic hydrogels at physiological temperatures- driven by the packing of polymeric micelles in a crystalline lattice. (C) The aqueous pharmaceutical formulation can be injected non-invasively into localized tissues/cavities using syringe and 27 gauge needle where a hydrogel depot of the polymer/DNA complexes is formed. The hydrogel can dissolve in tissue fluid to release nanoplexes for prolonged period of times, circumventing repeated administration of bolus dose.

CHAPTER 3

NOVEL CATIONIC PENTABLOCK COPOLYMERS AS NON-VIRAL VECTORS FOR GENE THERAPY

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Abstract

New cationic pentablock copolymers of poly(diethylaminoethylmethacrylate) (PDEAEM), poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO) -(PDEAEM-b-PEO-b-PPO-b-PEO-b-PDEAEM)- synthesized in our laboratory were investigated for their potential as non-viral vectors for gene therapy. Agarose gel studies showed that the copolymers effectively condensed plasmid DNA to form polyplexes, and also protected plasmids against nuclease degradation. Light scattering and transmission electron microscopy were used to analyze the apparent size, molecular weight and morphology of these polyplexes. Lactate dehydrogenase assay was employed to find the cytotoxicity limits of the polymers and polyplexes on a human ovarian cancer cell line. The polymers showed much less cytotoxicity than commercially available ExGen 500 (linear polyethyleneimine). By changing the relative lengths of the blocks in the copolymers, it was found that the cytotoxicity of these copolymers could be tailored. The micellar structures of these copolymers in aqueous solutions and their pH-sensitive protonation were added advantages. *In-vitro* transfection efficiencies of the polymers using green fluorescent protein (pEGFP-N1)

and luciferase (pRL-CMV) reporter genes were found comparable to the commercially available Ex-Gen 500. Besides, aqueous solutions of these pentablock copolymers have been shown to exhibit thermodynamic phase transitions and thermoreversible gelation, a quality that could allow subcutaneous/intramuscular injections of these polymers for controlled gene delivery over time.

1. Introduction

Non-viral gene therapy using cationic copolymers has recently gained increased interest as a potential treatment for cancer and several other genetic diseases[1-3]. It can overcome problems encountered with viral-based therapies, such as immunogenicity, toxicity, mutagenicity and potential danger of oncogenicity [2]. Non-viral gene therapy involving polymers provides flexibility to design a carrier having well defined structural and chemical properties on a large scale. The positively charged groups of the polycation enable formation of "polyplexes" with the negatively charged phosphates of DNA via electrostatic interactions. This results in DNA condensation, protection from the nuclease digestion and more efficient delivery of plasmid into the cell [4]. A variety of polycations have been proposed and investigated for polyplex formation [5], such as poly-L-lysine (PLL) [6], polyethylenimine (PEI) [7], polyamidoamine dendrimer [8], and (poly(2-dimethylamino)ethyl-methacrylate) (PDMAEMA) [9]. However, these systems are very toxic and they tend to aggregate *in vivo* [10, 11]. Also, they need to be administered repeatedly for sustained gene expression.

Amine methacrylate-based polymers have previously been reported as efficient cationic condensing agents for gene delivery [12, 13]. We have designed novel pentablock copolymers of PDEAEM and Pluronics®, which are triblock copolymers PEO-b-PPO-b-PEO [14, 15].They retain the thermoreversible gelation properties of the triblock Pluronics® [16, 17], while providing pH-sensitive groups for DNA condensation and endosomolysis [18].

The tertiary amine groups of PDEAEM are responsible for DNA condensation and providing pH buffering capacity to the polymer. Also, as it has been noted by Ferruti et al.[19] and others[20] that macromolecules with tertiary amine groups exhibit a lower toxicity than those with primary and secondary residues, our pentablock copolymers are expected to be more biocompatible than commonly used vectors such as ExGen. These pentablock copolymers form micelles in aqueous solutions [14] which is an added advantage as the micellar structure of Pluronics® has been shown to facilitate cellular entry and has been found to sensitize multi drug resistant tumors [21, 22]. The copolymers can be mixed with the therapeutic gene in an aqueous phase at low temperatures (below 4 °C) where they exist as sols and can form injectable polyplexes. On subcutaneous/intramuscular injection and subsequent heating to body temperatures, the copolymers self-assemble into gel [14, 15] that can act as reservoirs for sustained-release of polyplexes. These injectable delivery systems have several advantages over other common gene delivery systems, such as simple preparation without organic solvents; the lack of surgical procedures to implant matrices; easy storage at 4°C; ability to vary polymer fractions to tailor and minimize cytotoxicity; and lastly, controlled release of the polyplexes to circumvent repeated administrations needed with other polymers.

2. Materials and Methods

2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, 0.25% Trypsin-EDTA solution and Hank's Buffered Salt Solution (HBSS) were purchased from Invitrogen (Carlsbad, USA). Lactate dehydrogenase (LDH) assay kit was purchased from Takara Mirus Bio (Madison, USA). The Renilla luciferase assay system was purchased from Promega Corporation (Madison, USA). The Qiagen Maxi Prep kit was purchased from Qiagen (Valencia, USA). Ex-Gen 500, linear polyethyleneimine (PEI), *in vitro* transfection reagent was purchased from Fermentas Life Sciences (Vilnius, Lithuania). DNase I was purchased from Ambion (Austin, USA). Agarose was purchased from FMC/ BioWhittaker Molecular Applications, USA. All water used in procedures was ultrapure water with at least 18 megaohm resistivity, prepared using a nanopore ultrafiltration unit fed with distilled, deionized water.

2.2 Plasmid DNA

A 4.7 kb plasmid DNA encoding enhanced green fluorescent protein (pEGFP-N1) (ClonTech, USA) under the regulatory control of the cytomegalovirus (CMV) promoter was used as the reporter gene. To measure the levels of protein expression, we used a luciferase transfection assay, employing a 4.1 kb plasmid encoding *Renilla* luciferase (pRL-CMV) (Promega Corporation, Madison, USA). Plasmids were inserted into DH5α *E.coli*, incubated in selective Luria-Bertani (LB) medium and purified using the Maxi-Prep DNA Purification Kit from Qiagen (Valencia, USA). The concentration and purity of the resulting DNA in a buffer (pH 7.5) of tris-HCl and ethylenediaminetetraacetic acid (EDTA) was measured by measuring the absorbance at 260 nm and 280 nm. All DNA used had a 260/280 ratio of at least 1.80.

2.3 Synthesis of pentablock copolymers

The pentablock copolymers (Scheme 1) were synthesized using oxyanionic or ATRP reaction schemes, which are discussed in detail elsewhere [14, 15]. Pentablock copolymers with different wt% of PDEAEM were synthesized and investigated for gene delivery. Molecular weights of the pentablock copolymers (Table 1), as determined by NMR and gel permeation chromatography, varied from 15,000 Da to 22,000 Da with polydispersity up to 1.4.

2.4 Cell Culture

The SKOV3 human ovarian carcinoma cell line was used for cytotoxicity and transfection experiments. Cell cultures were maintained in a humidified environment with 5% CO_2 at 37°C and passaged regularly to remain subconfluent. Cells were fed with DMEM containing 10% fetal bovine serum (FBS), unless otherwise stated. Neither antibiotics nor antimycotics were used, to avoid the possibility of artificial membrane permeabilization effects from these agents.

2.5 Polymer-DNA complexes (Polyplexes)

Copolymer to DNA ratios are expressed as molar ratios of nitrogen (N) in the DEAEM of the pentablock copolymer to phosphate (P) in DNA, and written as N:P. The molecular weight of the DEAEM monomer is 171 and the average molecular weight of a nucleotide is approximately 308. All polyplexes were formed by the same procedure. The copolymer was dissolved in ultrapure water to achieve a concentration of 1mg/mL and then diluted with the desired media, buffer or water in a polypropylene tube. After incubating for 5 minutes at 20°C, this solution was added to the appropriate solution of DNA. The tube was gently agitated and allowed to incubate for 30 minutes at room temperature.

2.6 Molecular weight analysis and particle size determination

Multi-angle light scattering (MALS) analysis using a DAWN MALS detector (Wyatt Corporation, Santa Barbara, USA) was conducted to analyze the apparent size and molecular weight of the synthesized copolymers and their polyplexes dissolved in ultrapure water. All ultrapure water used was triple filtered using 0.22-μm syringe-filters (Millipore, Billerica, USA) to eliminate any dust particles. Samples were again filtered through 0.22-μm syringe filters into clean scintillation vials to prevent particulate contamination. Vials were cleaned by rinsing with triple-filtered ultrapure water followed by rinsing with triple-filtered methanol and, then ethanol and allowed to dry in a sterile laminar flow environment. Since the polysulfone membranes exhibit low protein binding affinity and the 0.22-μm pore size is on the order of the size of hydrated DNA , no plasmid and polymer loss was expected due to membrane adsorption. DNA concentration in the samples was measured before and after filtration by measuring absorbance at 260nm to ensure no loss of polyplexes. The experiments were conducted in micro-batch configuration with the samples being delivered to the flow cell of the DAWN EOS with a syringe pump at 0.5 ml/min. MALS measurements were performed at 5×10^{-4} g/ml and 27°C

The refractive index increments for each pentablock copolymer and the polyplexes were measured independently using an Optilab DSP Interferometric Refractometer (Wyatt Corporation, Santa Barbara, USA). Measurements were conducted at a wavelength of 690nm in vacuo. The temperature was set at 40°C to minimize fluctuations. Samples used were same

as those for MALS. Clean solvent was injected before and after each refractive index determination to confirm the baseline voltage and check for baseline drift.

A JEOL 1200EX2 scanning/transmission electron microscope (Tachikawa, Japan) was used to visualize the morphology of polyplexes, prepared at varying N:P ratios in 0.1mM phosphate buffer saline. A 10μL drop of the sample was placed onto a formvar-coated copper grid and allowed to adsorb. After 5 minutes, the liquid was wicked with filter paper. The grid was then placed immediately into a solution of 4% w/v uranyl acetate in 50% ethanol and allowed to stain for 30 minutes. The sample was rinsed by repeated immersion in 50% ethanol followed by two rinses in deionized water. After rinsing, the samples were loaded into the vacuum stage of the microscope and visualized at 80kV under magnifications of 40,000X to 250,000X. Naked DNA (pEGFP-N1) and the copolymer alone were also examined.

2.7 Gel retardation assay

To study the pentablock copolymer condensation with DNA, agarose gel electrophoresis of the self- assembled polyplexes at different N:P ratios were conducted, comparing their mobility. A total of 1μg of pEGFP-N1 DNA per lane was used. After adding 10x sucrose loading buffer to the samples, 15 μL of each sample was loaded to an appropriate well in a 0.7% agarose gel containing 0.1 μg/mL ethidium bromide. The gel was run in TBE buffer at 60V for approximately an hour. Visualization and image capture was accomplished using a UV-transilluminator under a Kodak EDAS 290 digital imaging suite (Fisher Scientific; Pittsburg, USA). A 1kb+ DNA ladder and only DNA served as controls.

2.8 Nuclease resistance assay

To investigate the ability of the pentablock copolymers to protect DNA from enzymatic degradation, polyplexes at different N:P ratios were incubated with DNase I for an hour, and run on an agarose gel. Appropriate buffers and the enzyme DNase I (RNase-free) were added to yield either 4 or 50 IU per micrograms of DNA. Naked pEGFP-N1 DNA served as a negative control, and naked DNA with DNase I served as a positive control for DNase 1 activity.

In addition, a change in the absorbance of polyplex solutions at 260nm was observed to detect DNA fragmentation by DNase I. Polymer solution in nanopure water was added to plasmid DNA in TE buffer (pH 7.5) to get desired N:P ratios with 20μg of DNA/ml final concentration, (i.e. 0.4 OD of DNA at 260nm). After incubating the polyplexes for an hour, 100IU of DNase I were added (yielding 5 IU/μg DNA) with 10x DNase I buffer to make the final volume of polyplex solution to 1ml, and the change in absorbance at 260nm was monitored.

The gelation of polyplexes at 37^0C is important for the ability of these polymers to act as controlled gene delivery devices. To confirm that polyplexes do show gel-sol transitions like the polymers themselves, polymer-DNA complex solutions in TE buffer at 4°C containing 22.5% by wt pentablock copolymer B and plasmid at N:P ratio of 25:1 were warmed to room temperature to form gels. Further, these gels were dissolved at room temperature into TE buffer, and small aliquots of the samples were run on a 0.7% agarose gel, both in the presence and absence of 10IU of DNase I to investigate the ability of the gel-forming polyplexes to protect the DNA even after dissolution of the gels.

2.9 Cytotoxicity assay

Lactate dehydrogenase (LDH) is an integral cytosolic enzyme that is secreted out in the medium following the rupture of cell membrane [20], since it is a potential site of interaction of cationic macromolecules [23]. Cells were cultured into 96-well tissue culture plates at a density of approximately 27,500 cells per well. After incubation overnight, the DMEM was removed, and replaced with appropriate polymer solutions in 200μL fresh DMEM. Cells were allowed to incubate in the presence of the test substances for 24 to 48 hours. 100μL of media was then collected in an optically clear 96-well microtiter plate, and LDH concentration was assayed using a commercial kit (Takara Bio LDH cytotoxicity detection kit, Otsu, Japan). The absorbance at 500nm was measured for each well using a BioTek EL-340 plate reader (Winooski, USA). Media alone and media with only cells were used to obtain a background LDH level for normalization. Cells exposed to 0.1% Triton X-100 in DMEM were used as a positive control, and set as 100% LDH release. The relative LDH release is defined by the ratio of LDH release over total LDH in the intact cells. Less than

10% LDH release was regarded as an acceptable level in our experiments. All samples were run in four replicates, and experiments were repeated twice. After incubation with polymers, changes in morphology and detachment of cells from the dish were also observed using an Olympus IMT-2 (Melville, USA) inverted, phase-contrast light microscope equipped with an objective of 100x magnification.

2.10 In-vitro transfection

2.10.1 Detection of Green Fluorescent Protein

Green fluorescent protein (GFP) was used for the assessment of *in-vitro* transfection efficiency of the copolymers in SKOV-3 cell line. Various formulations of polyplexes were made in serum free media OptiMEM at different N:P ratios with a fixed amount of pEGFP-N1 (6μg of DNA per well in a 6-well multiwell plate). Cells seeded in 6-well plates were incubated overnight up to 70% confluency prior to transfection. Growth medium was then removed and replaced with the test solutions of polyplexes along with 1 ml OptiMEM. The cells were incubated for 5 to 12 hours at 37°C, after which the transfection medium was replaced with fresh DMEM containing 10% FBS, and cells were incubated for another 44hrs to express the reporter gene. The advantage of using pEGFP as a reporter gene is that it can be observed both qualitatively and quantitatively. Cells expressing GFP were visualized directly on an Olympus IMT-2 (Melville, USA) inverted, phase-contrast light microscope. Images were recorded using an attached Nikon Coolpix 990 digital imaging system, without disrupting the cells. Flow-cytometry was used to obtain the percentage of cells transfected with the pEGFP-N1. Cells were harvested from the plates using HBSS and trypsin-EDTA treatment, and were suspended in 3ml HBSS in centrifuge tubes. These were centrifuged at 1200 rpm for 10 minutes. The supernatant was removed and the cell pellet was washed in 3ml HBSS to remove background fluorescence from the media. After repeating the centrifugation, cells were finally suspended in 0.5ml HBSS and were transferred to flow cytometry cuvettes for analysis. Flow cytometry was performed using a Beckman-Coulter Epics ALTRA Fluorescence-activated cell sorter (Fullerton, USA). ExGen 500 was used as a positive control, and was expected to yield high efficiency of transfection. Cells exposed only to DNA (without polymer) were used as negative controls.

2.10.2 Detection of Luciferase activity

In order to determine the total protein expressed by a reporter gene per total cellular protein, a luciferase assay was employed, using pRL-CMV as the reporter gene. Cells were seeded in a 96-well plate up to 70% confluency prior to transfection, and were then transfected with various polyplexes solution in 200µl OptiMEM using 1.5µg of DNA per well. After 5 hours incubation in OptiMEM, the solution was replaced with fresh DMEM containing 10% FBS, and incubated for 44hrs. Cells were then lysed using a lysis buffer (*Renilla* Luciferase Assay Lysis Buffer, Promega) and the luminescence of the expressed reporter protein was measured on an automated Berthold Mithras LB940 (Bad Wildbad, Germany) multilabel luminometer using the Promega *Renilla* Luciferase Assay System (Madison, USA). Positive and negative controls similar to those for pEGFP were used. Total recovered cellular protein content of the cells was determined by a modified Bradford assay, using a CB-Protein Assay^{TM} Kit. Bovine serum albumin standards were prepared. Twenty microliters of the samples from luciferase detection protocol were placed in individual wells of a 96-well plate and diluted with nanopure water to 100µl. CB-Protein Assay^{TM} reagent (100µl) was added into each well and mixed well. The plate was allowed to incubate for 15 minutes and absorbance was measured at 595nm. The amount of protein was read from the standard curve.

The statistical significance of the results of transfection was evaluated by Student's *t*-test for the transfection study. To compare the mean and standard deviations of more than 2 cases, Tukey's procedure was used. Software JMP 5.1 was used for statistical analysis. Four replicates of each case were done in each experiment, and all experiments were repeated twice.

3. Results and Discussion

3.1 Molecular weight analysis and particle size

Zimm plots were used to analyze the MALS data. For the pentablock B, containing 26% PDEAEM (Table 1), the weight average molecular weight was found to be $(3.118 \pm 0.097) \times 10^6$ g/mol, and the radius of gyration as 18.3 ± 2.0 nm. The refractive index

increment, dn/dc value, for the copolymers was found to be 0.0155(ml/g). Light scattering data from polymer solutions in triple filtered ultrapure water with concentrations of 0.4, 0.8, 1.0 and 1.2mg/ml was used for analysis. The second virial coefficient, which quantifies the polymer-solvent interaction, was found to be $(-9.457 \pm 0.387)x10^{-5}$ mol mL/g^2. The negative value suggests a poor interaction between polymer and the solvent, indicating that the polymer has hydrophobic components. This further supports evidence [14] that the pentablock copolymers self-assemble to form micelles due to hydrophobic interactions with water. Dividing the molecular weight of these polymer aggregates obtained from MALS by the molecular weight of a polymer unimer suggests that each micelle is made of approximately 132 unimers of the pentablock copolymer.

For the polyplexes of pentablock B with pRL-CMV plasmid at N:P ratio 5:1, wt average mol weight was found to be $(2.151 \pm 0.091)x10^7$ g/mol, with a radius of gyration of 32.3 $\pm$ 1.6 nm. The dn/dc value for these polyplexes was earlier found to be 0.0074ml/g. Light scattering data from polyplex solutions of 0.6, 0.8, and 1.0 mg/ml concentrations was used for analysis. The second virial coefficient was found to be $(-2.004 \pm 0.147)x10^{-6}$ mol mL/g^2. This indicates that polyplexes too, which are formed by the condensation of DNA by polymer micelles in solution, do not have favorable interactions with water. The negative value suggests that attractive interactions between the polyplexes are strong enough to pull the molecules into an aggregates from the dilute solution[24].

TEM images were obtained to observe the morphology of polyplexes. Fig 1 shows an image of polyplexes with pentablock C and pEGFP at N:P ratio of 5:1. In the image, condensates appear to be in two forms; first, toroidal ring structures, and second, structures with a linear morphology, which usually have loops at either end. Similar structures were obtained irrespective of the ratio of the polymer to DNA. Real time images of such toroidal and rod-like condensates formed with cationic polymers like PEG-poly(amidoamine) triblock copolymer and plasmid DNA have been reported earlier in other studies as well [25, 26]. The average diameter of rings was approximately 50nm and the average length of rods was approximately 100nm. This is very much in compliance with the MALS data shown above. However, dehydration of the samples for TEM imaging disrupts the micelles of polyplexes which otherwise would have formed in an aqueous environment, affecting the apparent size

and morphology of the condensates. To appreciate the complex architecture of the polymer-DNA condensates, images of uncondensed pEGFP plasmids, and only polymer were also taken (images not shown here). TEM images show distinct differences among the polyplexes, DNA and polymer, with these toroidal and linear structures seen only in the polyplexes.

3.2 DNA complexation

Experiments were performed to investigate whether the pentablock copolymers form complexes with plasmid DNA pEGFP-N1. Fig. 2 shows the results from a gel retardation assay. DNA was visualized by fluorescence of ethidium bromide. Lane 7 containing naked plasmid pEGFP-N1 showed at least three distinct bands, corresponding to the different states of supercoiling in the double-stranded plasmid DNA. The movement of plasmid DNA was retarded as the amount of pentablock copolymer in a complex increased, suggesting that pentablock copolymer formed a strong complex with plasmid DNA (lanes 1-6). Lanes 1-3 had pentablock A and lanes 4-6 had pentablock C. Almost complete retardation was achieved at N:P ratio of 1:1, which reflects that negatively charged DNA was completely neutralized and complexed by the cationic polymer, forming self-assembled polyplexes via ionic interactions. At N:P ratios of 5:1 and 10:1, the band appeared to move slightly upward, suggesting that these polyplexes had an overall positive charge.

3.3 Protection against nuclease degradation

The agarose gel in Fig 3 shows that the pentablock copolymer protected plasmid DNA against DNase I digestion. Naked plasmid pEGFP-N1 in lane 1 served as a negative control, and naked plasmid with DNase I in lane 2 served as a positive control. Lane 8 contained 1kb+ ladder for the control. Lanes 3 to 7 contained polyplexes of pentablock C with Dnase I.. Dark bands of DNA at the top of lanes 5 and 6 showed that plasmid DNA was still present and was not digested by DNase I. Thus, N:P ratios of 5:1 and above were sufficient to complex the DNA and protect it against nuclease degradation. Lane 7 showed that at unusually high concentrations of DNase I (50 IU / μg of DNA, as compared to 4 IU in other lanes) all DNA was digested. Lanes 5 and 6 showed slightly weaker bands in fluorescence

intensity as compared to naked plasmid in lane 1, probably because ethidium bromide could not efficiently intercalate the already complexed DNA in polyplexes.

We also monitored the change in absorbance of polymer-DNA complexes solution at 260nm to detect any increase in nuclease resistance. Upon, addition of 100 IU of DNase I to native DNA solutions (5 IU/ μg of DNA), an immediate increase in the absorbance of solution was observed due to the fragmentation of plasmid DNA molecules (Fig 4). However, the rate of degradation decreased significantly on addition of polymer to the DNA solution. At N:P ratio of 8:1 and above, scarce change in absorbance was detected upon addition of DNase I. From a comparison of the slope of the curves, reflecting a rate of degradation, suppression in DNase I activity was estimated as 73.7% at N:P ratio 2:1, 78.9% at N:P ratio 4:1, 99.9% at N:P ratio 8:1, and 100% at N:P ratio 12:1. This indicated that the pentablock copolymer is effective in protecting the plasmid DNA against degradation by DNase I at N:P ratios of 2:1 and above.

The gelation studies showed that the polyplexes did indeed form gels at room temperature at high concentrations. The gel dissolution studies showed, as seen in Fig 5, that polyplexes were indeed released from this gel. Lane 1 had naked DNA, lane 2 had released polyplexes, and lane 3 had released polyplexes with 10IU of Dnase I. We found that all the DNA in lanes 2 and 3 were intact at the top of the lanes, and the excessive unbound cationic polymer was seen to move in upward direction towards the negative electrode. This result showed that polyplex gels dissolved to release complexed DNA (polyplexes), and not just polymer or naked DNA.

3.4 *Cytotoxicity*

The pentablock copolymers with different weight percentages of PDEAEM were tested for their toxicity on the SKOV3 cell line. ExGen 500 was also tested. A cut-off concentration was found for each polymer at which less than 10% cell death was observed. Later, all transfection experiments were performed within these cut-off concentrations of the polyplexes. As shown in Fig 6, cell viability increased as weight-percentage of PDEAEM in the pentablock decreased, due to a dilution effect of the cationic groups in copolymers. The results were also expressed in terms of the cut-off molar concentrations of the cationic groups

(nitrogen residues) of the polymers. These results show that at lower wt% of PDEAEM in the pentablock copolymers, higher molar concentrations of the cationic groups can be tolerated by cells, and these cationic groups are actually responsible for all DNA condensation and protection. This also reflects the fact that the Pluronics® have a beneficial role in improving the biocompatibility of the pentablock beyond just diluting the number of cationic groups, because an increase in wt% of Pluronics® in the pentablock results in an increased cut-off molar concentration of cationic groups, which otherwise should have been same for all pentablock copolymers. This is presumably due to the shielding effect of the PEO in the Pluronics®. Besides, our pentablock copolymers were found to be much less toxic than the commercially available *in vitro* transfecting reagent ExGen 500. ExGen 500 was found to have a lower cut-off molar concentration of the cationic groups due to absence of these shielding groups.

Microscopic images of the SKOV3 cells in Fig 7 show how the cell morphology changed as polymer concentration increased in toxicity. In the presence of no polymer (Fig 7A), cell bodies were large, confluent and covered the entire surface of the plate. However, at higher concentrations (0.05mg/ml) of the polymer (Fig 7C), cell bodies were small, sparse and dispersed, indicating cell death. At an optimum concentration of 0.03mg/ml (Fig 7B), cells appeared to be healthy and sub-confluent. As seen from Fig 6, this concentration of pentablock C is close to the cut-off concentration measured by the LDH assay.

3.5 In-vitro transfection

3.5.1 Green Fluorescence Protein assay

Flowcytometry was used to measure the percentage of cells transfected by pEGFP-N1. Transfected cells showed transient expression of the reporter gene over time. Fig 8 shows the percentage of cells transfected at different N:P ratios using the pentablock B (copolymer with 26% PDEAEM) measured 44 hours after removing the polymer. Up to 17% transfection was achieved by the pentablock copolymer, which is very much comparable to the 20% transfection obtained from the commercially available and much more toxic ExGen 500. Also, higher transfection was obtained at higher N:P ratios of the polymer. However, at

very high N:P ratios, the toxicity increased due to increased polymer content, and the cells died before getting transfected or expressing the protein from the transfected gene.

3.5.2 Luciferase assay

In order to determine the total amount of reporter protein expressed by the cells, a renilla luciferase assay was employed. Polymers at different N:P ratios with pRL-CMV plasmid were tested for the amount of transfection obtained. Fig 9 shows the amount of luciferase expressed in terms of relative luminescence units (RLU). The experiments were conducted in a 96-well plate with 1.5µg of DNA/well. At this DNA dose, the amount of protein expressed increased on increasing the N:P ratio from 1:1 to 2:1 and 3:1 for pentablock copolymer B. However, at N:P ratios of 4:1 and above, the amount of polymer used surpassed the cut-off concentration at which cells could be viable, and hence a decrease in protein expressed was observed. Positive control ExGen 500 also showed an increase in transfection with increase in N:P ratio from 1:1 to 2:1. However, at higher N:P ratios, an increase in cell death decreased the protein expression considerably. Hence the values shown in Fig. 9 are for ExGen N:P ratios of 2:1, and compared to higher ratios for the pentablock copolymers, for the same amount of DNA. It is appreciable that RLUs obtained from the much more biocompatible pentablock copolymers are of the same order as RLUs from ExGen 500. Total cellular protein content of the cells was assayed using a Bradford assay kit, and was not found to be significantly different in different cases. On an average, total amount of cellular protein was found to be 3.56µg in each well.

Since the pentablock copolymers have the ability to undergo thermoreversible gelation, with slow release of polyplexes over time, the effect of exposure time on transfection was investigated. Fig 10 shows a time dependent study of the transfection efficiencies of the polymers. At a N:P ratios of 2:1, pentablock copolymer B showed a significant increase in transfection if cells were incubated with polyplexes for 12 hours instead of just 5 hours. Further, the amount of transfection thus obtained was comparable to the transfection by ExGen 500 (N:P 1:1) for 12 hours exposure. Statistically, the transfection efficiencies of ExGen 500 and the pentablock copolymers were not significantly different. This shows that the pentablock copolymers are equally efficient at transfecting cells *in vitro* as Ex-Gen 500.

The N:P ratios of 3:1 for the pentablock copolymers and 2:1 for Ex-Gen resulted in increased cell death with reduced transfection for this extended 12 hours incubation.

4. Conclusions

The key challenge for plasmid based gene therapy is to surmount the limiting steps in intracellular movement including endosomal release, cytoplasmic transport, and nuclear uptake, while enhancing the retention of plasmids in the nucleus.

The new pentablock copolymers synthesized in our laboratory show great promise as non-viral vectors for gene therapy. The copolymers are water soluble, pH-sensitive and have thermo-reversible gelation properties. Their ability to effectively condense DNA into polyplexes, and protect plasmids from nuclease degradation is an important first step towards use as vectors for gene delivery. The condensed polyplexes are small enough to allow cellular uptake. MALS showed that polymer exists in the form of micelles, which complex with the plasmid DNA to form condensed polyplexes. TEM images of the polyplexes show that plasmid was condensed into ring (diameter ~ 50nm) or rod like structures (length ~ 100nm). Since samples were dehydrated for obtaining the TEM images, disrupting the micelles formed by polyplexes in aqueous environment, cryo-TEM studies in the future will preserve the micellar structure and provide a more accurate image.

The pentablock copolymers have minimal toxicity which can be altered by changing the percentage of the cationic component. Increasing the wt% of Pluronics® in the copolymer was found to have a beneficial effect on the biocompatibility of the copolymers beyond just a dilution effect. We hypothesize that this may be due to the shielding of cationic charges of the pentablock copolymers by the PEO in the Pluronics®, making the polymers less toxic to cells. Also, our pentablock copolymers were found to be much less toxic (up to 50 times in wt concentration) than the commercially available *in vitro* transfecting reagent Ex-Gen 500. Effective transfection of reporter genes was observed within the toxicity limit of the copolymers. The amount of transfection increased with extended time of incubation if polymer concentration was within the cell viability limit. Our observed transfection efficiency using the pentablock copolymers was not significantly different from that of Ex-Gen 500 when cells were incubated for 12 hours with the polyplexes. Besides, the presence

of PEO blocks in the pentablock copolymers has the potential to reduce non-specific interactions *in vivo* when targeting moeties such as epidermal growth factor (EGF) [27, 28] are attached to the ends of the chains, significantly increasing the cellular uptake by neoplastic cells expressing increased amounts of EGF receptors on their cell membranes. Nuclear localization sequences (NLS) [29] may also be conjugated to the pentablock copolymers, which would increase the nuclear uptake of the polyplexes, overcoming a major hindrance in transfection. The biggest potential advantage of our new pentablock copolymers is their ability to form gels at body temperatures and enable localized delivery to tumors with a slow release of polyplexes for sustained gene expression without multiple administrations. The gelation properties and drug-release studies from the polymer using a dye have been reported in other papers from our group[14, 15]. We have also verified that polymer-DNA complexes do form gels at appropriate concentrations and temperature; and agarose gel electrophoresis retardation study show that these gels dissolve to release polyplexes (complexed DNA), and not just polymer or naked DNA. Development of a safe, efficient synthetic gene delivery vector system that can be used to transport suicide genes to neoplastic cells will provide an effective alternative for cancer therapy [30, 31].

Acknowledgements

References

1. G.D. Schmidt-Wolf, I.G.H. Schmidt-Wolf, Non-Viral and hybrid vectors in human gene therapy: an update, TRENDS in Molecular Medicine 9 (2) (2003) 67-72.
2. T. Merdan, J. Kopecek, T. Kissel, Prospects of cationic polymers in gene and oligonucleotide therapy against Cancer, Adv. Drug Delivery Reviews 54 (2002) 715-758.

3.   P. Felgener, et al., Nomenclature for synthetic gene delivery systems, Hum. Gene ther. 8 (1997) 511-512.

4.   S.D. Smedt, J. Demeester, W. Hennink, Cationic polymer based gene delivery systems., Pharm. Res. 5 (2000) 1425-1433.

5.   S. Han, M.R.I., Y.K. Sung, S.W. Kim, Development of Biomaterials for Gene Therapy, Molecular Therapy 2 (4) (2000) 302-317.

6.   A. Gondsho, K. Irie, H. Susaki, H. Iwasawa, S. Okuno, T. Sugawara, Tissue-targetting ability of saccharide-poly(L-lysine) conjugates, Biol. Pharma. 17 (1994) 275-282.

7.   O. Boussif, et al., A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethyleneimine., Proc. Natl. Acad. Sci. USA 92 (1995) 7297-7301.

8.   M.X. Tang, C.T. Fedemann, F.C. Szoka Jr, In vitro gene delivery by degraded polyamidoamine dendrimers, Biocnjug. Chem. 7 (1996) 703-714.

9.   P. Van De Wetering, J.Y. Cherng, H. Talsma, D. Crommelin, W.E. Hennik, (2-dimethylamino)ethyl-methacrylate based copolymers as gene transfer agents., J control Rel 53 (1998) 145-153.

10.   G.A. Brazeau, S. Attia, S. Poxon, J.A. Hughes, In vivo myotoxicity of selected cationic macromolecules used in non-viral gene delivery, Pharm. Res. 15 (1998) 680-684.

11.   P.R. Dash, M.L. Read, L.B. Barret, M.A. Wolfert, L.W. Seymour, Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery., Gene therapy 6 (1999) 643-650.

12.   P. Van de Wetering, E.E. Moret, N.M.E. Schuurmans Nieuwenbroek, M.J. van Streenbergen, W.E. Hennink, Structure-activity relationships of water-soluble cationic methacrylate/methacrylamide polymers for nonviral gene delivery, Bioconjug. chem. 10 (1999) 589-597.

13.   P. Van De Wetering, N.M.E. Schuurmans-nieuwenbroek, M.J. van Streenbergen, D.J.A. crommelin, W.E. Hennink, Co-polymer of 2-(dimethylamino)ethyl methacrylate with ethoxytriethylen glycol methacrylate or N-vinyl-pyrroliodone as gene transfer agents., J. Control. Release 64 (2000) 193-203.

14.   B.C. Anderson, S.M. Cox, P.D. Bloom, V.V. Sheares, S.K. Mallapragada, Synthesis and Characterization of Diblock and Gel Forming Pentablock Copolymers of Tertiary Amine Methacrylates, Poly(Ethylene Glycol) and Poly(Propylene Glycol), Macromolecules 36 (2003) 1670-76.

15.   M. Determan, Seifert Soenke, Thiyagarajan P., S.K. Mallapragada, Synthesis and characterization of temperature and pH responsive pentablock copolymers, In preparation (2004).

16.   A.V. Kabanov, E.V. Batrakova, Alakhov, Valery Yu., Pluronic(R) block copolymers as novel polymer therapeutics for drug and gene delivery, Journal of controlled release 82 (2002) 189-212.

17.   B.C. Anderson, S.M. Cox, A.V. Ambardekar, S.K. Mallapragada, The effect of salts on the micellization temperature of aqueous Poly(ethylne oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide) solutions and the dissolution rate and water diffusion coefficient in their corresponding gels, Journal of Pharmaceutical Sciences 91 (1) (2002) 180-188.

18.   R.A. Kristensen, J.P. Clamme, C. Vuilleumier, Y.M. Kurthy, Role of endocytosis in the transfection of L929 fibroblasts by polyethyleneimine/DNA complexes, Biochim, Biophys. Acta 1514 (2001) 21-31.

19.   Ferruti P, Knobloch S, Ranucci E, G. E, D. R, A novel chemical modification of poly-L-lysine reducing toxicity while preserving cationic properties, Proc Int Symp Control Rel Bioact Matter 24 (1997) 45-46.

20. D. Fischer, Y. Li, B. Ahlemeyer, J. Krieglstein, T. Kissel, In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis, Biomaterials 24 (2003) 1121-1131.
21. A.V. Kabanov, E.V. Batrakova, V.Y. Alkhov, Pluronic® block copolymers as novel polymer therapeutics for drug and gene delivery, J control Rel 82 (2002) 189-212.
22. V.Y. Alakhov, E.Y. Moskaleva, E.V. Batrakova, A.V. Kabanov, Hypersensitization of multidrug resistant human ovarina carcinoma cells by pluronic P85 block copolymer, Bioconjug. chem. 7 (1996) 209-216.
23. S. Choksakulnimitr, S. Masusa, H. Tokda, Y. Takakura, M. Hashida, In vitro cytotoxicity of macromolecules in different cell culture systems, J control Rel 34 (1995) 223-41.
24. R.P. Sear, Distribution of the second virial coefficients of globular proteins, Europhys. Lett. 60 (6) (2002) 398.
25. A.L. Martin, M.C. Davies, B.J. Rackstraw, C.J. Roberts, s. Stolnik, S.J.B. Tendler, P.M. Williams, Observation of DNA-polymer condensate formation in real time at a molecular level, FEBS letters 480 (2000) 106-112.
26. B.J. Rackstraw, A.L. Martin, S. Stolnik, C.J. Roberts, M.C. Garnett, M.C. Davies, S.J.B. Tendler, Microscopic Investigations into PEG-Cationic Polymer-Induced DNA condensation, Langmuir 17 (2001) 3185-3193.
27. M. Ogris, G. Walker, T. Blessing, R. Kircheis, M. Wolschek, E. Wagner, Tumor-targeted gene therapy: strategies for the preparation of ligand-polythethylene glycol polytheyleneimine/DNA complexes, J control Rel 91 (2003) 173-81.
28. M. Ogris, E. Wagner, Tumor-Targeted Gene Transfer with DNA Polyplexes, Somatic Cell and Molecular Genetics 27 (1/6) (2002) 85-95.
29. E. Hebert, Improvement of exogenous DNA nuclear importation by nuclear localization signal-bearing vectors: a promising way for non-viral gene therapy?, Biology of the Cell 95 (2003) 59-68.
30. M. Aghi, F. H., X.O. Breakfield, Prodrug activation enzymes in cancer gene therapy., J. Gene Med. 2 (2000) 148-164.
31. M. Iwai, Y. Harada, et al., Polyethylenimine-mediated suicide gene transfer induces a therapeutic effect for hepatocellular carcinoma in vivo by using an Epstein-Barr virus-based plasmid vector., Biochim, Biophys. Res. Commun. 291 (2002) 48-54.

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Table I: Molecular weight and polydispersity indices (PDI) of the pentablock copolymers used in this study.

Pentablock copolymer	Mn (NMR)	Mn (GPC)	PDI	%PDEAEM
A	15,000	40,112	2.36	17
B	17,300	23,516	1.235	26
C	22,000	30,664	1.206	40
D	19,973	20,365	1.338	62

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Scheme 1: Structure of pentablock copolymers. The number of repeating units of the PDEAEM blocks, n, was varied to control the properties of the copolymer.

Fig 1: TEM image of the polyplexes obtained from the condensation of pEGFP by pentablock C at 5:1 N:P ratio.

Fig 2: Agarose gel electrophoresis showing DNA complexation. Lanes 1-3 contain polyplexes formed at increasing N:P ratios with the pentablock A (1) 1:1 (2) 5:1 (3) 10:1. Lanes 4-6 contain polyplexes formed at increasing N:P ratios with pentablock C (4) 1:1 (5) 5:1 (6) 10:1. Lane 7 contains naked pEGFP-N1 DNA, and lane 8 contains a 1kb+ DNA ladder.

Fig 3: Agarose gel electrophoresis showing protection of plasmid pEGFP-N1 against DNase I digestion. Each lane has 1μg of DNA. Lane 1 contains only naked DNA. Samples in lanes 2 to 6 were incubated with 4 IU of DNase I / μg of DNA for an hour before running the gel. Lane 2 contains DNA with DNase I. Lanes 3 to 7 contain polyplexes formed at increasing N:P ratios with pentablock C:(Lane 3) 1:2 (Lane 4) 1:1 (Lane 5) 5:1 (Lane 6) 10:1 (Lane 7) 5:1. Polyplexes in lane 7 were incubated with 50 IU of DNase I. Lane 8 contains a 1kb+ DNA ladder.

Fig 4: Nuclease resistance of polyplexes against DNase I activity at different N:P ratios. 20μg of DNA/ml final concentration was used, and each sample was incubated with 5 IU DNase I / μg DNA.

Fig 5: Agarose gel electrophoresis of polyplexes released from a polymer-DNA complex gel. Lane1 contains naked plasmid, lane 2 has released polyplexes, and lane 3 has released polyplexes treated with 10IU of DNase I.
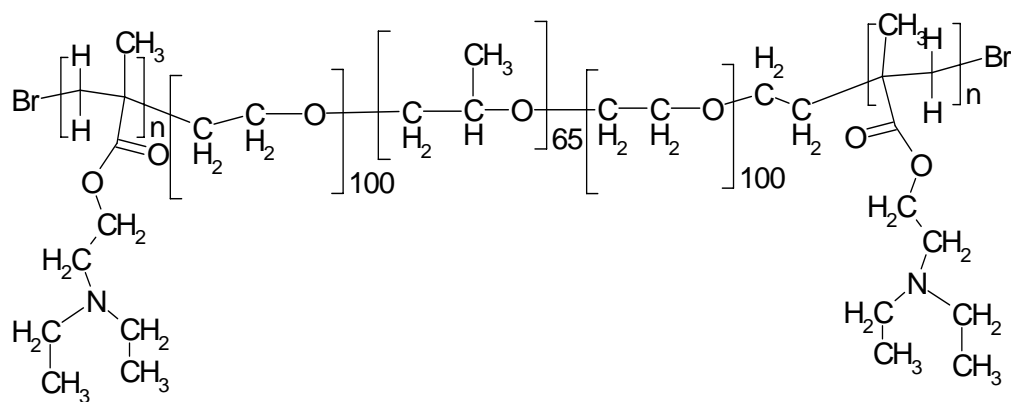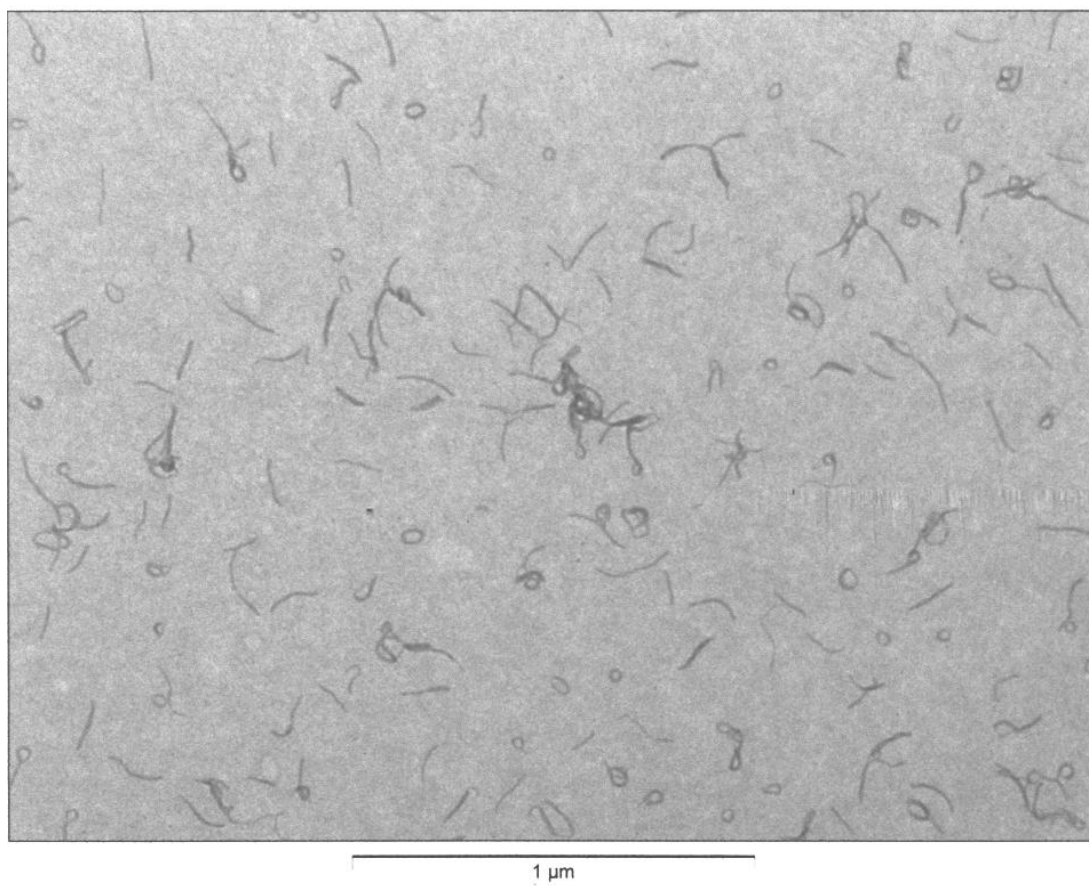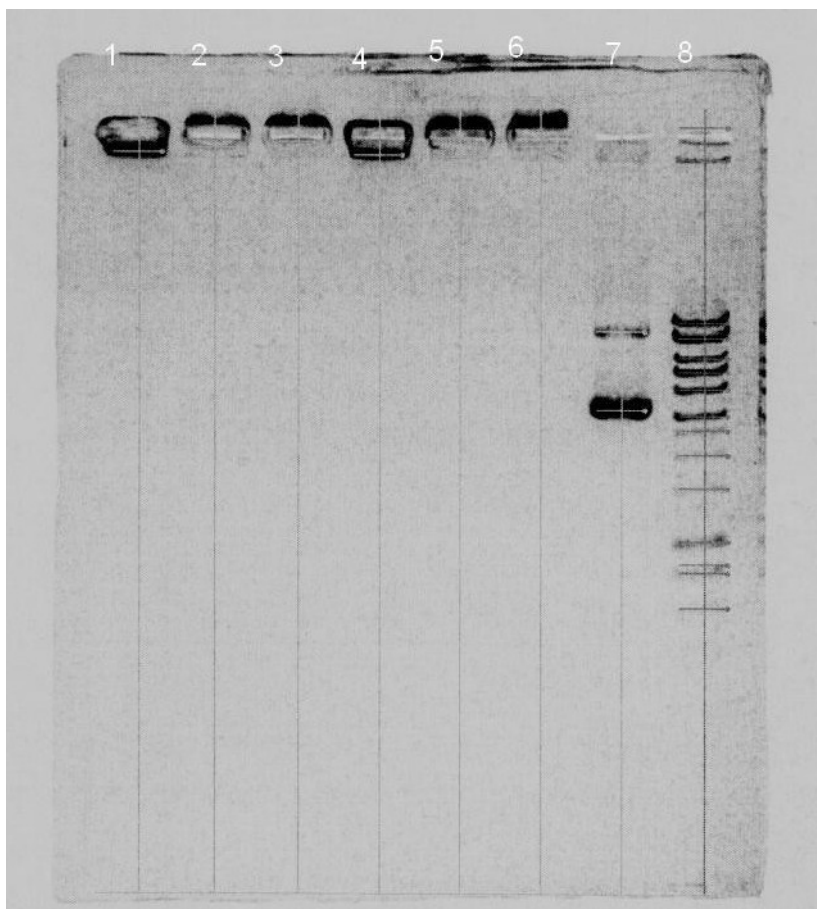
Fig 6: Cut-off concentrations of different polymers under which they are not toxic to SKOV3 cell lines for up to 48 hr. The left axis has molar concentrations (moles/μl) of nitrogen residues of the polymer, while the right axis has weight concentrations (mg/ml) of the polymers.

Fig 7: Morphology of the SKOV3 cells after incubation for 48 h with pentablock C. (A) No polymer (B) 0.03 mg/mL (C) 0.05 mg/mL.

Fig 8: *In vitro* transfection of pEGFP into SKOV3 cells using pentablock B and ExGen 500 at various N:P ratios

Fig 9: *In vitro* transfection of pRL-CMV in SKOV3 cell line using pentablock B and ExGen 500. Effect of N:P ratio on transfection of SKOV3 cells.

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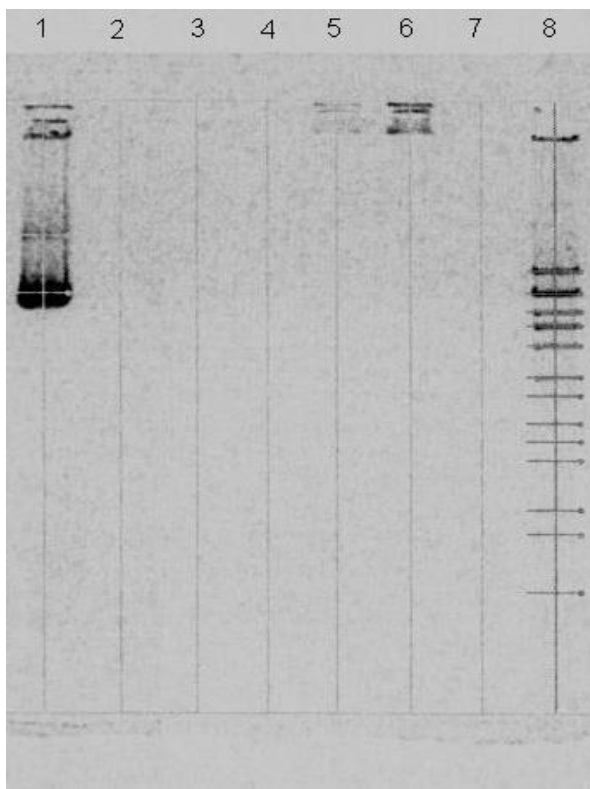
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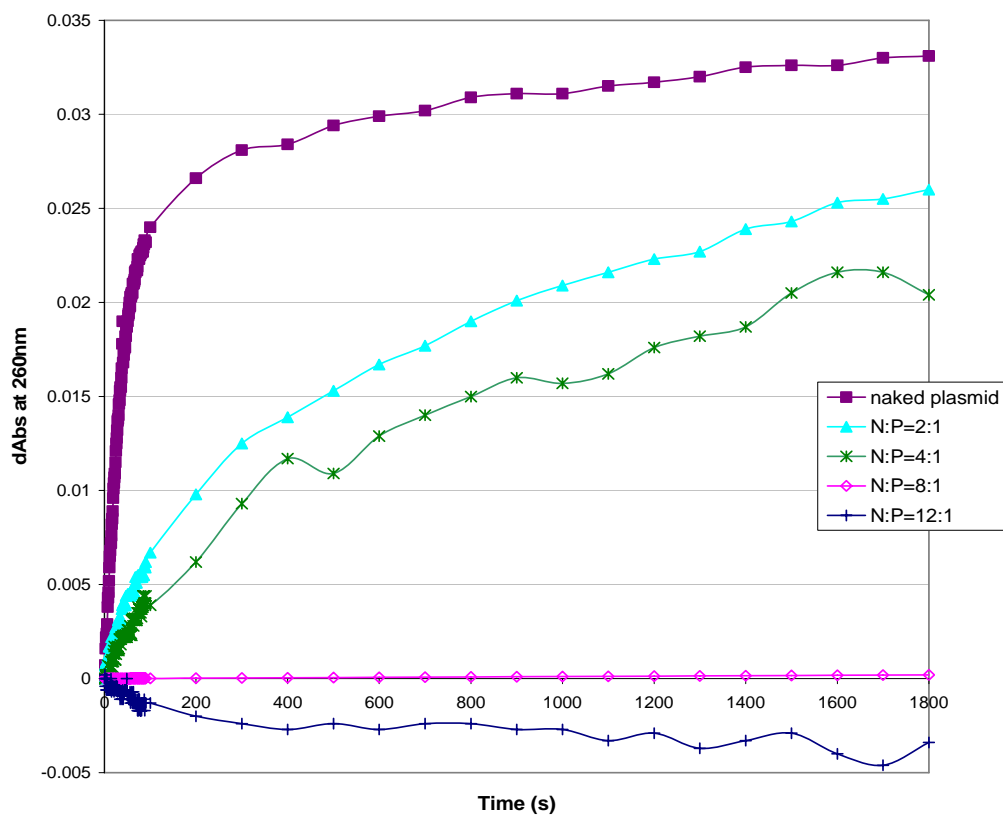
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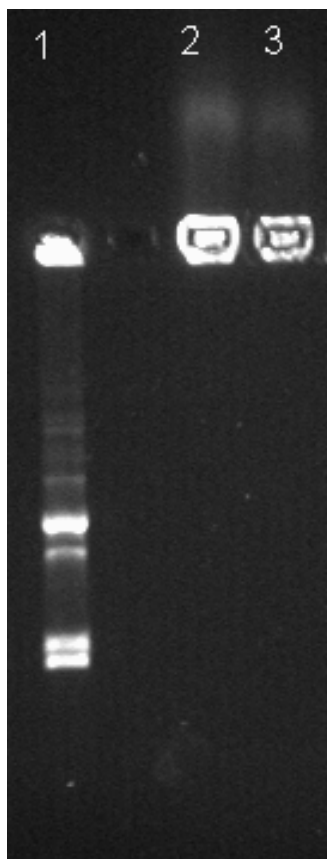
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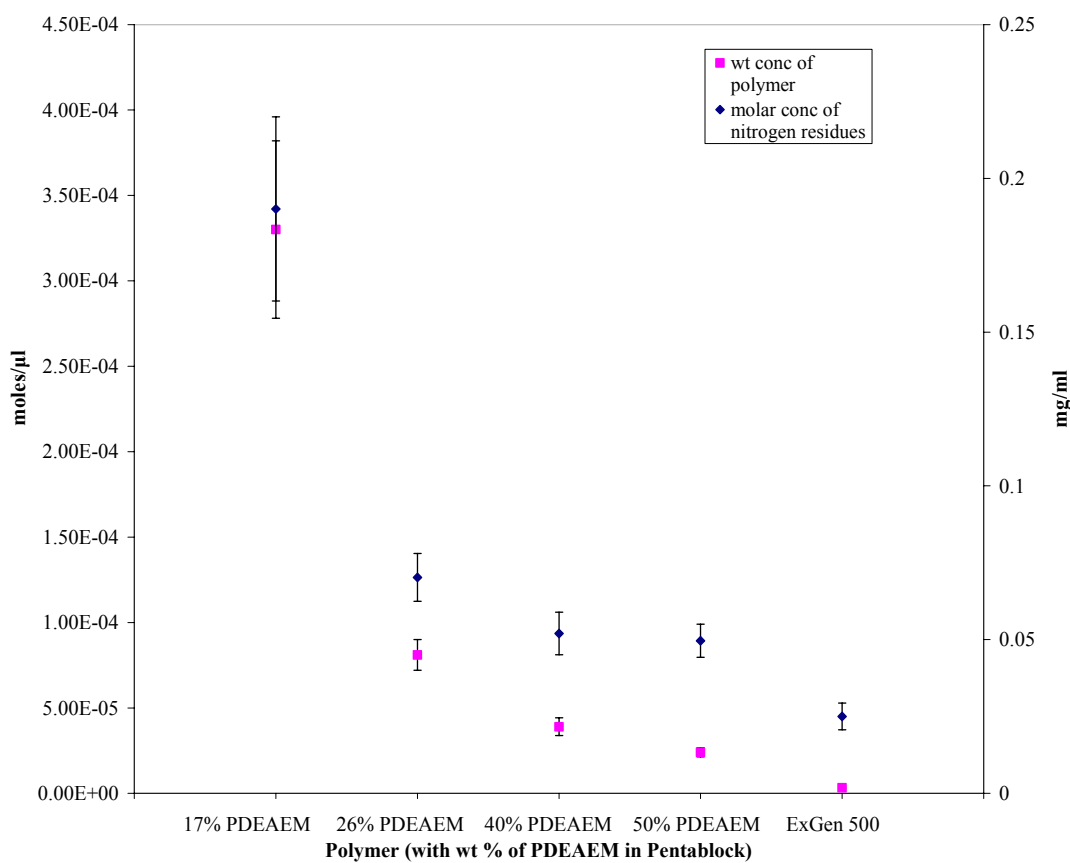
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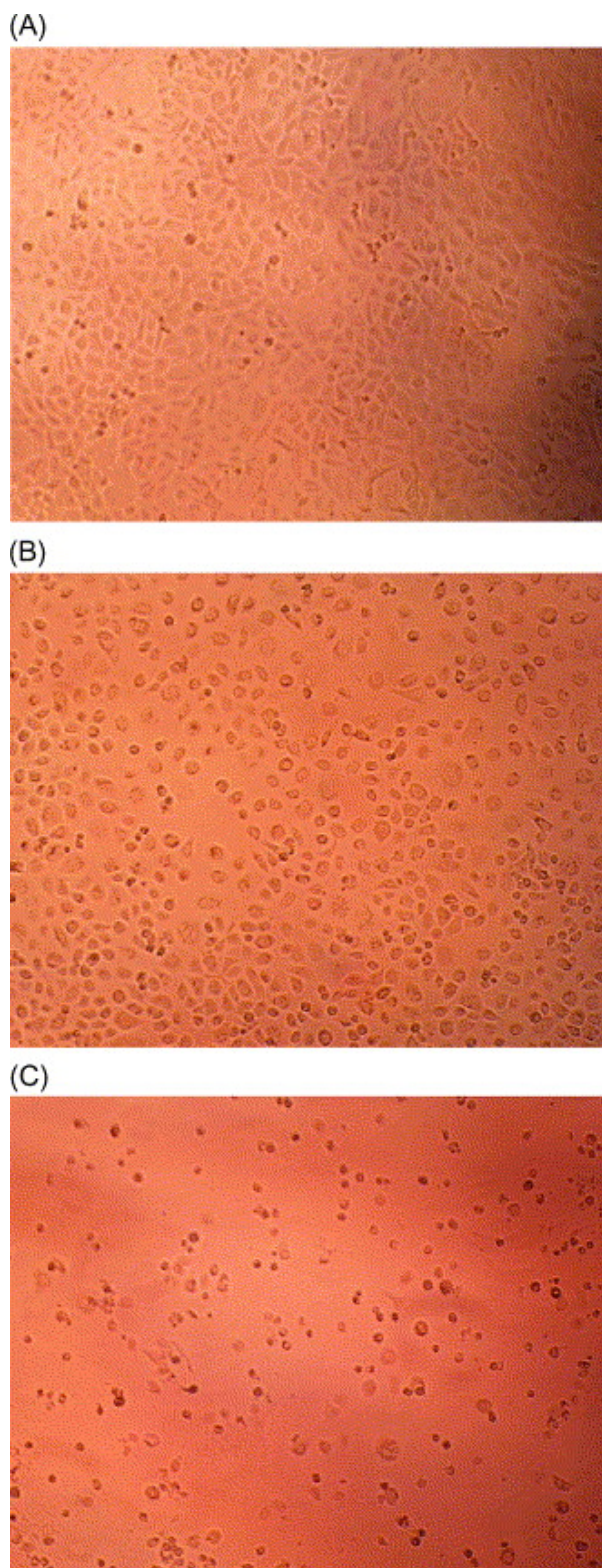
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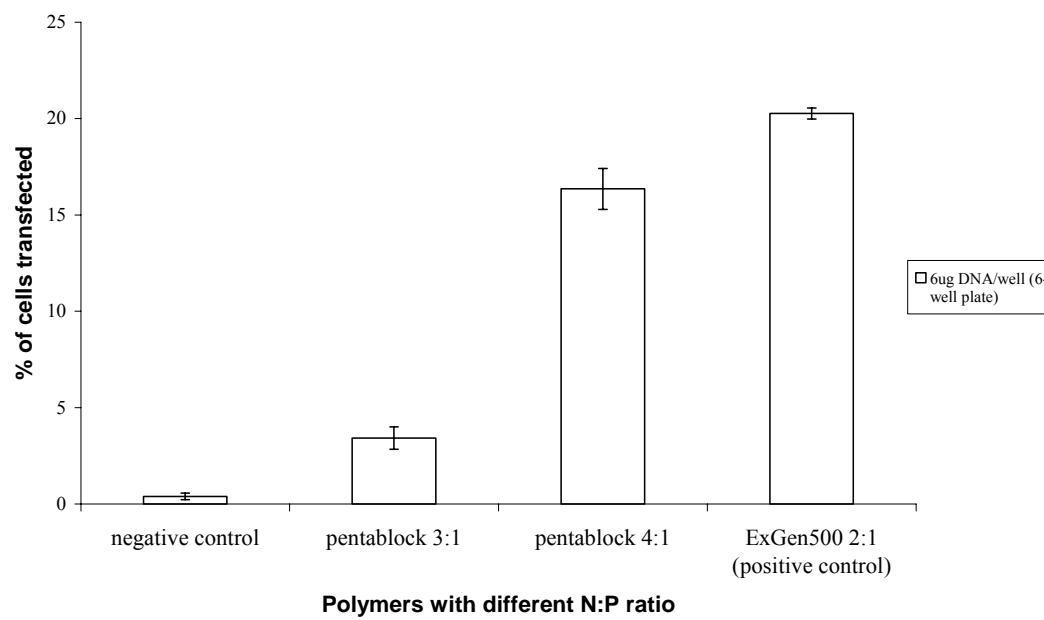
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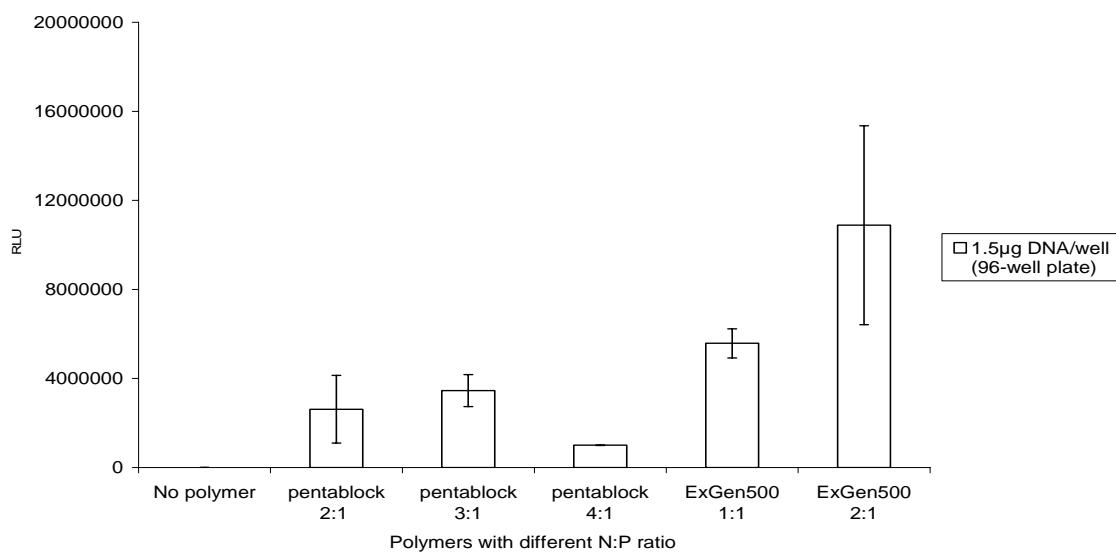
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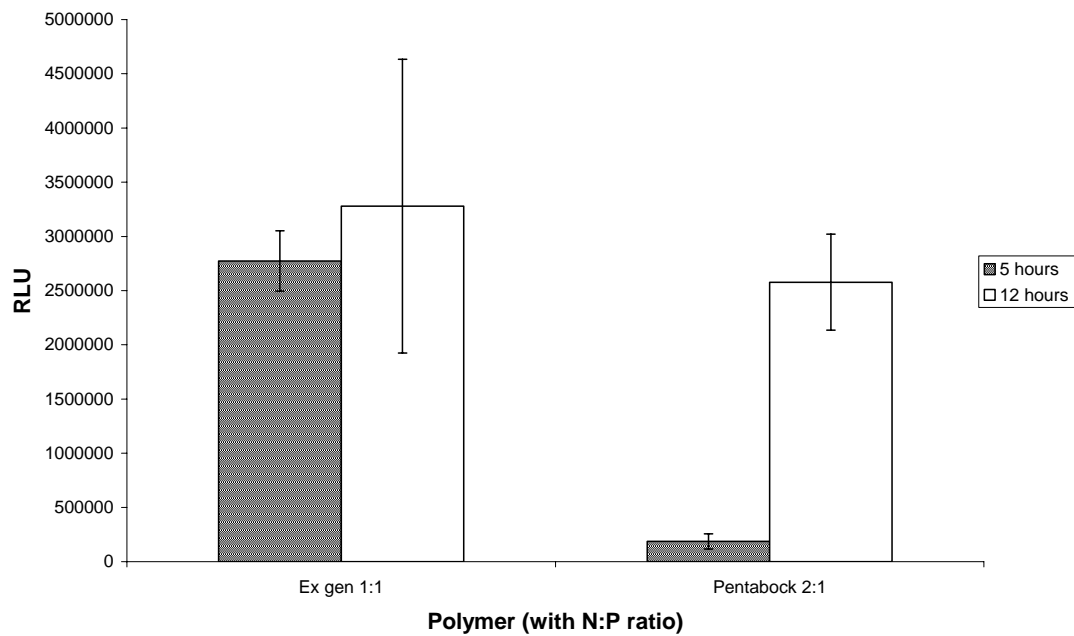
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CHAPTER 4

INVESTIGATION OF *IN VITRO* BIOCOMPATIBILITY OF NOVEL PENTABLOCK COPOLYMERS FOR GENE DELIVERY

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Abstract

Novel pentablock copolymers of poly(diethylaminoethylmethacrylate) (PDEAEM), poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), (PDEAEM-b-PEO-b-PPO-b-PEO-b-PDEAEM) were synthesized as vectors for gene delivery, and were tested for their biocompatibility on SKOV3 (human ovarian carcinoma) and A431 (human epidermoid cancer) cell lines under different *in vitro* conditions using various assays to elucidate the mechanism of cell death. These copolymers form micelles in aqueous solutions and can be tuned for their cytotoxicity by tailoring the weight percentage of their cationic component-PDEAEM. Copolymers with higher PDEAEM content were found to be more cytotoxic, though their polyplexes were less toxic than the polycations alone. Pentablock copolymers displayed higher cell viability than commercially available ExGen 500[®] at similar N:P ratios. While cell death with ExGen[®] was found to be accompanied by an early loss of cell membrane integrity, pentablock copolymers caused very little membrane leakage. Caspase-3/7 assay confirmed that none of these polymers induced apoptosis in the cells. These

pentablock copolymers form thermo-reversible gels at physiological temperatures, thereby enabling controlled gene delivery. Toxicity of the polymer gels was tested using an agarose-matrix, simulating an in vivo tumor model where injected polyplex gels would dissolve to release polyplexes diffusing through the tumor mass to reach the target cells. 25wt% copolymer gels were found to be non-toxic or mildly cytotoxic after 24hr incubation. Transfection efficiency of the copolymers was found to be correlated to cytotoxicity and depended on DNA dose, polymer concentration and N:P ratios. Transgene expression obtained was comparable to that of ExGen[®], but ExGen[®] exhibited greater cell death.

Keywords: Block-copolymers; Cytotoxicity; Cationic; Gene-delivery; Temperature- sensitive

1. Introduction

In the recent past, biocompatible polymers have been widely explored for biomedical applications such as DNA and RNA delivery to targeted cells[1-3], drug carrier systems for proteins and peptides[4,5], and scaffolds for tissue regeneration[6]. The term biocompatibility implies that these polymers are non-cytotoxic, non-immunogenic, and demonstrate an appropriate host response in specific applications[7,8].

Gene therapy treats a genetic deficiency by delivering genetic information in the form of nucleic acids to the targeted cells. Although great advances have been made in identifying target structures for gene therapy, and in the biotechnological production of nucleic acids, the progress has been mainly hampered by the lack of safe gene delivery systems that are efficient and non-toxic[1,9]. A variety of cationic polymers have been proposed and investigated recently for gene delivery[10,11]. Prominent examples include poly-l-lysine (PLL)[12-14], polyethyleneimine (PEI)[15-17], polyamidoamine (PAMAM) dendrimers[18], chitosan[19,20], and methacrylate/methacrylamide polymers[21,22]. Cationic polymers condense negatively charged DNA through electrostatic interactions forming stable complexes called polyplexes. For an efficient DNA-delivery vector, these polyplexes should deliver their genetic payload with minimum damage to the cells such as cell membrane rupture,

inflammation, or apoptosis. However, the existing polymeric gene-delivery systems are either toxic, aggregate *in vivo*, or they do not show good transfection efficiencies[23,24].

Mechanisms and reasons for toxicity caused by polycationic macromolecules are not yet fully understood. It has been noted that the toxicity is dependent on the polymer molecular weight, surface charge density, structure, flexibility, and three-dimensional arrangement of cationic charges [7,25]. Whether this toxicity is mediated by interaction of polycations with the cell membrane, or by activation of some intracellular signal transduction pathway after cellular uptake, is an issue of debate[7,26-28]. Contradictory studies have been reported and there is no general agreement on the causes of polymer toxicity. While Gebhart and co-workers[29] showed that molecular weight (MW) of chitosans did not affect cell viability, a very recently published report[30] shows that chitosan derivatives show dependence on size and MW for both toxicity and transfection efficiency. Florea et al.[26] showed that branched PEI produced similar toxicities across a range of molecular weights in COS-1 and Calu-3 cells, contradicting other studies on PEI done with different cell lines that showed that PEI toxicity increased with an increase in MW[7,31]. Researchers have also shown that transfection efficiency of polymer vectors is correlated to their toxicity[29,32]. Hill et al. studied poly(amidoamine)s (PAAs) and showed that only polymers that exhibited some toxicity were able to transfect A549 cells. Florea et al.[26] found that transfection efficiency of PEI was correlated with toxicity in Calu-3 cells, but not in COS-1 cell line. These studies suggest that toxicity of a particular polymer should be evaluated individually, and on more than one cell line.

Homopolymers of DMAEM (dimethylaminoethylmethacrylate) have previously been shown to complex DNA and transfect COS-7 and OVCAR cells[22]. However, they were toxic and exhibited only up to 3-6% transfection. In addition, good transfection was obtained only at high molecular weights ($M_w > 300$kDa), which is not suitable for renal clearance in *in vivo* applications. Recently, we had reported novel pentablock copolymers PDEAEM-b-PEO-b-PPO-b-PEO-b-PDEAEM synthesized in our laboratory as promising non-viral vectors for gene delivery[33]. While their cationic component PDEAEM is responsible for condensation of DNA and endosomal escape of polyplexes, hydrophilic PEO chains in the copolymer shield the cationic surface charges of PDEAEM thereby decreasing their toxicity. Furthermore,

these copolymers form micelles[34] which facilitate transport across the lipid bilayer of cell membranes. At higher concentrations and physiological temperatures, these micelles self-assemble to form thermo-reversible gels[35]; a characteristic property that can potentially be used to form subcutaneously injectable systems for long-term gene delivery. Self assembly of these copolymers and the properties of their macroscopic gels have been discussed in detail earlier[36,37]. In the present study, we have investigated the effect of various factors such as wt% of PDEAEM in the copolymers, their concentration in media, time of incubation, and N:P ratios (molar ratios of nitrogens (N) in pentablock copolymer to phosphates (P) in DNA), on the toxicity of the polymers and, have attempted to understand the mechanism by which these cationic copolymers cause cell death. Commercially available *in vitro* transfection reagent ExGen 500® has been used as a control for the study. The results obtained are intended to be used to tailor the formulations for *in vivo* studies, where suicide genes can be delivered to the localized tumors in a sustained fashion using injectable thermo-reversible gels as depot of polyplexes, circumventing repeated administration to maintain the therapeutic levels of the protein.

2. Materials and Methods

2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), OptiMEM I®, fetal bovine serum (FBS), 0.25% trypsin-EDTA solution and Hank's Buffered Salt Solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). HEPES salt was obtained form Sigma-Aldrich Co. (#H4034) to make Hepes buffer saline. Lactate dehydrogenase (LDH), and MTT assay kits were also purchased from Sigma-Aldrich Co. (Tox-7 and Tox-1, respectively). *Renilla* luciferase assay system and Caspase-Glo® 3/7 assay kit were purchased from Promega Corporation (Madison, WI). ExGen 500® (written as ExGen henceforth), the *in vitro* transfection reagent, was purchased from Fermentas Life Sciences (Hanover, MD). DNase I was purchased from Ambion (Austin, TX). Low melting agarose of PCR grade from Fisher Scientific (cat#BP2410) was used for making agarose matrices over the cells. Ultrapure water with at least 18 megaohm resistivity was used in all studies.

2.2 Polymers

The pentablock copolymers (Table I) were synthesized using oxyanionic or ATRP reaction schemes, which are discussed in detail elsewhere[34,35]. The chemical structure of pentablock copolymers is shown in scheme 1. Pluronic® F127 $[(PEO)_{100}$-b-$(PPO)_{65}$-b-$(PEO)_{100}]$ was used as the macroinitiator in pentablocks A, B, and C and E, while pentablock D used Pluronic® F68 $[(PEO)_{78}$-b-$(PPO)_{30}$-b-$(PEO)_{78}]$. Pentablock copolymers with different wt% of PDEAEM were investigated for gene delivery. Molecular weights of the pentablock copolymers (Table I), as determined by NMR and gel permeation chromatography (described elsewhere[35]), varied from 15kDa to 22kDa with polydispersities of up to 1.4. The pentablock A (containing 17% PDEAEM by wt) used in this study however had a higher polydispersity of 2.36.

2.3 Cells

The SKOV3 human ovarian carcinoma cell line, and A431 (ATCC CRL-1555), a human epidermoid carcinoma cell line obtained from ATCC™ (Virginia, USA), were used for cytotoxicity and transfection experiments. DU145, a human prostrate cancer cell line, obtained from Iowa Cancer Research Foundation, was also used for some transfection experiments. Cell cultures were maintained in a humidified environment with 5% CO_2 at 37°C and passaged regularly to allow them to remain sub-confluent. Cells were fed with DMEM supplemented with 10% fetal bovine serum (FBS) and 1μM L-glutamine, unless otherwise stated. Neither antibiotics nor antimycotics were used to avoid the possibility of artificial membrane permeabilization effects from these agents.

2.4 Plasmid DNA

A 4.1 kb plasmid encoding *Renilla* luciferase (pRL-CMV) (Promega Corporation, Madison, WI) was used as the reporter gene. DH5α *E.coli* cells were transformed with the plasmid DNA and incubated in selective Luria-Bertani (LB) medium. Amplified plasmid DNA was purified using the Maxi-Prep DNA Purification Kit from Qiagen (Valencia, CA). The concentration and purity of the resulting DNA in a buffer (pH 7.5) of Tris-HCl and

ethylenediaminetetraacetic acid (EDTA) was determined by measuring the absorbance at 260 nm and 280 nm. All DNA used had a 260/280 ratio of at least 1.80.

2.5 Polyplexes

Copolymer to DNA ratios are expressed as molar ratios of nitrogens (N) in pentablock copolymer to phosphates (P) in DNA, and written as N:P. The molecular weight of the DEAEM monomer is 185 and the average molecular weight of a nucleotide is approximately 308. Using the fact that 1μg of DNA contains 3nmol of phosphates, the amount of polymer required for corresponding N:P ratios was calculated (Table I). All polyplexes were formed by the same procedure. Copolymers were first dissolved in Hepes buffer saline (HBS - 20mM of HEPES with 145mM NaCl) pH 7.4, unless otherwise stated, to obtain a concentration of 1mg/ml. This polymer solution was then diluted with the desired media or buffer in a polypropylene tube. After incubating for 5 minutes at room temperature, this diluted polymer solution was added to DNA (in TE buffer) contained in another tube. The tube was gently agitated and allowed to incubate for 30 minutes at room temperature.

2.6 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is an integral cytosolic enzyme that is secreted out in the medium following the rupture of cell membrane[25]. Since the potential site of interaction of cationic macromolecules is the cell membrane, measuring the amount of LDH released in the medium has long been a preferred way to estimate membrane damage[7]. Cells were cultured in 96-well tissue culture plates at a density of approximately 1.2×10^4 cells per well. After incubation overnight, growth media was removed, and replaced with 200μl polymer solutions in appropriate media. Cells were incubated with polymer solutions for 6 to 48 hours, after which 100μL of media was then collected in an optically clear 96-well microtiter plate, and LDH concentration was assayed using a commercial kit (Tox-7 from Sigma-Aldrich Co) according to the supplier's protocol. The absorbance of each well was measured at 490nm using a BioTek EL-340 plate reader (Winooski, USA). Background absorbance at 630nm was subtracted from the main readings. Media alone and media with only cells were used to obtain a background LDH level for normalization. Cells exposed to 0.1% Triton X-100 in

DMEM were used as a positive control, and set as 100% LDH release. The relative LDH release is defined by the ratio of LDH released over total LDH in the intact cells. Less than 10% LDH release was regarded as an acceptable level in our experiments. All samples were run in four replicates, and experiments were repeated twice.

2.7 MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used for the quantitative determination of cell viability. The assay is based on the cleavage of the cell membrane permeable yellow tetrazolium salt MTT into purple formazan by the "succinate-tetrazolium reductase" system (EC 1.3.99.1) which belongs to the respiratory chain of the mitochondria, and is active only in metabolically intact cells.

MTT assay was performed according to the method of Edmondson[38]. After incubating the monolayer of cells with polymer solutions for 6 to 48hrs in a 96-well plate, as described above for the LDH assay, polymer solutions were aspirated and replaced with 200μl of fresh DMEM without serum. 20μl of MTT stock solution prepared in phosphate buffer saline (PBS) pH 7.4 was then added to each well giving a final MTT concentration of 0.5mg/ml. After 4hr of incubation in a CO_2 incubator, the unreacted dye was removed by aspiration and the insoluble formazan crystals were dissolved by incubating with 200μl dimethylsulfoxide (DMSO) for 2h in a humidified atmosphere (37°C, 5% CO_2). Finally, the MTT absorbance was measured at 570nm. Background absorbance measured at 630nm was subtracted from the main readings. Viability was reported relative to control cells not exposed to the polymers.

2.8 Microscopic observations

After incubation with polymers, changes in morphology and detachment of cells from the dish were also observed using an Olympus IMT-2 (Melville, USA) inverted, phase-contrast light microscope equipped with objectives of 10x and 4x magnification.

2.9 Agarose diffusion assay

Our novel pentablock copolymers show sol-gel transitions. At w/w concentrations of 20% or more, aqueous solutions of the copolymers form a gel at physiological temperatures. The gels dissolve in presence of excess water, as the polymer concentration decreases. Polymer concentration in the gels is around 1000 times higher than the concentrations in aqueous solutions at which about 80% of the cells are metabolically viable. However, the polymer gels dissolve slowly, and in an *in vivo* situation where the polymer gel complexed with the therapeutic gene would be subcutaneously injected at the site of tumor, polyplexes would have to diffuse through a mass of tissues to reach the targeted cells. Therefore, to mimic this situation, the polymer gels (containing 25 wt% of polymer) were placed on the top of an agarose gel layer covering SKOV3 cells underneath. The agarose matrices were made in DMEM, containing 10% FBS and 1% agarose. The polymer was allowed to diffuse to the cells for 24 hrs. The experimental model is shown in Fig 1.

This method, derived from the work of Guess et al.[39], was adapted from ISO procedures for cytotoxicity testing (ISO 109993-5. Biological evaluation of medical devices- Part 5: Tests for cytotoxicity: in vitro methods). Monolayers of SKOV3 cells were grown to confluence in 6 well plates, after which the culture medium was replaced with 3ml serum supplemented (10%) DMEM, containing 1% agarose, to generate a protective agarose layer. To avoid heat denaturation of serum proteins, agar was melted and cooled to 45°C before adding it to DMEM containing 10% FBS. The plates were left at room temperature for 15 minutes to let the agarose solidify (melting point 32°C). After the agarose layer was formed, 3ml of vital stain neutral red solution (0.01% in PBS) was added to each well, and the plate was left to incubate for 30min at 37°C and 5% CO_2. Excess dye was then removed and polymer gels were placed at the center on top of these solidified agarose matrixes. The cells were then incubated for another 24hrs. This assay is based on the migration or diffusion of toxic substances from the test article through the agarose to the cellular monolayer. The slow diffusion of leachable substances through the agarose results in a concentration gradient around the test article and a zone of dead cells if the leachable substances are toxic. Sample biocompatibility was estimated by observing cell lysis and zone of dead cells (marked by

decolorized zones) under and around the specimen by light microscopy at 150X magnification.

The decolorized zones were scored as follows: 0 = no decolorization detectable; 1 = decolorization only under the specimen; 2 = zone not greater than 5 mm from the specimen; 3 = zone not greater than 10 mm from the specimen; 4 = zone greater than 10 mm from the specimen; 5 = the total culture is decolorized. Cell lysis was defined as loss of cell membrane integrity, visible in light microscopy. Cell lysis was scored as follows: 0 = no cell lysis detectable; 1 = less than 20% cell lysis; 2 = 20% to 40% cell lysis; 3 = > 40% to < 60% cell lysis; 4 = 60% to 80% cell lysis; 5 = more than 80% cell lysis.

For each specimen, one score was given, and the median score value for all parallels from each specimen was calculated for both the decolorization zone and the lysis zone. The cytotoxicity was classified as follows: 0-0.5 = non-cytotoxic; 0.6-1.9 = mildly cytotoxic; 2.0-3.9 = moderately cytotoxic; 4.0-5.0 = markedly cytotoxic. The median (instead of the mean) was calculated to describe the central tendency of the scores because the results are expressed as an index in a ranking scale.

2.10   Characterization of polymer induced cell death

To elucidate whether the cell death induced by these polymers and polyplexes is apoptotic or necrotic in nature, staurosporine (Sigma #S-6942) was employed as a positive control for apoptosis. Staurosporine is an alkaloid that is a potent inhibitor of phospholipid/calcium-dependent protein kinase (protein kinase C), selectively inducing apoptosis[40]. SKOV3 cells were incubated in 96-well plates with polymers, polyplexes (as described earlier), and 200nM staurosporine for 5h. The effect of staurosporine on LDH release and metabolic activity of the cells was compared to that of the polymers and polyplexes. The Caspase-Glo^{TM} 3/7 Assay was employed to measure caspase-3 and -7 activities in the treated cell cultures. These caspases play key effector roles in apoptosis in mammalian cells, and their presence confirms the induction of apoptosis. The assay provides a proluminescent caspase-3/7 substrate that contains the tetrapeptide sequence DEVD (Asp-Glu-Val-Asp), a caspase-3/7 recognition site. The presence of activated caspases in the cells will result in the cleavage of the substrate, generating a "glow-type" luminescent signal

produced by luciferase. Luminescence is proportional to the amount of caspase activity present. The assay was performed in a 96-well plate with SKOV3 cells, according to the supplier's protocol. Briefly, after incubating the cells with the test compounds for specified times in a white-walled 96-well plate, 100μl of Caspase-Glo^{TM} 3/7 Reagent was added to each well containing 100μl of treated cells in culture medium. Prior to this, culture plates and reagent were allowed to equilibrate to room temperature. Luminescence was measured on a Veritas^{TM} Microplate Luminometer after incubating the treated cells with the reagent for 1 to 3hrs.

2.11   Luciferase Transfection

In order to determine the total protein expressed by a reporter gene per total cellular protein, a luciferase assay was employed, using pRL-CMV as the reporter gene. Cells were seeded in a 96-well plate up to 70% confluency prior to transfection, and were then transfected with various polyplex solutions in 200μl OptiMEM I® using 1μg of DNA per well, unless otherwise stated.   After 4 to 10 hrs incubation in OptiMEM I®, the solution was replaced with fresh DMEM containing 10% FBS, and incubated for 44hrs. Cells were then lysed using a lysis buffer (*Renilla* Luciferase Assay Lysis Buffer, Promega) and the luminescence of the expressed reporter protein was measured on an automated Veritas^{TM} Microplate Luminometer using the Promega *Renilla* Luciferase Assay System (Madison, USA). ExGen was used as a positive control, and was expected to yield high efficiency of transfection. Cells exposed only to DNA (without polymer) were used as negative controls.

Total recovered cellular protein content of the cells was determined by a modified Bradford assay, using a CB-Protein Assay^{TM} Kit. Bovine serum albumin standards were prepared. Twenty microliters of the samples from luciferase detection protocol were placed in individual wells of a 96-well plate and diluted with nanopure water to 100μl. CB-Protein Assay^{TM} reagent (100μl) was added into each well and mixed well. The plate was allowed to incubate for 15 minutes and absorbance was measured at 595nm. The amount of protein was read from the standard curve.

2.12   Statistics

Where appropriate, the data is presented as mean and standard deviation (SD). Four samples were used for each case in all the experiments and, mean and SD were calculated over them. Significant differences between two groups were evaluated by Students' t-test and between more than two groups by one-way ANOVA analysis of variance, followed by Tukey's test. The level of significance was set at $p<0.05$, unless otherwise stated.

3   Results

3.1 Effect of different wt% of PDEAEM on pentablock copolymer cytotoxicity

Pentablock copolymers containing different wt% of PDEAEM were incubated with SKOV3 and A431 cell lines in DMEM (10% FBS) for an extended period of 48hr to determine the effect of the cationic block on the polymers' cytotoxicity. After 48hrs, the damage to cell membranes, and their residual metabolic activity were evaluated using LDH and MTT assays, respectively. Figs 2a and 2b show trends in the membrane leakage and cell viability of SKOV3 cells respectively, and Figs 3a, 3b give the same trends for A431 cell line respectively. Two things are evident from these graphs. First, as the wt% of PDEAEM increases in the copolymers, there is a gradual increase in the damage to the cell membrane and a decrease in cell viability. Second, for each copolymer (except pentablock D containing 60% PDEAEM for A431 cells) a concentration can be noted from the graphs below which the polymer causes less than 10% membrane damage and/or allows more than 80% of the cells to still be metabolically viable. The copolymer containing 60% PDEAEM was found to be cytotoxic to A431 cells at almost all concentrations. The copolymers provide a unique way to tune the cytotoxicity for efficient use in gene therapy, as opposed to cationic polymers such as ExGen whose cytotoxicity can be controlled only by reducing the amount of polymer used. These results suggest that below a certain concentration, pentablock copolymers can have extended tissue-contact times of up to 48hrs without causing significant damage to the cells, which is of significance for *in vivo* studies. Also, it can be observed that A431 cells are slightly more sensitive to the copolymers than SKOV3, exhibiting more toxicity at similar polymer concentrations.

3.2 Toxicity increases with exposure time

During *in vitro* transfection, cells were incubated with polyplexes in a low serum media OptiMEM I® to avoid polymer loss due to binding with serum proteins. Since cells need to be grown in 10% serum supplemented media for good protein expression of the transfected gene, they were first incubated with polyplexes for a limited time, and then the polymer solutions were replaced with fresh media containing FBS to let the transfected gene be expressed.

Fig 4 shows the effect of incubation time of polyplexes with the cells on their toxicity. Polyplexes of pentablock copolymer B (26% PDEAEM), containing 1μg plasmid pRL-CMV and different amounts of copolymer, were incubated with the SKOV3 cells for 6 to 14hr in OptiMEM I®. For all polymer concentrations, it was observed that cell viability decreased on extending the incubation time of polyplexes with the cells. Typically at 50μg/ml, it decreased from 100% to 60% when incubation time was extended from 6 to 14hr. After 14hr of exposure, cell viability was reduced to 60% even at lower copolymer concentrations of 30μg/ml.

Cells were also incubated with just polymer solutions to examine how DNA complexation affects their cytotoxicity. Figs. 5a,b show that polyplexes were less toxic to the cells than the cationic polymers alone. The cellular membrane damage was significantly reduced by the DNA complexation to the polymer, possibly due to the shielding of positive charges on the polycations. At N:P ratio of 8, with polymer concentration of ~80μg/ml, the LDH release reduced from 86% to 7% after DNA complexation. Significant increase in the metabolic activity was also found at higher polymer concentrations corresponding to N:P ratios of 6 and above. This is in agreement with results seen by researchers for other polymers[7,30,41].

Toxicity of pentablock copolymers was compared to ExGen at concentrations corresponding to same N:P ratios, thus actually comparing the concentration of their cationic components that are responsible for DNA condensation and endosomolysis. SKOV3 cells were incubated for 6hrs with the polyplexes of two polymers in OptiMEM I®. Fig 6a shows that polyplexes of ExGen caused extensive cell membrane damage, as compared to that caused by pentablock copolymers. At a high N:P ratio of 8, while pentablock copolymers

showed only 10% LDH release, ExGen showed up to 85% LDH release. Further, as seen in Fig 6(b), MTT assay showed that at all N:P ratios, cell viability was significantly higher in the presence of the pentablock copolymers than with ExGen. These results clearly suggest that pentablock copolymers are less cytotoxic than ExGen, and perhaps the two polymers interact with the cells in a different fashion, inducing cell death by different mechanisms.

3.3 Apoptosis vs necrosis

Apoptosis is the carefully regulated process of cell death[42]. In contrast to the swelling and membrane rupture in necrosis, a cell undergoing apoptosis rapidly condenses into small enclosed fragments, which can then be phagocytosed by neighboring cells. Apoptosis can be characterized by the loss of mitochondrial membrane potential, activation of caspases, loss of plasma membrane asymmetry, and the condensing and eventual fragmentation of the cellular DNA[43].

Damage to the cell membrane, which can be determined by the LDH assay, is known to occur either in necrotic cells or in the late stage of apoptosis. On the other hand, loss of mitochondrial inner transmembrane potential is often associated with the early stages of apoptosis and may be one of the central features of the process[44]. Collapse of this potential results in the decoupling of the respiratory chain, which reduces the ability of dying cells to reduce compounds such as tetrazolium salt MTT into colored formazan product, as can be determined by the MTT assay. Since staurosporine selectively induces apoptosis into the cells, its effect on the LDH and MTT assays can be compared to those of ExGen and pentablock copolymers to try to understand the mechanism of cell death.

As expected in apoptosis, Fig 7 shows that cells incubated with 200nm staurosporine for 5hrs gave little LDH release, even though their metabolic activity was almost reduced to zero. Similar to this, as the concentration of pentablock copolymers increased, there was a significant decrease in metabolic activity of cells, but no significant change in the cell membrane damage. This hints that cell death in the presence of the pentablock copolymers might not be by necrosis, but might be through an apoptotic route. In contrast, as the concentration of ExGen increased, the decrease in metabolic activity of the cell population was accompanied with large LDH release. This indicates that cell death caused by the rapid

loss of membrane integrity (necrosis) primarily accounted for the decrease in number of viable cells in the presence of ExGen.

The Caspase-Glo[TM] 3/7 Assay was performed to check the induction of apoptosis in the cells. SKOV3 cells were incubated with polymer-DNA complexes at different N:P ratios, and the activity of caspases was measured at three different times of incubation- 3hrs, 4hrs, 6hrs. Different polymer concentrations and times of incubation were used to empirically find the conditions that would activate caspases in the cells. Staurosporine was used as the positive control. Fig 8 shows the results of these caspase assays. Staurosporine induced apoptosis by activating caspases after 4hrs and 6hrs of incubation, but no caspase activity was detected after 165 minutes incubation. However, no significant caspase activity was detected at any incubation time for any tested N:P ratio of pentablock copolymers' or ExGen polyplexes. At higher incubation times or higher polymer concentrations, increased cell death was clearly visible using light microscopy, as it has been shown in LDH and MTT assays above.

These results indicate that neither ExGen nor pentablock copolymers induced apoptosis in the treated cells. However, they did affect the cells differently. Unlike ExGen, that extensively ruptured the cellular membrane leading to cell death, pentablock copolymers appear to cause cell death by some alternative mechanism[45-47]. This has implications in minimizing the inflammatory processes accompanying cell death in the presence of the pentablock copolymers as opposed to the presence of ExGen.

3.4 Luciferase Transfection

The transfection efficiency of polymers depends on the amount of polymer used (N:P ratio) to condense the DNA, the amount of the DNA dose, and time for which cells are incubated with the polyplexes. The upper limit of all these factors in turn depends on the cytotoxicity of the polymer. As N:P ratio increases, amount of free polymer in the media increases, increasing the cell death, thereby effectively decreasing reporter protein expression. A higher DNA dose would generate more reporter protein, but will require higher amount of polymer for condensation, thus again being limited by the polymer toxicity profile. Also, increased exposure of cells to the polyplexes would allow polyplexes an

extended period of time to enter into the cells, but that would again increase the toxicity, as shown above in Fig 4.

Keeping these above stated factors in mind, several different carcinoma cell lines were transfected with luciferase plasmid using pentablock copolymers, while ExGen was used a positive control. Ideal N:P ratios and ideal incubation times of polyplexes with the cell lines were obtained empirically for both the polymers, following manufacturer's protocol for ExGen. Fig 9 shows the transfection obtained with pentablock B using 1μg pRL in SKOV3, A431 and DU145 cells. Polyplexes were incubated for 10hrs with the cells. As can be observed, pentablock copolymers were able to transfect all three cell lines. However, amount of gene expression obtained at similar N:P ratios was different in different cell lines. While luciferase expression increased significantly in all three cell lines by increasing N:P ratio from 4 to 6, the increase was much higher in DU145 cells than in SKOV3 or A431 cell lines. Also, while in SKOV3 cells luciferase expression peaked off at N:P ratio 8 and then decreased, it was almost the same (not significantly different) for different N:P ratios in A431 cells.

As observed in Figure 9, the amount of luciferase protein expressed in the SKOV3 cells increased with the amount of copolymer used to condense the DNA (N:P ratio). However, after a certain polymer concentration, the luciferase expression decreased on further increasing the N:P ratio. Similar trends were observed in Figure 10 for ExGen. Toxicity of the polymers at these N:P ratios can be correlated from Figures 5 and 6.

In Figure 11, comparing the transfection efficiency of pentablock copolymers and ExGen, it can be observed that maximum amount of luciferase expressed using pentablock copolymer E was approximately only 19 times less than that by given by ExGen. Polyplexes of both the polymers were incubated in OptiMEM I® with the cells for 3.5hrs using 0.6μg of pRL/well in a 96-well plate. It should be noted here that ExGen has been optimized over the years to give this good transfection, while optimum formulation of pentablock copolymers is still being investigated. Again, this figure also shows that there is an optimum concentration for both the polymers at which they give maximum transfection. Above that concentration, increased toxicity camouflages expression of the transfected gene.

Luciferase activity(RLU) in each well is not normalized by the total amount of protein(mg) as that gives artificially high values(RLU/mg) in the samples where total protein level has been reduced by the cell death. Instead, since all experiments were performed with same initial number of cells per well (~1.2 x 10^4) in a 96-well plate, luciferase expression is reported as RLU/well for each case.

3.5 Polymer gel cytotoxicity studies

Since the agarose matrix was transparent, images of the cells around the polymer gel and directly below the gel were taken using a light microscope after 24hrs of incubation. Fig 12 shows these images of cells at 60x and 150x magnification. Since the cells were treated with the vital stain Neutral Red before placing the polymer gel on them, a decolorized zone on the plate can be observed if there were any cell deaths. The images show that there was a small decolorized spot (diameter < 1mm) directly below the polymer gel. However, the cells around it were stained red and seemed to be as healthy as those far away from the polymer gel. There was also no visible significant cell lysis around the polymer gel. The polymer gels of pentablock copolymers A, B and C were tested, and all of them appeared to be either non-cytotoxic, or mildly cytotoxic. The results with their cytotoxicity scores have been summarized in Table II. The modulus and dissolution rate of these polymer gels depend on their molecular weight (MW)[35]. Copolymers with higher MW chains form stronger gels that dissolve over a longer period. Therefore pentablock B, which had lowest M_w (weight average MW), did not have a high modulus at 25 wt%, and dissolved and spread faster on the agarose matrix thus, causing cell death over larger radii.

4   Discussion

In this study we have evaluated cytotoxicity of new pentablock copolymers under different *in vitro* conditions. Different assays and cell types were used to determine various aspects of toxicity. Molecular weight of the synthesized copolymers was maintained below 20kda, as that is the cut-off mass for renal excretion by the kidney, thus assuring final removal of copolymer from the body in an *in vivo* study. Copolymers with different wt% of PDEAEM blocks were studied to assess the effect of this cationic group on copolymers'

toxicity. PDEAEM blocks of the copolymers, containing tertiary nitrogens, condense DNA and, are responsible for their pH buffering capacity that helps in the release of polyplexes from endosomes. Therefore, increasing PDEAEM content in the copolymers would increase both the amount of DNA they can condense, and their transfection efficiency. However, it was observed that as the wt% of PDEAEM increased from 17% to 60%, cell viability decreased significantly in SKOV3 and A431 cancer cell lines. Copolymers were incubated with cells in FBS supplemented DMEM for 48hrs at different concentrations. Copolymers with higher percentages of PDEAM (pentablock C and D) caused more leakage of the cell membrane, followed by decrease in metabolic activity, while those with up to 26% PDEAEM did not cause much cell membrane damage even at high concentrations. This increase in toxicity of copolymers can be explained by the fact that copolymers with higher wt% of PDEAEM block have higher cationic surface charge, and thus higher charge density. This higher cationic charge resulted in increased damage to the anionic cell membranes, as has been suggested by researchers for other polycations[7]. However, for all the copolymers (except for pentablock D on A431 cells) a concentration can be determined from the graphs below which they exhibited less than 10% LDH release and/or more than 80% cell viability even after 48hrs of incubation. This is of great significance for gene therapy applications involving longer tissue-contact time *in vivo*.

A431 cells were found to be more sensitive to the copolymers, exhibiting comparatively less cell viability than SKOV3 cells at same polymer concentrations, especially in terms of cell membrane damage. This might be due to the different compositions of the membranes and glycocalyx of different cell lines[7].

Since pentablock copolymers C and D, containing 40 and 60wt% PDEAEM respectively, showed good transfection only at concentrations where high cell death was also observed (transfection data not shown), further detailed screening of other *in vitro* conditions was reported only with the pentablock copolymer B(26% PDEAEM).

Polyplexes were found to be less toxic than the polycations alone. Complexing DNA with the polymer reduced the LDH release by up to 80% at N:P ratio of 8. A significant increase in cell metabolic activity was also observed. This indicates that free cationic copolymers perhaps damage cells due to their positive surface charge interacting with

cellular lipid membranes and other internal cell organelles. On binding with DNA, some of this surface charge is shielded, thus reducing their toxicity. Changes in the conformation of polycationic macromolecules on binding with DNA might also be a reason for this reduced toxicity. This is very much in agreement with other researchers[7,41], but in contrast with Gebhart et al[29], who reported using an MTT assay with Cos-7 cells that polyplexes of PEI(50K) and ExGen reduced the percent survival of cells by 40% compared to the polycations alone. At higher N:P ratios, though some of the polycation is used to condense DNA, the rest of it is available in the free charged form to interact with the cells, thus explaining decrease in cell viability.

Toxicity of the polyplexes of pentablock copolymers was found to increase as their time of incubation with the cells in OptiMEM I® increased from 6 to 14hrs. Though at lower concentrations, polyplexes were not toxic even up to 10hr of incubation time, at higher concentrations, cell metabolic activity decreased significantly. This suggests that to transfect cells *in vitro*, there is an upper time-limit for which polymer-DNA solutions can be incubated with the cells in OptiMEM I®. After that, the polymer-DNA solution should be replaced with fresh media containing FBS for good growth of the cells.

The toxicity of pentablock copolymers was compared to ExGen (22kda linear PEI). Both polymers seemed to affect the cells in different ways. While ExGen caused extensive damage to the cell membrane integrity, followed by a decrease in metabolic activity of the cells, pentablock copolymers showed no significant cell membrane damage even when the metabolic activity decreased below 80%. At the same N:P ratios, however, pentablock copolymers exhibited significantly higher cell viability than ExGen.

These results led to investigation of the different mechanisms by which the two polymers interact with the cells. The cytotoxicity of polycationic macromolecules with different structures is influenced by various properties such as molecular weight, charge density, three dimensional arrangements of the cationic residues, structure, and conformational flexibility[25,48]. The types of amines in the polymer have also been reported to play a role in the toxicity. Ferruti et al[13] had reported, based on his study with modified PLL, that polymers with tertiary amines exhibit lower toxicity that those with primary and secondary residues. Dekie et al[49] had also noted that presence of primary amines on the poly

L-glutamic acid derivatives had a significant toxic effect on red blood cells. The observations presented here agree with other studies and show that that the pentablock copolymers, that have tertiary nitrogens, are significantly less toxic than ExGen, which has series of primary nitrogens, even though their molecular masses are almost the same (close to 20kda).

Charge density of the polycations, resulting from the number and three dimensional arrangement of the cationic residues, together with the flexibility of the polycations, is another important factor influencing cytotoxicity. These factors determine the accessibility of the cationic charges to the cell surface[50,51]. Rigid molecules have more difficulty in attaching to the cell membrane than flexible ones. Interaction of cationic macromolecules with membrane proteins and phospholipids disturbs membrane function and structure[28,52]. ExGen is a linear and flexible polycation with a very high charge density (248nmol of nitrogen residues per μg), thus causing more damage to the anionic cell membranes. On the other hand, pentablock copolymers which exist as spherical micelles in an aqueous environment, have more of a globular structure with comparably less charge density (1-3nmol of nitrogen residues per μg), thus causing less damage to the cell membrane. Other such examples of polymers that show good biocompatibility because of their globular structures are PAMAM and cHSA (cationized human serum albumin), as reported by Fischer et al[7]. Another advantage of pentablock copolymers is that they have hydrophilic chains of PEO that shield the surface charge of the cationic PDEAEM, further decreasing their toxicity. In addition to this, the hydrophobic chains of PPO, which are known to interact with the cellular lipid membranes inducing structural changes[53,54], enable easy access of pentablock copolymers into the cells and help in their translocation within the cells[55,56]. Furthermore, Pluronic® micelles had been shown to enhance sealing of permeablized membranes damaged by ionizing radiations or electroporation, thus preventing cell necrosis[57,58]; and increasing the rate of wound and burn healing[59,60]. Since pentablock copolymers form similar micelles, they might also be exhibiting these biological-response modifying activities of Pluronic®, thus explaining less cell membrane damage caused by the copolymers. These characteristics of pentablock copolymers are advantageous for *in vivo* studies, since less leakage of cell membranes of the treated cells would cause less inflammation to the surrounding cells.

The mechanism of cytotoxicity caused by polycations is not fully understood. In this study, two different possible mechanisms were observed. While the toxic effects of ExGen seemed to principally result from its interaction with the cell membrane, causing rapid rupture of cell membrane, followed by decrease in metabolic activity; the cytotoxicity of pentablock copolymers appeared to involve some mechanism other than just a membrane lytic effect.

To obtain further insight into the mechanism of cell death, and differentiate between apoptotic and necrotic routes, a Caspase-Glo[TM] 3/7 assay was employed. Demonstration of biochemical changes in the cells such as activation of caspases is commonly used to characterize apoptosis. Cells treated with positive control Staurosporine showed high activity of caspases, thus confirming induction of apoptosis. Staurosporine induced cell death showed typical features of apoptosis in MTT and LDH assays, such as complete loss of metabolic activity while cell membrane was still intact. For ExGen and pentablock copolymers, different polymer concentrations were tested for different incubation times with the cells. However, no significant caspase activity was detected for any of them, indicating that apoptosis was not occurring with either of the polymer. MTT assays however indicated reduced cell viability at these concentrations. This suggests that cells were dying, but not by apoptosis. An early and rapid loss of plasma membrane integrity by ExGen suggests a necrotic type of cell death, as noted by other researchers[7]. However, since pentablock copolymers neither cause damage to the cell membrane, nor induce apoptosis, but still reduce metabolic activity at high concentrations, additional mechanism of cytotoxicity involving an intra-cellular route seems to be involved. There is a possibility that these polycations have specific interactions with a membrane component after cellular uptake[45,46], that activate some signal transduction pathways inside the cell[47], leading to cell death.

Pentablock copolymers were tested for their transfection efficiency on different cell lines under various *in vitro* conditions using a luciferase plasmid. Pentablock copolymer B, containing 26% w/w PDEAEM, gave appreciable transfection in all the three cell lines tested. Since different cell lines have different composition of their membranes and glycocalyx, and different cell division rates, the rates of entry of polyplexes across the cell membrane and nuclear membrane differ in each of them. This affects both, the toxicity and

the transfection efficiency of the polymers, and explains why the level of luciferase expression was different in different cell lines at similar N:P ratios. While transfection in SKOV3 cells peaked at N:P ratio 8 and then decreased at N:P 10, it was not significantly different in A431 cells at N:P ratios of 6, 8, and 10. These results suggest that different cell lines have different optimum conditions under which they give best transfection, and should therefore be evaluated individually.

Fig 9 and 10 show that the transfection efficiency of polymers in SKOV3 cell line peaks at certain N:P ratios and decreases after that. This means that above these N:P ratios, the toxicity of polymers increases to the extent that the cells die before they can express the transfected gene. Thus, at those high N:P ratios, even though transfection is good, not too many cells are left viable to express the transfected gene, thereby displaying less luciferase expression per well. The only way to work at higher N:P ratios and obtain high transfection while not increasing the polymer toxicity is to reduce the amount of DNA dose, as demonstrated in experiments with pentablock copolymer E (containing 28% w/w PDEAEM) (fig 11). The maximum amount of total luciferase expression obtained in SKOV3 cells with pentablock E was only 19 times less than the maximum given by ExGen. Two reasons can well explain this high gene expression by pentablock E in figure 11 compared to pentablock B in figure 10(a). First, it had slightly higher content of PDEAEM than pentablock B, thus being able to condense more DNA per polymer micelle. Second, in experiments with pentablock E, only 0.6μg of pRL/well was used (instead of 1μg used otherwise). Since lesser amount of polymer was required to condense 0.6μg DNA than 1μg DNA, it was possible to work at high N:P ratios (up to 10:1) with 0.6μg DNA and get higher transfection without compromising toxicity. It should be noted that with pentablock B complexed with 1μg plasmid (fig 10a), luciferase expression peaked at N:P 8:1, and it decreased beyond that value. These results clearly indicate that transfection efficiency of the polymers is critically correlated with their cytotoxicity, and that it can be optimized by adjusting the DNA dose and corresponding N:P ratios. Another thing to be noted is that though ExGen gives higher transfection than pentablock copolymers, it is also accompanied with high toxicity, as shown in figure 6 for corresponding N:P ratios. So some of the transfection observed might be from the cells that had subsequently died.

The agarose-matrix experiments simulating a tumor-model suggested that polymer gels, containing up to 1000 times higher polymer concentration than that used for experiments in liquid growth medium, did not kill the cells after diffusing through the agarose-matrix. Pentablock copolymers A, B and C, containing different wt% of PDEAEM block were tested on these agarose-gel matrices. Polymer gels of all these polymers were found to be non-toxic or mildly toxic. This indicates that in an *in vivo* experiment, a polymer gel can be implanted at the site of tumor, releasing polymer micelles over a period of time, without causing significant cell death in the vicinity. Thus, such a gel could even deliver genes complexed with the polymer in a sustained fashion to the targeted cells, providing extended gene expression, and maintaining desired level of the expressed therapeutic proteins without repeated injections. Complexes of DNA and pentablock copolymers have also been found to form gels, as reported earlier by our group[33], and these gels were found to dissolve in excess buffer to release complexed plasmids, and not free plasmid.

It should be clearly noted here that in our polymer-DNA gel system, gels dissolve to release polyplexes, which are subsequently up taken by the targeted cells. Recently, several polymer systems, like hydrogels of gelatin[61], implantable polymer matrices (EVAc: poly (ethylene-co-vinyl acetate)) and injectable microspheres (PLGA and PLA)[62], have been reported in the literature that encapsulate naked DNA and release it in a controlled fashion to the cells. In these cases, however, the encapsulation materials are inert and do not aid in the transfection. No system has been reported till date to our knowledge that delivers complexed DNA (polyplexes) to the cells in a sustained fashion. Since polymer-DNA complexes give much higher transfection than naked DNA, it's evident that our novel polymer-DNA gels can be instrumental in improving the gene therapy.

5   Conclusions

Pentablock copolymers were tested for their cytotoxicity under various *in vitro* conditions. Toxicity of these copolymers was found to increase relative to the wt% of their cationic block PDEAEM, and can thus be tuned by tailoring the cationic content in the copolymers while still preserving their DNA complexation properties. Below certain

concentrations, different pentablock copolymers could be incubated with cells in complete media for up to 48hr without exhibiting significant cell death. Polyplexes were found to be less toxic than polycations alone, as DNA condensation shields their surface charges. However, polyplexes caused more cell death at longer incubation time with the cells. Polyplexes of pentablock copolymers were found to be much less toxic than ExGen. While ExGen caused rapid loss of cell membrane integrity, followed by decrease in cell viability; pentablock copolymers caused less than 10% membrane leakage even at high concentrations where metabolic activity was reduced to less than 80%. None of the polymers however were found to induce apoptosis in the cells. Pentablock copolymers were hypothesized to cause cell death by activating some signal transduction pathway once they get into the cells. Optimum conditions that showed maximum transgene expression with minimal cell death were obtained by varying the DNA dose, the polymer concentration and N:P ratios. The transfection was found to be correlated to the toxicity of the polyplexes. Transfection obtained with pentablock copolymers was comparable to that shown by ExGen. Pentablock copolymers form thermo-reversible gels at higher concentrations and physiological temperatures. The agarose-matrix experiments with the 25wt% polymer gels proved that they were non-toxic or mildly toxic. This suggests that if formed subcutaneously at the site of tumors, pentablock copolymer gels can release polyplexes over a period of time, which can then diffuse through tumor tissues to the targeted cancer cells without damaging neighboring healthy tissues. The thermo-reversible gelation features along with the good transfection efficiencies and tunable cytotoxicities make these new copolymers promising vectors for gene delivery.

References

1. Schmidt-Wolf GD, Schmidt-Wolf IGH. Non-Viral and hybrid vectors in human gene therapy: an update. TRENDS in Molecular Medicine 2003;9(2):67-72.
2. Merdan T, Kopecek J, Kissel T. Prospects of cationic polymers in gene and oligonucleotide therapy against Cancer. Adv. Drug Delivery Reviews 2002;54:715-758.
3. Brigger I, Dubernet C, Couvreur P. Nanoparticles in cancer therapy and diagnosis. Advanced Drug Delivery Reviews 2002;54(5):631-651.
4. Jeong B, Kim SW, Bae YH. Thermosensitive sol-gel reversible hydrogels. Advanced Drug Delivery Reviews 2002;54(1):37-51.
5. Kost J, Langer R. Responsive polymeric delivery systems. Advanced Drug Delivery Reviews 2001;46(1-3):125-148.
6. Lauffenburger DA, Schaffer DV. The matrix delivers. Nature Medicine 1999;5(7):733-734.
7. Fischer D, Li Y, Ahlemeyer B, Krieglstein J, Kissel T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials 2003;24:1121-1131.
8. De Groot CJ, Van Luyn MJA, Van Dijk-Wolthuis WNE, Cadee JA, Plantinga JA, Otter WD, Hennink WE. In vitro biocompatibility of biodegradable dextran-based hydrogels tested with human fibroblasts. Biomaterials 2001;22(11):1197-1203.
9. Verma IM. A Tumultuous Year for Gene Therapy. Molecular Therapy 2000;2(5):415-416.
10. Liu F, Huang L. Development of non-viral vectors for systemic gene delivery. Journal of Controlled Release 2002;78(1-3):259-266.
11. Schatzlein AG. Non-viral vectors in cancer gene therapy: Principles and progress. Anti-Cancer Drugs 2001;12(4):275-304.
12. Gondsho A, Irie K, Susaki H, Iwasawa H, Okuno S, Sugawara T. Tissue-targetting ability of saccharide-poly(L-lysine) conjugates. Biol. Pharma. 1994;17:275-282.
13. Ferruti P, Knobloch S, Ranucci E, Gialnasi E, Duncan R. A novel chemical modification of poly-L-lysine reducing toxicity while preserving cationic properties. Proc Int Symp Control Rel Bioact Matter 1997;24:45-46.
14. Zauner W, Brunner S, Buschle M, Ogris M, Wagner E. Differential behaviour of lipid based and polycation based gene transfer systems in transfecting primary human fibroblasts: a potential role of polylysine in nuclear transport. Biochimica et Biophysica Acta (BBA) - General Subjects 1999;1428(1):57-67.
15. Boussif O, et al. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethyleneimine. Proc. Natl. Acad. Sci. USA 1995;92:7297-7301.
16. Iwai M, Harada Y, et al. Polyethylenimine-mediated suicide gene transfer induces a therapeutic effect for hepatocellular carcinoma in vivo by using an Epstein-Barr virus-based plasmid vector. Biochim, Biophys. Res. Commun. 2002;291:48-54.
17. Godbey WT, Barry MA, Saggau P, Wu KK, Mikos AG. Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. J Biomed Mater Res 2000;51(3):321-8.
18. Tang MX, Fedemann CT, Szoka Jr FC. In vitro gene delivery by degraded polyamidoamine dendrimers. Biocnjug. Chem. 1996;7:703-714.
19. Richardson SCW, Kolbe HVJ, Duncan R. Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA. International Journal of Pharmaceutics 1999;178(2):231-243.
20. Erbacher P, Zou S, Bettinger T, Steffan AM, Remy JS. Chitosan-based vector/DNA complexes for gene delivery: biophysical characteristics and transfection ability. Pharmaceutical Research 1998;15(9):1332-1339.

21. P. Van De Wetering, N.M.E. Schuurmans-nieuwenbroek, M.J. van Streenbergen, D.J.A. crommelin, W.E. Hennink. Co-polymer of 2-(dimethylamino)ethyl methacrylate with ethoxytriethylen glycol methacrylate or N-vinyl-pyrroliodone as gene transfer agents. J. Control. Release 2000;64:193-203.

22. van de Wetering P, Cherng JY, Talsma H, Crommelin DJA, Hennik WE. (2-dimethylamino)ethyl-methacrylate based copolymers as gene transfer agents. J control Rel 1998;53:145-153.

23. Brazeau GA, Attia S, Poxon S, Hughes JA. In vivo myotoxicity of selected cationic macromolecules used in non-viral gene delivery. Pharm. Res. 1998;15:680-684.

24. Dash PR, Read ML, Barret LB, Wolfert MA, Seymour LW. Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery. Gene therapy 1999;6:643-650.

25. Choksakulnimitr S, Masusa S, Tokda H, Takakura Y, Hashida M. In vitro cytotoxicity of macromolecules in different cell culture systems. J control Rel 1995;34:223-41.

26. Florea BI, Meaney C, Junginger HE, Borchard G. Transfection efficiency and toxicity of polyethylenimine in differentiated Calu-3 and nondifferentiated COS-1 cell cultures. AAPS PharmSci 2002;4(3):E12.

27. Hill IRC, Garnett MC, Bignotti F, Davis SS. In vitro cytotoxicity of poly(amidoamine)s: relevance to DNA delivery. Biochimica et Biophysica Acta (BBA) - General Subjects 1999;1427(2):161-174.

28. Morgan DML, Larvin VL, Pearson JD. Biochemical characterisation of polycation-induced cytotoxicity to human vascular endothelial cells. J. CELL SCIENCE 1989;94(3):553-559.

29. Gebhart CL, Kabanov AV. Evaluation of polyplexes as gene transfer agents. Journal of Controlled Release 2001;73(2-3):401-416.

30. Kean T, Roth S, Thanou M. Trimethylated chitosans as non-viral gene delivery vectors: Cytotoxicity and transfection efficiency. Journal of Controlled Release 2005;103(3):643-653.

31. Fischer D, Bieber T, Li Y, Elsässer H-P, Kissel T. A Novel Non-Viral Vector for DNA Delivery Based on Low Molecular Weight, Branched Polyethylenimine: Effect of Molecular Weight on Transfection Efficiency and Cytotoxicity. Pharmaceutical Research 1999;16(8):1273-1279.

32. van de Wetering P, Cherng J-Y, Talsma H, Hennink WE. Relation between transfection efficiency and cytotoxicity of poly(2-(dimethylamino)ethyl methacrylate)/plasmid complexes. Journal of Controlled Release 1997;49(1):59-69.

33. Agarwal A, Unfer R, Mallapragada SK. Novel cationic pentablock copolymers as non-viral vectors for gene therapy. Journal of Controlled Release 2005;103(1):245-258.

34. Anderson BC, Cox SM, Bloom PD, Sheares VV, Mallapragada SK. Synthesis and Characterization of Diblock and Gel Forming Pentablock Copolymers of Tertiary Amine Methacrylates, Poly(Ethylene Glycol) and Poly(Propylene Glycol). Macromolecules 2003;36:1670-76.

35. Determan MD, Cox JP, Seifert S, Thiyagarajan P, Mallapragada SK. Synthesis and characterization of temperature and pH responsive pentablock copolymers. Polymer 2005;46(18):6933-6946.

36. Determan MD, Guo L, Thiyagarajan P, Mallapragada SK. Supramolecular Self-Assembly of Multiblock Copolymers in Aqueous Solution. Langmuir 2006;22(4):1469-1473.

37. Determan MD, Chieh-Tsung L, Thiyagarajan P, Mallapragada SK. Self-assembly of Temperature and pH-Responsive Pentablock Copolymers. Polymeric Materials:Science and Engineering 2005;23:808.

38. Edmondson J. A Rapid and Simple MTT-based Spectrophotometric Assay for Determining Drug Sensitivity in Monolayer Cultures. J. Tissue Cult. Methods 1988;11:15-17.

39. Guess WL, Rosenbluth SA, Schimdt B. Agar diffusion method for toxicity screening of plastics on cultured cell monolayers. J Pharm Sci 1965;54:1545-1547.

40. Koh J-Y, Wie MB, Gwag BJ, Sensi SL, Canzoniero LMT, Demaro J, Csernansky C, Choi DW. Staurosporine-Induced Neuronal Apoptosis. Experimental Neurology 1995;135(2):153-159.

41. Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine) and its role in gene delivery. J Control Release 1999;60(2-3):149-60.

42. Miller LJ, Marx J. Apoptosis. Science 1998;281(5381):1301.

43. Lincz LF. Deciphering the apoptotic pathway: All roads lead to death. Immunol Cell Biol 1998;76(1):1-19.

44. Green DR, Reed JC. Mitochondria and Apoptosis. Science 1998;281(5381):1309-1312.

45. Leroy D, Schmid N, Behr J-P, Filhol O, Pares S, Garin J, Bourgarit - JJ, Chambaz EM, Cochet C. Direct identification of a polyamine binding domain on the regulatory subunit of the protein kinase casein kinase 2 by photoaffinity labeling. Journal of Biological Chemistry 1995;270(29):17400-17406.

46. Arnold L.J. J, Dagan A, Gutheil J, Kaplan NO. Antineoplastic activity of poly(L-lysine) with some ascites tumor cells. PROC. NATL. ACAD. SCI. U. S. A. 1979;76(7):3246-3250.

47. Vepa S, Scribner WM, Natarajan V. Activation of endothelial cell phospholipase D by polycations. American Journal of Physiology - Lung Cellular and Molecular Physiology 1997;272(4 Pt 1):L608-L613.

48. Rihova B. Biocompatibility of biomaterials: hemocompatibility, immunocompatiblity and biocompatibility of solid polymeric materials and soluble targetable polymeric carriers. Advanced Drug Delivery Reviews 1996;21(2):157-176.

49. Dekie L, Toncheva V, Dubruel P, Schacht EH, Barrett L, Seymour LW. Poly--glutamic acid derivatives as vectors for gene therapy. Journal of Controlled Release 2000;65(1-2):187-202.

50. Ryser HJ. A membrane effect of basic polymers dependent on molecular size. Nature 1967;215(104):934-936.

51. Singh AK, Kasinath BS, Lewis EJ. Interaction of polycations with cell-surface negative charges of epithelial cells. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 1992;1120(3):337-342.

52. Morgan DML, Clover J, Pearson JD. Effects of synthetic polycations on leucine incorporation, lactate dehydrogenase release, and morphology of human umbilical vein endothelial cells. J. CELL SCIENCE 1988;91(2):231-238.

53. Batrakova EV, Miller DW, Li S, Alakhov VY, Kabanov AV, Elmquist WF. Pluronic P85 enhances the delivery of digoxin to the brain: In vitro and in vivo studies. Journal of Pharmacology and Experimental Therapeutics 2001;296(2):551-557.

54. Kostarelos K, Tadros TF, Luckham PF. Physical Conjugation of (Tri-) Block Copolymers to Liposomes toward the Construction of Sterically Stabilized Vesicle Systems. Langmuir 1999;15(2):369-376.

55. Melik-Nubarov NS, Pomaz OO, Dorodnych TY, Badun GA, Ksenofontov AL, Schemchukova OB, Arzhakov SA. Interaction of tumor and normal blood cells with ethylene oxide and propylene oxide block copolymers. FEBS Letters 1999;446(1):194-198.

56. Batrakova EV, Li S, Vinogradov SV, Alakhov VY, Miller DW, Kabanov AV. Mechanism of pluronic effect on P-glycoprotein efflux system in blood-brain barrier: Contributions of energy depletion and membrane fluidization. Journal of Pharmacology and Experimental Therapeutics 2001;299(2):483-493.

57. Hannig J, Zhang D, Canaday DJ, Beckett MA, Astumian RD, Weichselbaum RR, Lee RC. Surfactant sealing of membranes permeabilized by ionizing radiation. Radiation Research 2000;154(2):171-177.

58.   Lee RC, Hannig J, Matthews KL, Myerov A, Chen C-T. Pharmaceutical therapies for sealing of permeabilized cell membranes in electrical injuries. Annals of the New York Academy of Sciences 1999;888:266-273.

59.   Agren MS. An amorphous hydrogel enhances epithelialisation of wounds. Acta Dermato-Venereologica 1998;78(2):119-122.

60.   Cao YL, Lach E, Kim TH, Rodriguez A, Arevalo CA, Vacanti CA. Tissue-engineered nipple reconstruction. Plastic And Reconstructive Surgery 1998;102(7):2293-2298.

61.   Kushibiki T, Tomoshige R, Fukunaka Y, Kakemi M, Tabata Y. In vivo release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents. Journal of Controlled Release 2003;90(2):207-216.

62.   Luo D, Woodrow-Mumford K, Belcheva N, Saltzman WM. Controlled DNA Delivery Systems. Pharmaceutical Research 1999;16(8):1300-1308.

Table I: Molecular weights (MW) and polydispersity indices (PDI) of different pentablock
copolymers.

Pentablock copolymers	wt% PDEAEM	M$_w$ (GPC)	M$_n$ (NMR)	PDI	nmol of Nitrogen/µg	amount containing 3nmol of nitrogen*
A	17	40,112	15,000	2.36	1.01	2.96µg
B	26	23,516	17,300	1.23	1.45	2.08 µg
C	40	30,664	22,000	1.21	2.03	1.48 µg
D	60	20,365	19,973	1.34	2.73	1.10 µg
E	28	28,400	17,525	1.25	1.52	1.98 µg
ExGen	-	-	22,000	-	248.64	12.1 ng

*It is the amount of polymer required to condense 1µg of plasmid DNA such that molar ratio
of nitrogens of the polymer to the phosphates of DNA is 1, using the fact that 1µg of DNA
has 3nmol of phosphate. Synthesis of pentablock D used Pluronic® F68 as the macroinitiator
while all others used Pluronic® F127. M_w: Weight average MW, M_n: Number average MW.

Table II: Summary of test results from agarose overlay analysis. Approximately 4mg of polymer gels containing 25 wt% of the polymer were placed on the top of agarose layer.

Specimen	Zone Index	Lysis Index	Cytotoxicity score	Comment
Pentablock A	0.5	0	0.5	Non-cytotoxic
Pentablock B	2	1	1	Mildly cytotoxic
Pentablock C	1	1	1	Mildly cytotoxic

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Scheme 1: Structure of pentablock copolymers.

Fig 1: Diffusion of polymer through a 1% agarose matrix of DMEM with 10% FBS, simulating a tumor-model.

Fig 2: Cytotoxicity of pentablock copolymers with different wt% PDEAEM, on SKOV3 cell line after 48hr incubation in FBS supplemented DMEM, (a) percentage toxicity evaluated in terms of cell membrane damage using LDH assay, (b) residual metabolic activity evaluated by MTT assay, (n=4±SD).

Fig 3: Cytotoxicity of pentablock copolymers with different wt% PDEAEM, on A431 cell line after 48hr incubation in FBS supplemented DMEM, (a) percentage toxicity evaluated in terms of cell membrane damage using LDH assay, (b) residual metabolic activity evaluated by MTT assay, (n=4±SD).
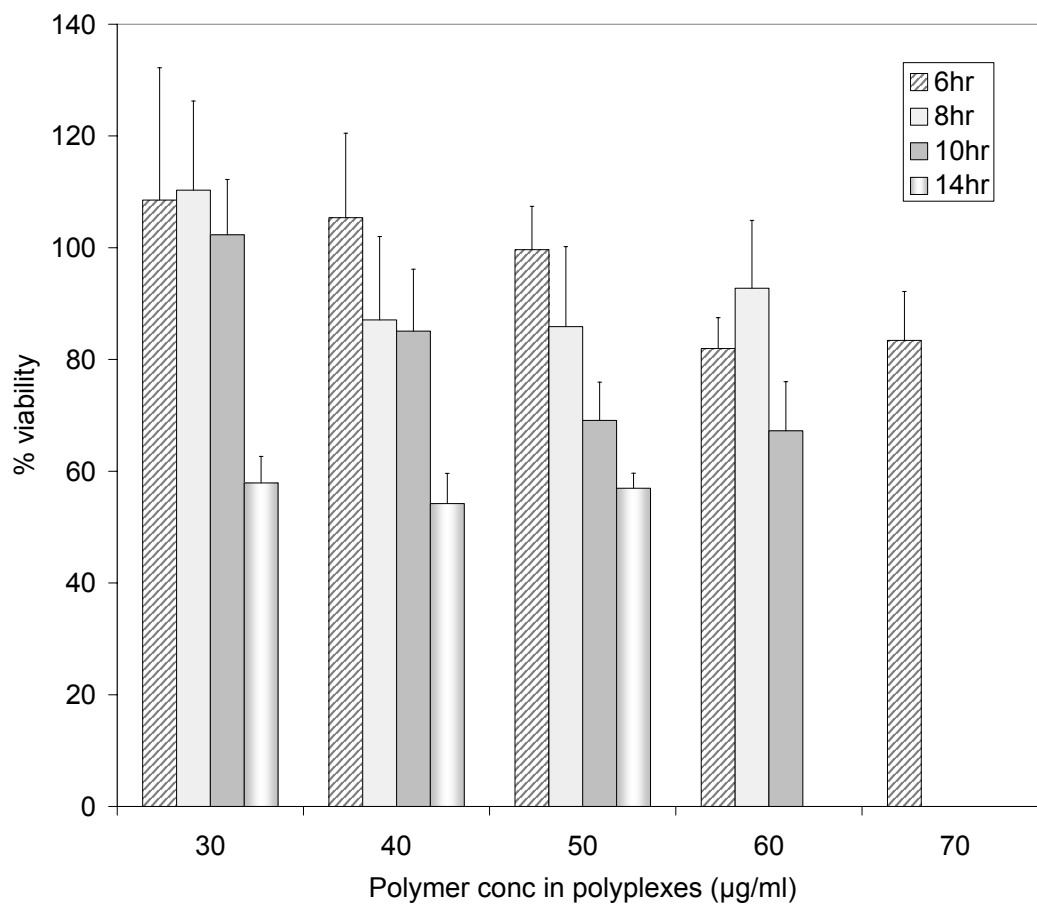
Fig 4: Dose and time dependent effect of the polyplexes of pentablock copolymer B on SKOV3 cells incubated in OptiMEM I[®]. Residual metabolic activity was evaluated by MTT assay. Polyplexes contained 1μg of pRL, (n=4±SD).

Fig 5: Comparing the cytotoxicity of polyplexes of pentablock copolymer B with that of the polycations alone on SKOV3 cells after 6hr incubation in OptiMEM I[®], (a) LDH assay, (b) MTT assay. Polyplexes contained 1μg of pRL-CMV, (n=4±SD). * indicates $p<0.1$; ** indicates $p<0.05$; *** indicates $p<0.01$
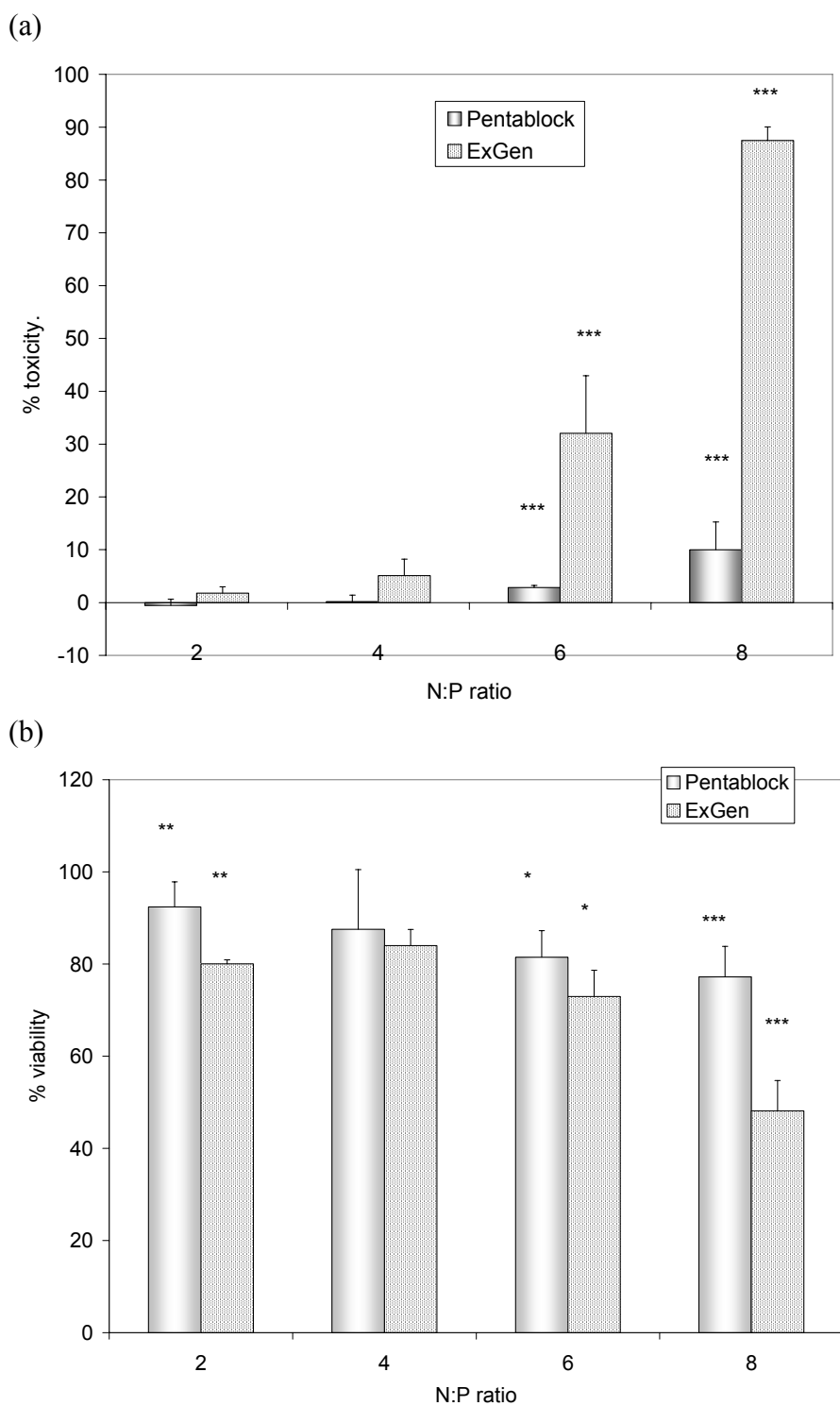
Fig 6: Cytotoxic effect of polyplexes of pentablock copolymer B and ExGen on SKOV3 cells after 6hr incubation in OptiMEM I[®], (a) LDH assay, or (b) MTT assay. Polyplexes contained 1μg of pRL-CMV, (n=4±SD). * indicates $p<0.1$; ** indicates $p<0.05$, ***indicates $p<0.01$

Fig 7: Dose dependent effect of different polycations incubated with SKOV3 cells for 5hr. *P*: pentablock copolymer B, *Ex*: ExGen 500, *strp*: Staurosporine (200nM), (a) membrane damage by LDH assay, (b) percentage viability evaluated by MTT assay, (n=4±SD). * indicates $p<0.1$
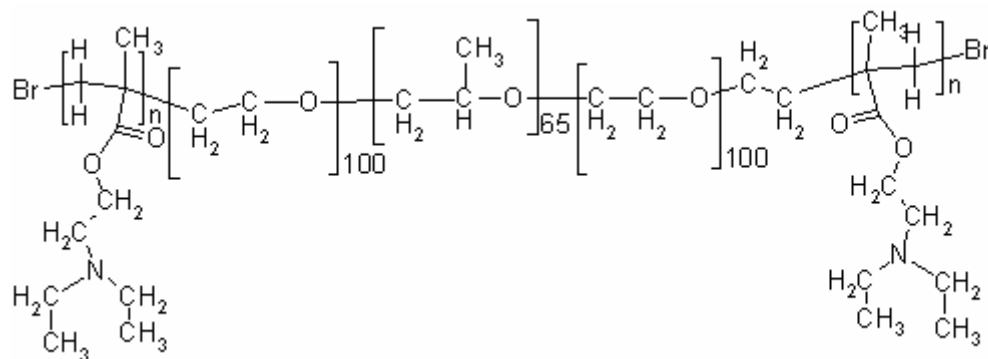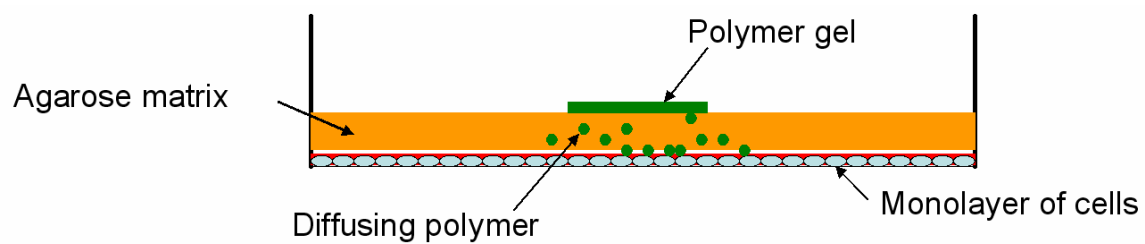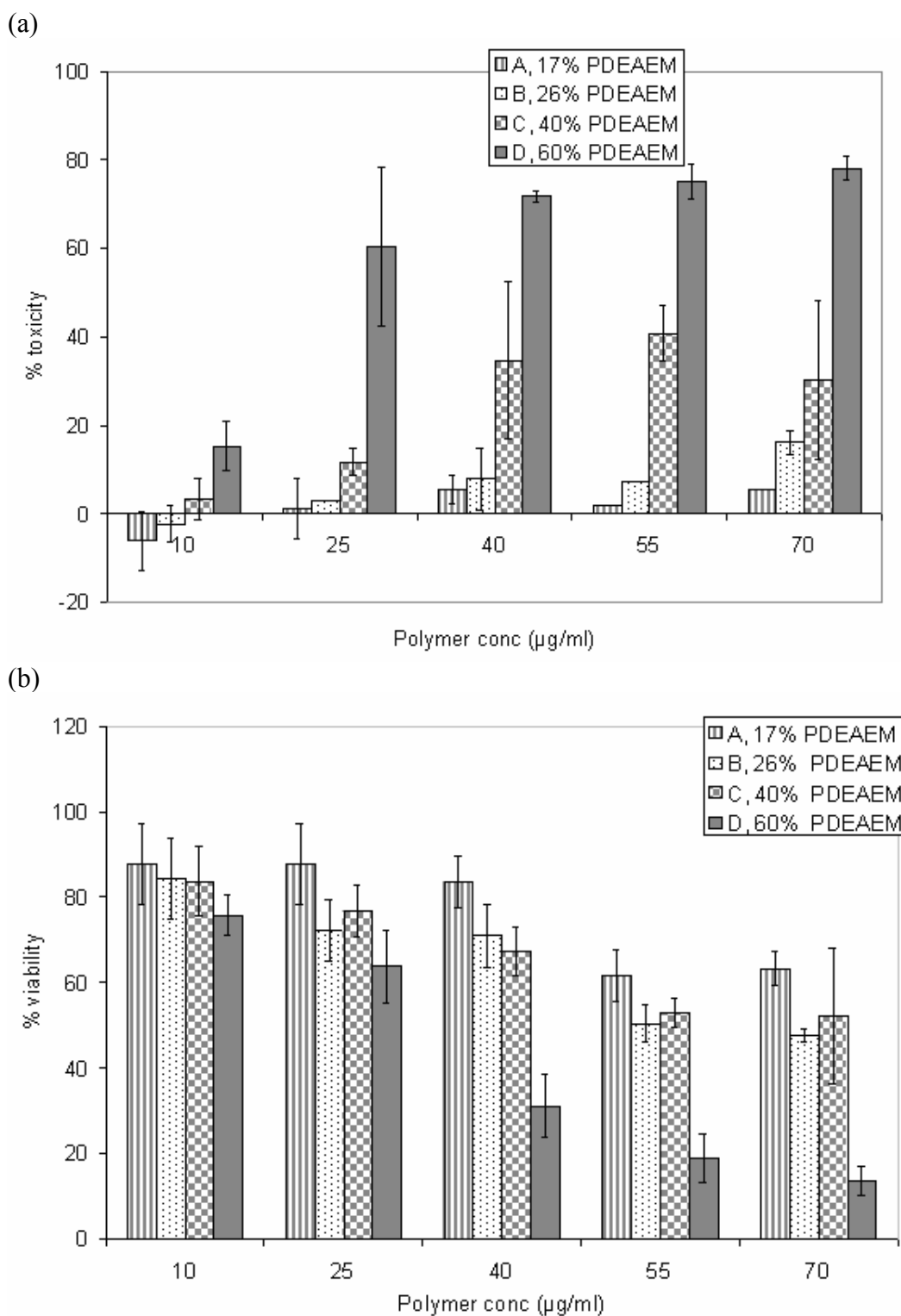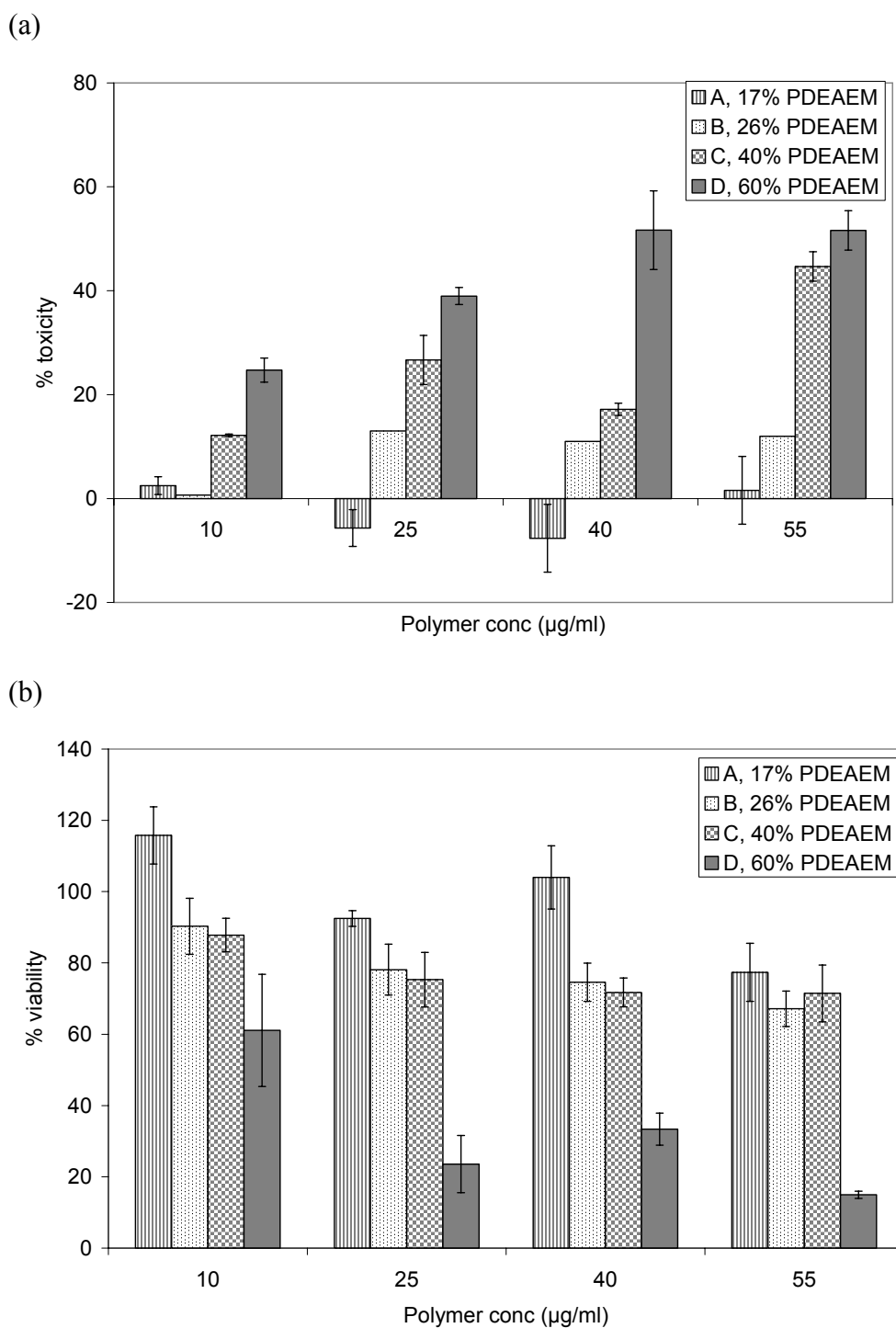
Fig 8: Activity of Caspases 3/7 in SKOV3 cells after incubation with different compounds for specified times, as found using Caspase-Glo[TM] 3/7 assay. *Ex:* ExGen 500, *P:* Pentablock copolymer B, *Strsp*: 200nm Staurosporine. All solutions were made in OptiMEM I[®] media. (n=4±SD).

Fig 9: Luciferase expression obtained in DU145, SKOV3 and A431 cell lines by transfecting them with polyplexes of pentablock copolymer B and 1μg DNA at different N:P ratios, (n=4±SD).

Fig 10: Luciferase expression in SKOV3 cells after transfecting 1µg of DNA per well in a 96-well plate with ExGen, at different N:P ratios. Polyplexes were incubated with cells for 11hrs in OptiMEM I®, (n=4±SD).

Fig 11: Luciferase expression in SKOV3 cells after transfecting 0.6µg of DNA with pentablock copolymer E and ExGen at different N:P ratios. Polyplexes were incubated with cells for 3.5hrs in OptiMEM I®, (n=4±SD).

Fig 12: SKOV3 cells under agarose-gel matrix after 24hr incubation with a 25wt% gel of pentablock copolymer A placed on top of agarose matrix, at (a) 60x magnification, (b,c) 150x magnification- regions right below and around the polymer gel. Scale bar=1mm.

Scheme 1: Structure of pentablock copolymers.

Fig 1: Diffusion of polymer through a 1% agarose matrix of DMEM with 10% FBS, simulating a tumor-model.

(a)

(b)

Fig 2: Cytotoxicity of pentablock copolymers with different wt% PDEAEM, on SKOV3 cell line after 48hr incubation in FBS supplemented DMEM, (a) percentage toxicity evaluated in terms of cell membrane damage using LDH assay, (b) residual metabolic activity evaluated by MTT assay, (n=4±SD).

(a)

(b)

Fig 3: Cytotoxicity of pentablock copolymers with different wt% PDEAEM, on A431 cell line after 48hr incubation in FBS supplemented DMEM, (a) percentage toxicity evaluated in terms of cell membrane damage using LDH assay, (b) residual metabolic activity evaluated by MTT assay, (n=4±SD).

Fig 4: Dose and time dependent effect of the polyplexes of pentablock copolymer B on SKOV3 cells incubated in OptiMEM I[®]. Residual metabolic activity was evaluated by MTT assay. Polyplexes contained 1µg of pRL, (n=4±SD).

(a)

(b)

Fig 5: Comparing the cytotoxicity of polyplexes of pentablock copolymer B with that of the polycations alone on SKOV3 cells after 6hr incubation in OptiMEM I[®], (a) LDH assay, (b) MTT assay. Polyplexes contained 1μg of pRL-CMV, (n=4±SD). * indicates p<0.1; ** indicates p<0.05; *** indicates p<0.01
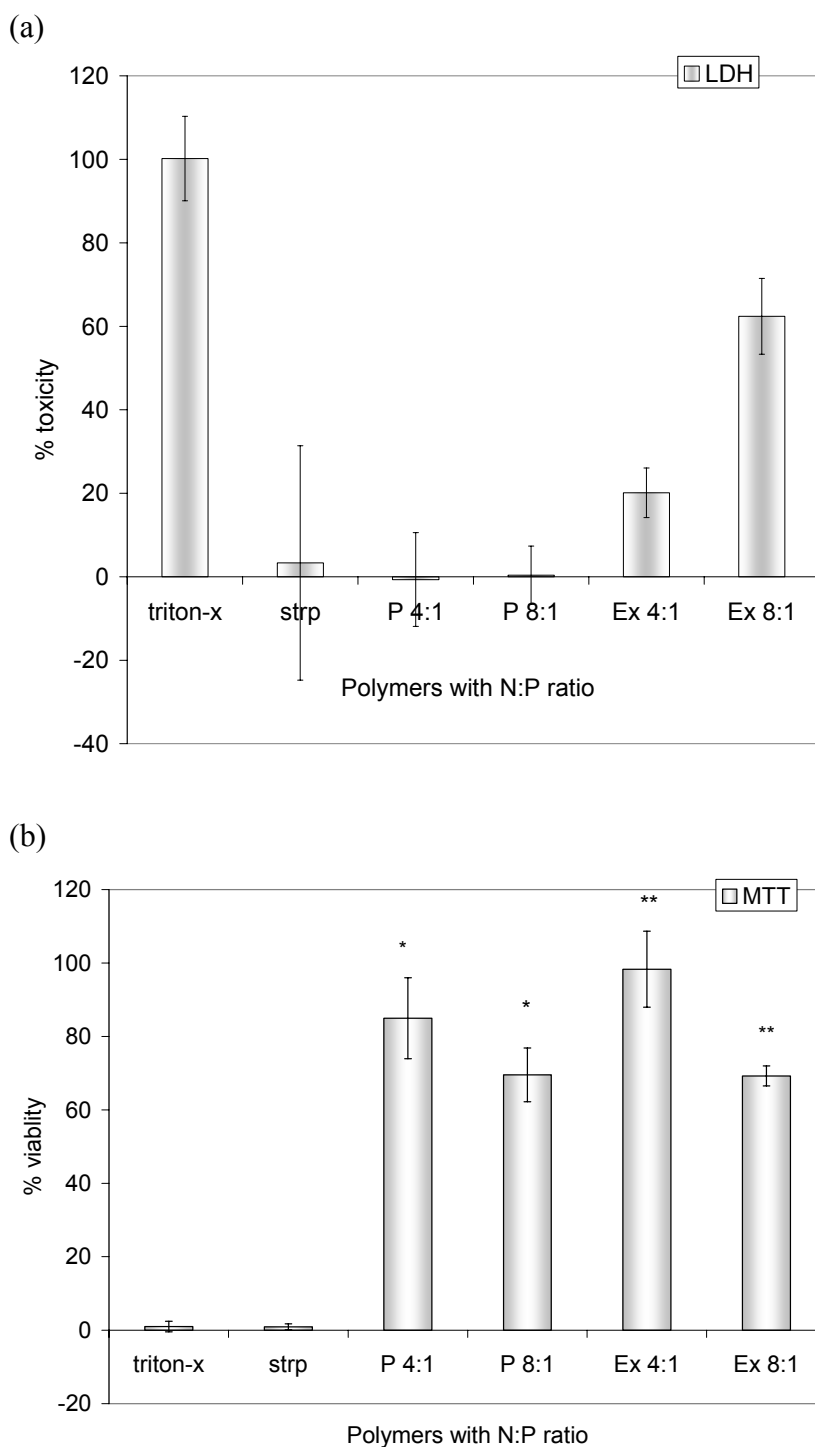
(a)

(b)

Fig 6: Cytotoxic effect of polyplexes of pentablock copolymer B and ExGen on SKOV3 cells after 6hr incubation in OptiMEM I®, (a) LDH assay, or (b) MTT assay. Polyplexes contained 1µg of pRL-CMV, (n=4±SD). * indicates $p<0.1$; ** indicates $p<0.05$, ***indicates $p<0.01$

(a)

(b)

Fig 7: Dose dependent effect of different polycations incubated with SKOV3 cells for 5hr. *P*: pentablock copolymer B, *Ex*: ExGen 500, *strp*: Staurosporine (200nM), (a) membrane damage by LDH assay, (b) percentage viability evaluated by MTT assay, (n=4±SD). * indicates p<0.1
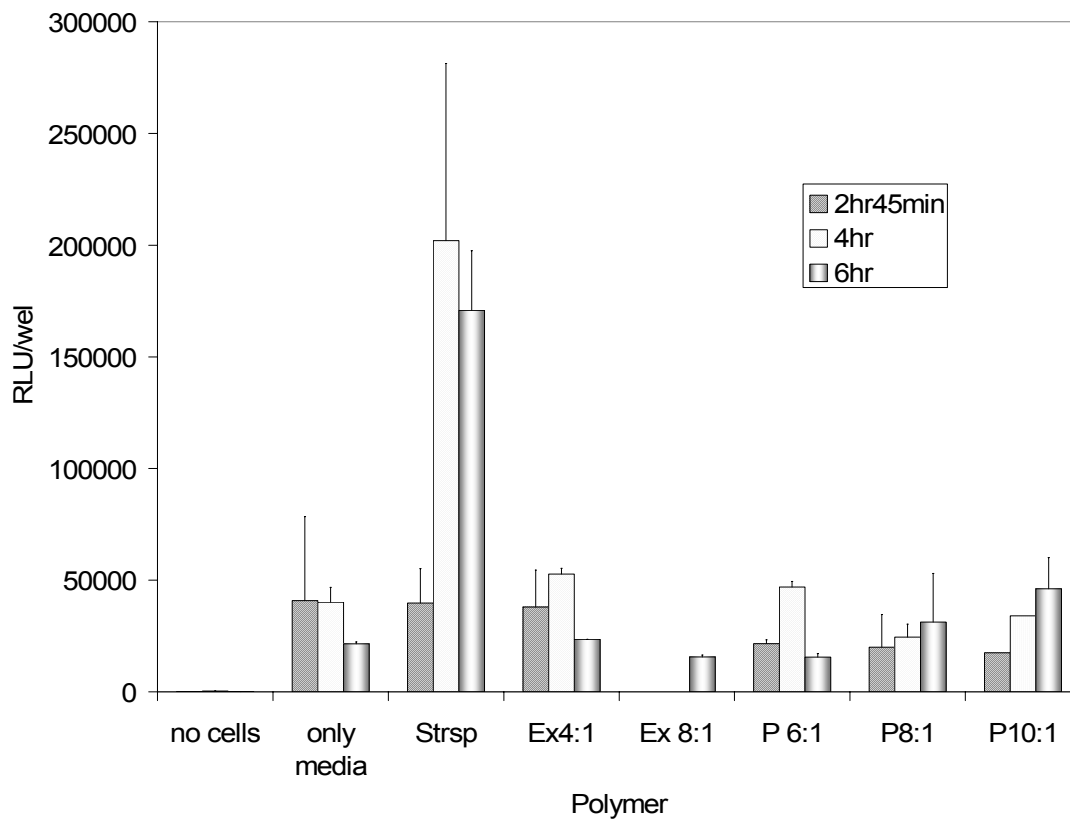
Fig 8: Activity of Caspases 3/7 in SKOV3 cells after incubation with different compounds for specified times, as found using Caspase-Glo^{TM} 3/7 assay. *Ex:* ExGen 500, *P:* Pentablock copolymer B, *Strsp*: 200nm Staurosporine. All solutions were made in OptiMEM I$^{®}$ media. (n=4±SD).
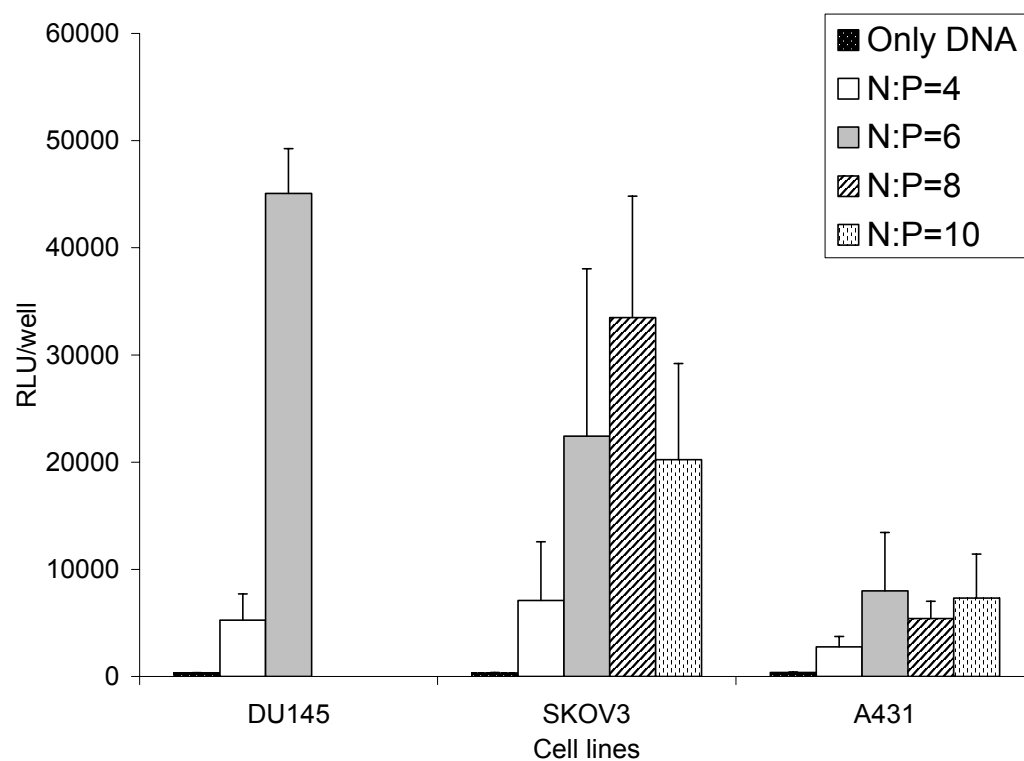
Fig 9: Luciferase expression obtained in DU145, SKOV3 and A431 cell lines by transfecting them with polyplexes of pentablock copolymer B and 1µg DNA at different N:P ratios, (n=4±SD).
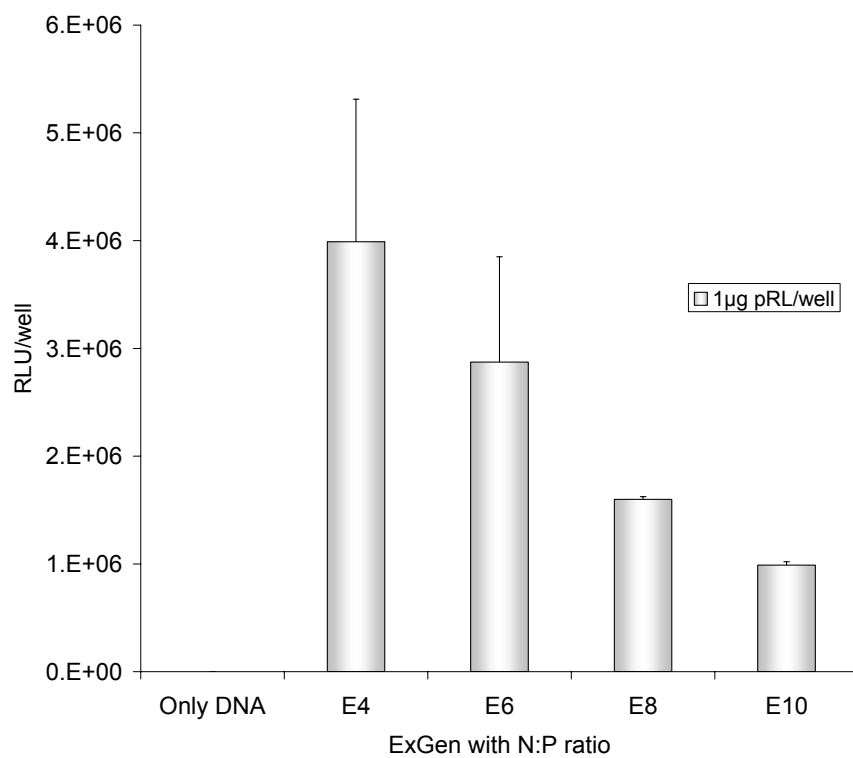
Fig 10: Luciferase expression in SKOV3 cells after transfecting 1μg of DNA per well in a 96-well plate with ExGen, at different N:P ratios. Polyplexes were incubated with cells for 11hrs in OptiMEM I®, (n=4±SD).
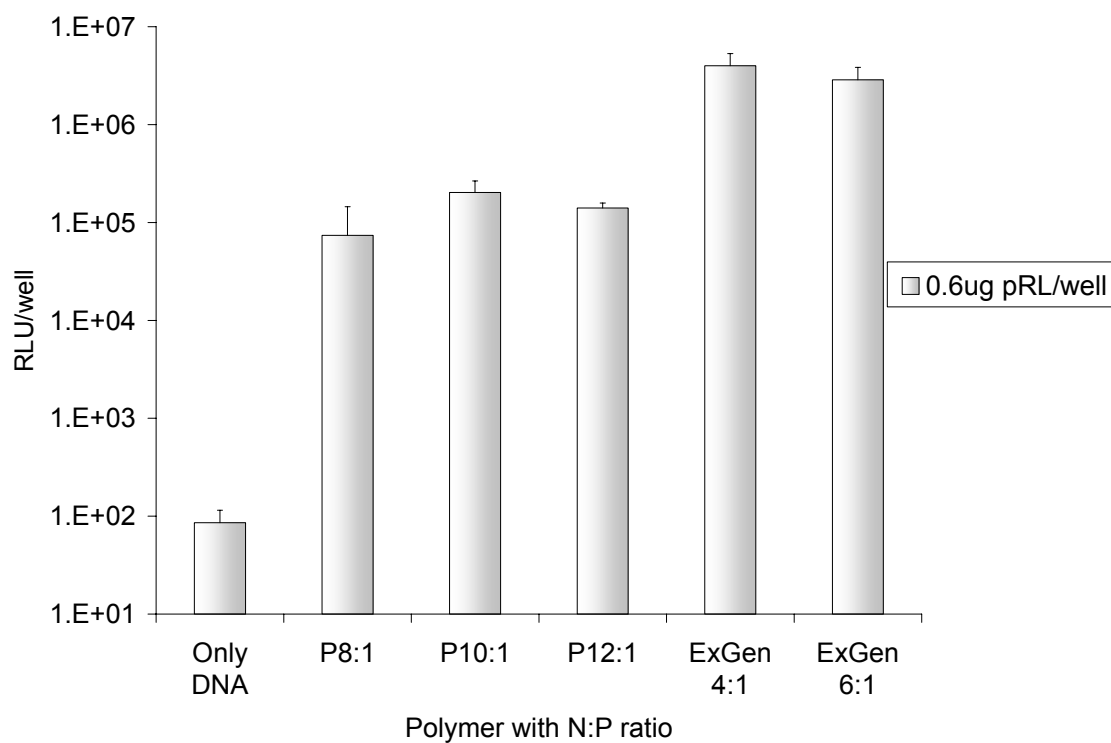
Fig 11: Luciferase expression in SKOV3 cells after transfecting 0.6μg of DNA with pentablock copolymer E and ExGen at different N:P ratios. Polyplexes were incubated with cells for 3.5hrs in OptiMEM I[®], (n=4±SD).
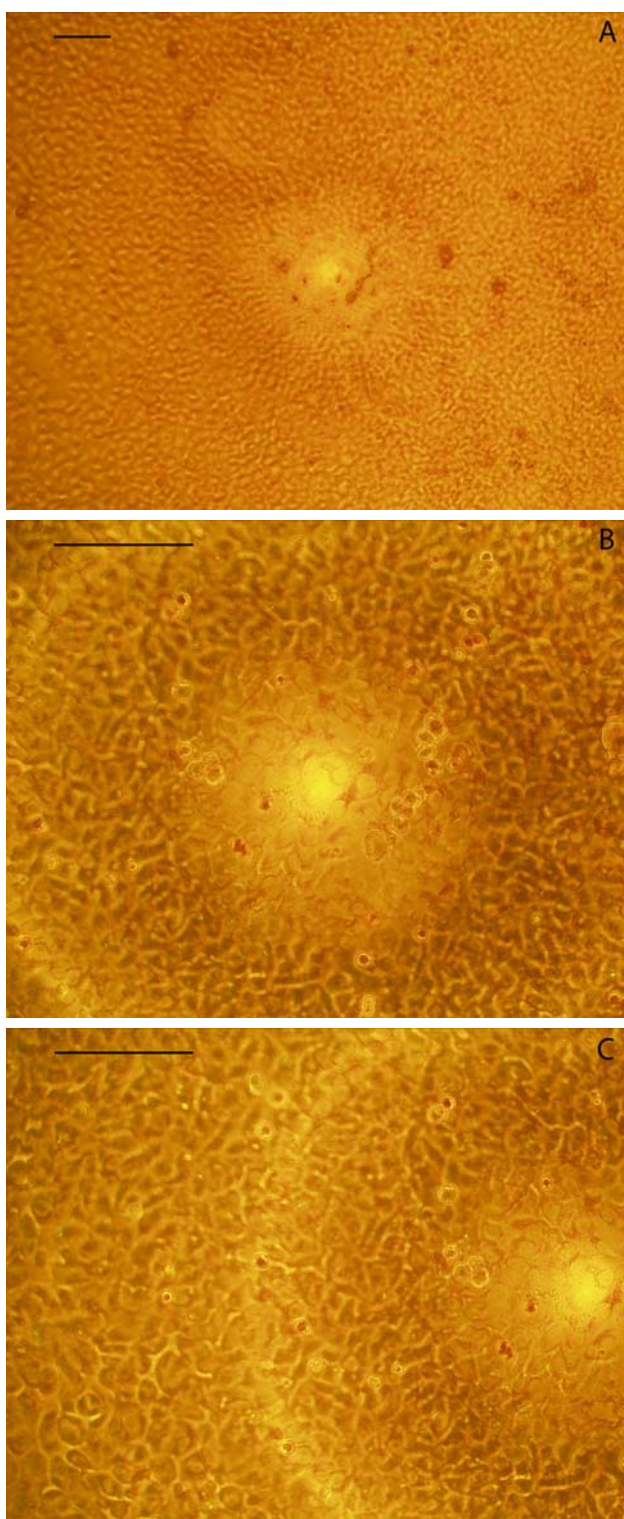
Fig 12: SKOV3 cells under agarose-gel matrix after 24hr incubation with a 25wt% gel of pentablock copolymer A placed on top of agarose matrix, at (a) 60x magnification, (b,c) 150x magnification- regions right below and around the polymer gel. Scale bar=1mm.

CHAPTER 5

MORPHOLOGY OF THE PLASMID DNA CONDENSATES OF NOVEL CATIONIC AMPHIPHILIC COPOLYMERS AND THEIR INTRA-CELLULAR TRAFFICKING

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AA is primary researcher

RV performed cryo-TEM

YT, RTD, SKM supervised the work

Abstract

This paper investigates the morphology of novel amphiphilic pentablock copolymers of poly(ethylene oxide) (PEO), poly(propylene oxide) (PPO), and poly(diethylamino ethyl methacrylate) (PDEAEM) and their condensates with plasmid DNA using cryo-TEM, and the intra-cellar path they take to reach the cell nuclei. The copolymers existed as spherical micelles of 20-30 nm diameters in the micrographs and condensed the plasmid DNA into compact, defined thread like structures with extended linear or ring like forms. At higher polymer/DNA ratios condensates were more compact, and were larger in number, decorated with the micelles of excess copolymer. Similar compact structures were observed at even

lower polymer/DNA ratios at endosomal pH 4.7 where copolymer had increased protonation. Intra-cellular tracking of copolymer/DNA polyplexes using fluorescent labeling and confocal microscopy revealed that they were taken up by the cells all along the perimeter of the cell membrane, with some prominent localized discrete spots. Polyplexes were found to be trapped in endo/lysosomal vesicles for up to 7 hrs after transfection. Labeled DNA could be detected in the nucleus of the transfected cells 10 hrs post transfection, with most of it in perinuclear region, and very little in the rest of the cytoplasm. The fluorescence of labeled DNA was more diffuse in the perinuclear region compared to discrete spots observed with cells transfected with using ExGen 500[®]. Complexes of ExGen/DNA could be detected in perinuclear region and inside cell nuclei by only 4.5 hr and 6 hr, indicating their diffusion through cytoplasm was faster and involved mechanisms other than those associated with pentablock copolymers. The nuclear import of polyplexes, and not their trafficking to the perinuclear region, was found to limit the transfection efficiency of the copolymers. Expression of green fluorescence protein (GFP) in the cells transfeced with GFP plasmid using pentablock copolymers confirmed the transfection. This study identifies critical steps upon which to focus for improving the DNA condensation process and enhance their intra-celluar trafficking which can lead to improvement in gene delivery technology using cationic polymers.

1. Introduction

Cationic polymers that can condense negatively charged DNA into nanoparticles have been reported to be efficient and versatile non-viral gene delivery vectors in a number of applications *in vivo*, and are currently being evaluated in several clinical gene therapy trials[1,2]. The critical problems that limit the gene delivery efficiency of existing polymeric systems are low and transient transfection efficiency, cytotoxicity, and colloidal stability of their complexes with DNA[3,4]. While several novel and second generation copolymers have

been reported to address these issues[5,6], understanding the the morphology of polymer/DNA complexes and identifying the limiting steps in their intra-cellular trafficking after cellular uptake is critical in improving the transfection efficiency of these copolymers.

Polycations condense negatively charged plasmid DNA into compact, ordered mononuclear or polynuclear complexes (20-200nm in diameter) via electrostatic interactions[7]. DNA condensation is a reversible, linear polymer to globule transition process. Approximately 90% of the electrostatic repulsion between DNA segments must be neutralized to allow condensation to occur[8]. Studies have demonstrated that at a certain critical ratio of the polycation to DNA, the latter undergoes localized bending and distortion, which facilitates formation of rods, toroids[7] and spheroids[9] like nanoparticles.

Cryo-TEM has been utilized in a number of applications in the study of DNA molecules[10,11], and micellar structures of amphiphilic block copolymers[12,13]. Rapid cryogenic vitrification of sample solutions in cryo-TEM enables direct real-space imaging of nanostructures in their native state in aqueous conditions, avoiding staining and drying artifacts involved in conventional TEM. This is of great significance in investigating multi-component self-assembled structures that are formed by a combination of interactions. Supercoiling of the plasmid DNA[14] have been visualized successfully using cryo-TEM. Gustafsson and colleagues revealed the structural features of complexes formed between plasmid DNA and cationic liposomes[15]. Simberg et al[10,16] displayed heterogeneity in size and structure of cationic lipid-DNA complexes, and their aggregation with serum proteins.

Our research into the cationic polymer agents for gene delivery has been focused on novel amphiphilic block copolymers of poly(ethylene oxide) (PEO), poly(propylene oxide) (PPO), and poly(diethylamino ethyl methacrylate) (PDEAEM), with a family of these synthesized[17] and subjected to various physiochemical[17,18] and biological characterizations[19,20]. These copolymers exist as micelles in aqueous solutions, formation driven by low lower critical solution temperature (LCST) of central PPO group that forms a hydrophobic core stabilized by a hydrophilic corona of PEO and PDEAEM chains. The hydrodynamic diameter of these polymeric micelles is around 25 nm[17] which is relatively small compared to other colloidal drug carriers such as liposomes and emulsions[21]. These cationic copolymers, with partially protonated tertiary nitrogen of PDEAEM blocks at pH

7.4, condense the DNA into nanoparticles of size around 100 to 150nm dia, as shown by DLS, MALLS and TEM, and protect it against degradation by nucleases[19,22]. We have shown that these copolymers successfully transfect several different cancer cell lines with transfection efficiencies comparable to linear PEI (ExGen 500[®]) while exhitbing significantly less cytotoxicity compared to ExGen 500[®20]. In this report we have attempted to understand the effect of polymer concentration and environmental pH on the formation of these polyplexes by studying their morphology using cryo-TEM. An increased knowledge of such structures will benefit the understanding of DNA condensation process and facilitate improvement of the gene delivery efficiency of these copolymers.

A lot of speculations have been made on the mechanism of transfection by polycations[23,24] but the processes governing their intracellular transport remain elusive. Several studies have been reported on the intra-cellular paths taken by DNA condensates of PEI and PLL[25-27] which, however, conflict on whether polyplexes of PEI get trapped into the endosomes to escape later, or they don't ever get into the endosomes at all. The studies also showed that polyplexes of PLL and PEI followed different paths from the cell membrane to the nucleus, and used that to explain the different transfection efficiency of the two polycations. These reports definitely suggest that a different polycation can have different mechanism of cell transfection and should therefore be investigated individually. Therefore, here we have attempted to examine using fluorescent labeling and confocal microscopy the intracellular paths taken by the pentablock copolymer/DNA complexes during gene transfection. This work would help identify the critical rate limiting steps in the intra-cellular trafficking of the copolymers, thereby allowing for the development of strategies to overcome these barriers.

2   Materials and Methods

2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), OptiMEM I[®], heat inactivated qualified fetal bovine serum (FBS), 0.25% trypsin-EDTA solution and Hank's Buffered Salt Solution (HBSS), ExGen 500[®] (written as ExGen henceforth), EDTA, TAE buffer, Lysotracker Red[®]

DND-26, DAPI (4'-6-Diamidino-2-phenylindole), and ethidium monoazide (EMA) were purchased from Invitrogen (Carlsbad, CA). HEPES salt was purchased from Sigma (St Louis, MO). Ultrapure water with at least 18 megaohm resistivity was used in all studies.

2.2 <u>Polymers</u>

Pluronic F127 [$(PEO)_{100}$-b-$(PPO)_{65}$-b-$(PEO)_{100}$] micro pastille surfactant was donated by BASF (Florham Park, NJ) and used without further modification. Pentablock copolymers of PDEAEM-b-PEO-b-PPO-b-PEO-b-PDEAEM were synthesized using an ATRP reaction scheme as explained in detail elsewhere[17]. Molecular weight and poly-dispersity of the copolymers were measured using H^1 NMR (in deuterated chloroform) and GPC (THF mobile phase, poly(methylmethacrylate) calibration standards). Copolymers reported in this study had 20wt% of PDEAEM, with architechture- $PDEAEM_8$-PEO_{100}-PPO_{65}-PEO_{100}-$PDEAEM_8$ and M_n = 18520 and M_w/M_n = 1.14. Copolymers were synthesized with a molecular weight less than 20KDa so that in an in vivo application they can be removed from the body via renal clearance system after gene delivery. It can be calculated that 1μg of this copolymer has 1.03 nM of nitrogen residues. The molecular weight of the DEAEM monomer is 185.

2.3 <u>Cells</u>

The SKOV3 human ovarian carcinoma cell line, obtained from ATCC[TM] (Manassas, VA), was used for all experiments. Cell cultures were maintained in a humidified environment with 5% CO_2 at 37°C and passaged regularly to allow them to remain sub-confluent. Cells were fed with DMEM growth media supplemented with 10% FBS and 1μM L-glutamine, unless otherwise stated. Neither antibiotics nor antimycotics were used to avoid the possibility of artificial membrane permeabilization effects from these agents.

For confocal microscopy, cells were grown onto glass coverslips coated with a cell adhesive poly(L-lysine) (PLL) film. The cover slips were placed in 6-well cell culture plates. Approximately 10,000 cells were plated on each coverslip by placing 500μl of growth media containing the cells. Once the cells adhered to the coverslips, growth media was in each well

was to made up to 2 ml. The cells were then incubated overnight to allow them to become 70% confluent before transfection.

2.4 Plasmid DNA

Plasmid DNA pEGFP-N1 with 6732bp (Clontech, Mountain View, CA), encoding for green fluorescence protein (GFP), was used as the reporter gene. DH5α *E.coli* cells were transformed with the plasmid DNA and incubated in selective Luria-Bertani (LB) medium. Amplified plasmid DNA was purified using the Maxi-Prep DNA purification kit from Qiagen (Valencia, USA). The concentration and purity of the resulting DNA in TE buffer, pH 7.4 was determined by measuring the absorbance (A) at 260 nm and 280 nm. All DNA used had a A_{260}/A_{280} ratio of at least 1.80.

DNA was labeled with the fluorescent probe ethidium monoazide (EMA, 8-azido-3-amino-6-phenyl-5-ethylphenanthradinium chloride). EMA can be photolysed in the presence of nucleotides to yield fluorescently labeled nucleic acids[23,28]. The labeled plasmid emits red fluorescence at around 600 nm when excited with a 488 nm laser. Covalently bound EMA-DNA was prepared as follows, adapted from procedures described previously[29,30]. To 200 μg of pEGFP-N1 in 2 mL water, 5 μg of EMA was added, giving a 50:1 molar ratio of nucleotide to probe. After a 30 min incubation period on ice in dark, the solution was exposed to UV light of principal wavelength 312 nm for 20min. Excess EMA and the intercalated but not covalently bound EMA was removed by performing ethanol precipitation three times. In each cycle, the DNA was precipitated using sodium acetate buffer, pH 4.7 by incubating at -20°C for an hour, then pelleted by centrifugation at 15000 rpm at 4°C for 10 min, and suspended in fresh TE buffer, pH 8.0. The finally obtained labeled DNA pellet was suspended in 100 μL TE buffer, pH 7.4 to be used for transfection experiments.

2.5 Polyplexes

Polymer/DNA complexes were prepared at different molar ratios of nitrogen (N) in the pentablock copolymer to phosphates (P) in DNA, written as N:P. The average molecular weight of a nucleotide is approximately 308. Using the fact that 1μg of DNA contains 3 nM of phosphates, the amount of polymer required for corresponding N:P ratios was calculated. Polyplexes were formed by following the precise order of mixing DNA and pentablock

copolymers, described in detail elsewhere[20]. Pentablock copolymers dissolved in 0.5x HBS, pH 7.0 (Hepes buffer saline- 20 mM of HEPES with 145 mM NaCl) were added to the plasmid DNA contained in a 1.5ml Eppendorf tube with final volume made to 200 uL using excess buffer. The tube was vortexed gently and allowed to incubate for 20 min at room temperature. For cryo-TEM, samples were prepared with different N:P ratios in 0.5x HBS buffer at desired pH such that final DNA concentration in the samples was 10 μg/mL.

For transfecting cells on cover slips to be used for confocal microscopy, all polyplexes were prepared with 3 μg plasmid at N:P ratio of 13 with final volume made up to 1ml using OptiMEM I[®] growth media. To test the transfection efficiency of copolymers, SKOV3 cells growin in 12-well cell-culture plates were transfected with polyplexes containing 3ug DNA/well in 1ml OptiMEM I[®]. In all cases, the polymer solutions were aspirated 3 hr after incubation with cells. Cells were washed with HBSS buffer and further incubated for desired period of time in complete growth media to detect expression of GFP. Cells incubated with naked DNA (without polymer) were used as controls. Number of cells expressing the GFP protein 48hrs after transfection with pEGFP was counted by Flow-Cytometry, as described before[19].

2.6 Cryo-TEM

Vitrified specimens of the polymer/DNA complexes were prepared for transmission electron microscopy (TEM) in a controlled environment vitrification system (CEVS) at 25°C and 100% relative humidity, as previously described [31]. Briefly, a drop of the solution was applied onto a perforated holey-carbon film, supported on an electron microscopy 200 mesh copper grid, and held by tweezers in the vitrification system chamber. The sample was blotted with a filter paper, and immediately plunged into liquid ethane at its freezing point (–183°C). Samples were examined in a Philips CM120 or an FEI T12 G^2 cryo-dedicated transmission electron microscopes (Eindhoven, The Netherlands), operated at 120 kV, using either an Oxford CT-3500 (for the CM120; Oxford Istruments, Abingdon, England) or a Gatan 626 (for the T12; Gatan, Pleasanton, CA) cooling holders and transfer stations. Specimens were equilibrated in the microscope below –178°C, examined in the low-dose imaging mode to minimize electron beam radiation damage, and recorded at a nominal

underfocus of about 2µm to enhance phase-contrast. Images were acquired digitally by a Gatan MultiScan 791 (CM120) or a US1000 (T12) cooled charge-coupled device camera (Gatan, Pleasanton, CA) using the Digital Micrograph 3.1 software package.

2.7 Labeling

Half an hour prior to observing cells under the confocal microscope, growth media was replaced with a 70 nM freshly prepared LysoTracker[TM] Red DND-26 solution in complete growth media. Cell incubation continued at 37°C until confocal imaging was performed. The lysosomal marker emits red light in far red region, peaking at 620 nm when excited at 568 nm. DAPI was used to stain the cell nuclei. Cells were washed with HBSS, and then incubated in 300 nM solution of DAPI in PBS buffer for 5 min before finally fixing them with 4% paraformaldehyde. Stained nuclei were excited using a mercury-arc lamp and observed under the fluorescence microscope.

2.8 Confocal microscopy

At different time points after transfection, each coverslip was removed from 6-well plates and washed in a stream of PBS. Cells were fixed with 4% paraformaldehyde. After drying the bottom of coverslip, it was mounted on glass slides, and one drop of PBS was placed in between to keep the cells from drying out. Imaging was done with a Hamamatsu Orca CCD camera on a Nikon Eclipse inverted microscope equipped with standard epifluorescence illumination and differential interference contrast (DIC) optics. Confocal images were collected with a Prairie Technologies Confocal Microscope (Prairie Technologies, Madison, WI). All imaging and physiological functions were controlled by Prairie Technologies software. Image analysis was done with MetaView software (Universal Imaging Corporation). An argon/krypton mixed gas laser with excitation lines at 488 and 568 was used to induce fluorescence. Excitation of EMA bound to DNA was achieved by using the 488 nm excitation line, with the resulting fluorescent wavelengths observed by using a 600/40 nm notch filter. Red fluorescence of lysotracker dye was induced by the 568 nm excitation line and detected again using a 600/40 nm notch fitler. A z-series of typically 25 images with a spatial resolution of 0.3 to 0.5µm was collected.

3 Results

3.1 <u>Morphology</u>

Amphiphilic pentablock copolymers with hydrophobic PEO blocks and hydrophilic PPO blocks form micellar structures in aqueous solutions. The cryo-TEM is an excellent technique to observe the conformation and morphology of such structures as the micelles are preserved in the sample preparation process. The vitrification process captures the micellar structures in a state as close as possible to the native state without the need for artifact-inducing staining-and-drying[32]. Fig. 1a show that micelles of pentablock copolymers have spherical or disc shaped morphology at pH 7.4 with a size range of 20 to 40 nm diameter. Since the pK_a of PDEAEM block is around 7.4, it displays good solubility in aqueous buffers at pH 7.4 and forms the part of the hydrophilic corona. However, when the copolymers are in a buffer of pH 4.7, mimicking the environment inside the lysosomes, there is increased protonation of tertiary nitrogens in PDEAM groups. Thus, the micelles with hydrophilic PDEAEM groups on their surface have increased positive surface charge on them. This results in the repulsion of positively charged polymer chains in the corona of micelles and among the neighboring micelles. As a result, as seen in Fig. 1b, the size of micelles at pH 4.7 is much smaller than that observed at pH 7.4 and they are more sparsely located.

PDEAEM blocks of the pentablock copolymer are partially protonated at pH 7.4, and can therefore electrostatically condense the negatively charged plasmid DNA into nanocomplexes. Representative images of DNA condensates formed using these amphiphilic copolymers at N:P ratio 13 and pH 7.4 are shown in Fig. 2a. It can be noticed that plasmid DNA is condensed into fine thread like nanostructures of around 100 nm size. Most of these long extended threads tend to enclose and form rings. It has been shown by several researchers that uncondensed plasmid DNA displays a relaxed, large open-loop structure with little twisting or fasciculation of the strands[33,34]. Compared to that relaxed morphology, the structures observed in Fig 2a are more defined, condensed, and compact, and it is apparent that DNA condensation has occured. Similar loose rings and extended linear plectonemic-like structures of polymer/DNA complexes have been reported previously using AFM and TEM with PEG-b-PLL[35] and, TEM on PEG-g-PEI[36] block copolymers. It should

be noted, as has also been argued previously[33], that compact toroids of DNA condensates seen in several studies with cationic polymers using conventional TEM[35,37] were formed as a result of constriction of loose rings, like the ones observed in images shown in this report, during dehydration. The hydrated natural form of the polyplexes, preserved in cryo-TEM and AFM samples, is much looser than that revealed by TEM. A comparison of AFM and TEM images of PEG-poly(amidoamine)-PEG copolymer / DNA complexes shown by Rackstraw and colleagues also supports this argument[33]. They suggested that dehydration of samples in TEM imaging reduced the size of structures obtained and alter the relative proportions of the condensate types (ring like or linear), as opposed to structures obtained from AFM imaging performed in PBS.

More condensed and compact nanostructures were obtained when larger amount of copolymer to DNA (N/P) ratio was used. As shown in Fig. 2b and c, at N/P 26 the thread like nanostructures are less extended, greater in numbers, and had a greater tendency to bend into ring like structures. This can be explained by the greater cationic charge provided by larger molar concentration of protonated nitrogens to electrostatically bind plasmid DNA. On further increasing the polymer concentration to get an N:P ratio of 52 in the formulation, more compact DNA condensates were formed, as seen in Fig. 2d. The thread like structures were more like rods, and tend to bend into smaller ring shaped structures. Small spherical micellar like structures (~25nm in diameter) were also noticed decorating these DNA condensates, also seen in Fig. 2c. These are the micelles of extra pentablock copolymer used at this high N:P ratio. This suggest not all copolymer is used up in condensing the plasmid DNA, and a lower N:P ratio would be enough to form DNA condensates of this size.

The pentablock copolymers presented here are pH sensitive polymers, with a good buffering capacity at low pH, a property that help polymer/DNA condensates in the escape from low pH endosomal vesicles during their intra-cellular trafficking[38]. The tertiary nitrogens of PDEAEM group in the copolymer get protonated at low pH, giving them enhanced positive surface charge. To observe the effect of this increased cationic charge on condensation of DNA, we examined the morphology of polyplexes formed in a buffer of pH 4.7, found in the acidic lysosomal vesicles of the cells. As shown in Fig. 3a and b, the DNA condensates formed similar thread like structures as observed at pH 7.4; however they were

less extended, and had greater tendency to form closed loops or rings. The number of condensates that could be seen in all the images were also significantly less than that observed with similar N:P ratio at pH 7.4 (Fig. 2a). A probable reason could be that since copolymers had greater cationic charge at lower pH, the subsequent polyplexes had higher positive surface charge as lesser amount of it was neutralized in DNA condensation. This would have resulted in repulsion between the condensates, and hence they were found in less density in any imaged region. Also to be noticed are free polymer micelles decorating these threads like structures of polyplexes (Fig. 3a). This further confirms that at pH 4.7 smaller amount of cationic copolymer was required to completely condense the plasmid DNA, and rest of it was left free in solution. Fig. 3c shows that at higher N:P ratio of 26 in pH 4.7 buffer, as expected, more compact condensates of polyplexes were formed and greater amount of free polymeric micelles decorating the thread like structures were present.

As had been suggested earlier that nucleic acid compaction rather than surface charge was critical for efficient nuclear trafficking[39], this cryo-TEM study indicates that DNA condensates of 100-150 nm formed by pentablock copolymers should have a good capability to deliver the DNA to the cell nuclei.

3.2 Intra-cellular trafficking

Cells grown on PLL coated coverslips were transfected with EMA-labeled DNA, or its condensates with linear PEI (ExGen) or pentablock copolymers. Previous biocompatibility studies have indicated that pentablock copolymers are non-cytotoxic at lower N/P ratios and provide efficient transfection in the SKOV3 cells[20]. Since efficient DNA condensation was observed at N/P ratio of 13 in cryo-TEM studies, all pentablock copolymer polyplexes used in presented confocal microscopy studies were made at N/P 13. Polyplexes at N/P ratio 26, though provide better transfection because of better DNA compaction, were found to have greater cytotoxicity[20] (unpublished data[22]). Cells were observed for the location of fluorescence from EMA-DNA at different time points after transfection. Lysosomes and nuclei were labeled in some of the slides to examine the entrapment of DNA inside these cell organelles. Since EMA is a membrane impermeable dye, all the fluorescence of EMA seen inside the cells is due to EMA covalently bound to the DNA. Efforts were made to make sure

that any EMA just intercalated into the grooves of DNA is removed during purification in DNA labeling process. Plasmid DNA containing covalently bound EMA is not capable of getting transcribed and produce reporter protein[40]. Therefore, unlabelled plasmids were used for gene expression experiments. Images were taken at settings, e.g. laser intensity, PMT voltage, and pin-hole size, where no background fluorescence from the cells could be detected (Fig. 4). Nuclei of SKOV3 cells stained with DAPI are shown in Fig 4 to give a perspective of their shape and size.

After incubating the cells with pentablock copolymer/DNA complexes for 30 min, a faint fluorescence could be seen homogenously all over the coverslip, outlining the cells, with little clumps forming on the cell membrane. Most of the polyplexes at this time point were expected to be removed from the coverslip during the washing step since they were not yet internalized by the cells. After 2 to 4 hr of incubation, faint fluorescence outside the cells disappeared (Fig. 5). All the fluorescence was on the inner surface of cell membrane, with most of it localized in small discrete spots. These spots may be some discrete features on the cell membrane, such as coated pits, where most of the polyplexes attached to the cell membrane, and were then up taken into the cells via endocytosis. The observation of fluorescence all along the perimeter of cell membrane suggests that some polyplexes did enter the cells by a mechanism other than endocytosis. It could be by fusion of positively charged complexes with the anionic plasma membranes followed by trafficking of the amphiphilic copolymer across lipid bilayer as observed with other amphiphilic lipids and micellar polymeric structures[41-43]. Between 2-6 hrs, the number of such discrete spots of fluorescence increased, and so did their size, as they moved away from the cell membrane toward the nucleus (Fig. 5). The increase in size may be due to the fusion of endosomes with lysosomes. By 6 hr post-transfection (Fig. 5, a central 0.35 μm thick x-y plane of the cells), the fluorescence from labeled DNA was dispersed all over in the cytoplasm enclosing a dark patch with no fluorescence at all; the patch with no fluorescence is the nucleus and is clearly outlined by fluorescence.. Some fluorescence in the cytoplasm was localized in discrete vesicles of defined shapes, most likely representing the polyplexes entrapped in endosomes or lysosomes. The diffused fluorescence in the cytoplasm suggests that some of the polyplexes had already escaped out of the endosomes. Most of this diffused fluorescence was

located around the nucleus, suggesting that the polyplexes escaped the lysosomes mainly in the peri-nuclear region. To ensure entrapment of labeled DNA in endosomes, the low-pH vesicles of endosomes/ lysosomes were labeled with LysoTracker dye, and confocal images of lysosomes and DNA in same planes were aligned. As shown in Fig. 6, central plane of an SKOV3 cell 7 hr after transfection had yellow spots in the cytoplasm that represent the red colored endosomes containing green fluorescence of entrapped labeled DNA. It should be noticed that there is green fluorescence outside these vesicles too. The color of lysosomes containing labeled DNA varies between various shades of yellow to red, indicating different amounts of DNA entrapped inside.

By 10 hr post-transfection, fluorescence from labeled DNA could be detected inside the nuclei of SKOV3 cells (marked with arrow in Fig 7). Different planes of an SKOV3 cell around a central plane are shown as a montage in Fig. 7 to confirm the localization of fluorescence inside the nucleus. A lot of labeled DNA could also be detected in the perinucear region, suggesting nuclear import of polyplexes is one of the rate limiting steps. Another representative image showing fluorescence from labeled DNA in a central plane of SKOV3 cells in presented in Fig. 8 along with their z-plane. Four distinct observations can be made in this image. First, fluorescence is localized inside the nuclei clearly marking the whole nuclei in the x-y plane. Second, most of the diffused fluorescence noticed in the cytoplasm at earlier time points has disappeared completely in some parts. Third, some fluorescence is seen on the peripheral cell membrane clearly outlining the cells. This might be due to the recycling endosomes that finally merge back with the cell membrane at the end of their cycle. Labeled-polyplexes still attached to the inner surface of these vesicles appear on the surface of the cell membrane when the vesicles fuse back with it. Fourth, the fluorescence in the cytoplasm is organized on one side of the nucleus in a distinct structure that resembled gogli-apparatus, and most likely involved microtubule organizing center. In a recent study by Suh et al[24] it was suggested that besides thermal-motion driven diffusion, PEI/DNA nanocomplexes were actively transported through cytoplasm along the microtubules by motor-proteins towards the micro-tubule organizing center located adjacent to the cell nucleus. Such active transport along the microtubules might also be involved in the trafficking of cationic pentablock copolymer/DNA polyplexes, resulting in the accumulation

of these polyplexes in the perinuclear region. The faster accumulation of ExGen/DNA complexes than those of pentablock copolymers in the perinuclear region and their subsequent earlier detection in the nuclei possibly indicates they have higher diffusion rate (thermal and active taken together) in the cytoplasm. Another possibility may be that microtubule associated motor protein-driven active transport is more involved in trafficking of ExGen/DNA complexes than with pentablock copolymers. The cells in Fig. 7 and 8 were stained with lysotracker dye but the fluorescence was too weak to be detected suggesting that most of the low pH vesicles have disappeared from cytoplasm after either being disrupted by copolymers, or by final fusion with the cell membrane at the end of their cycle.

Cells transfected with the EMA-DNA using ExGen were imaged to investigate any difference in the intra-cellular pathway of these polyplexes from those of pentablock copolymers. The fluorescence from labeled DNA was found to be localized in the low-pH vesicles of the cells up to 4.5 hr after transfection, as shown in an aligned central plane of an SKOV3 cell in Fig. 9. This suggests that ExGen polyplexes also get entrapped into the endosomes and need to escape them to get into the nucleus. Nuclei of these ExGen transfected cells in Fig. 9 can be seen as a dark patch outlined with the fluorescence in perinuclear region. Fluorescence of labeled DNA could be detected in the nuclei of cells by only 6 hr post-transfection (as compared to 10 hr with pentablock copolymers). One of such bright spots is marked with an arrow in Fig. 10 showing a montage of different planes of an SKOV3 cell, ensuring localization inside the nucleus. Fluorescence in the perinculear region was localized in discrete spots, suggesting the ExGen/DNA complexes were bound to some intra-cellular components, potentially lysosomes or micro-tubule organizing center[24]. This is in contrast to the diffused fluorescence observed in Fig 7 in the perinuclear region of cells transfected with pentablock copolymers. However, most of the fluorescence had disappeared from the cytoplasm of these cells too. This is more clearly visible in ExGen transfected SKOV3 cells shown in Fig. 11. To reiterate, labeled DNA can be detected either in the nucleus of the cells, or in few discrete patches in the cytoplasm. It can be ensured by looking at the x-y plane and z-plane that these bright spots are not inside the nuclei. The dark patches in the cytoplasm could also be noticed.

In the cells incubated with only EMA-DNA, all the fluorescence was always found in discrete patches in the cytoplasm, with none of it ever detected inside the nucleus. However, the fluorescence of labeled DNA could be seen in the cytoplasm even 15 hr post-transfection (data not shown). This may be due to the small fragments of degraded labeled DNA still trapped in the recycling endosomes/ lysosomes.

The present study suggests, within the confinement of variables studied, that polyplexes of both pentablock copolymers and ExGen get trapped in the acidic endosomal vesicles and their escape from these vesicles is not a limiting step in the final delivery of ferried DNA to the nucleus. The pentablock copolymers have a pKa of pH ~7.3 with a good buffering capacity at low pH[17]. This property potentially aids in the escape of their polyplexes from the endosomal vesicles via proton sponge hypothesis[38], similar to that hypothesized for ExGen[25]. The diffused fluorescence in the perinuclear region and inside the nucleus 10 hr post-transfection of cells does confirm their escape from endosomes. A critical barrier in the trafficking of polyplexes to the nucleus is getting passed the nuclear membrane. Though real mechanism of polyplex entry into the nucleus is still elusive[25,26], it may involve interaction of cationic copolymer/DNA complexes with anionic phospholipids located in the cytoplasm and on the nuclear membrane[44,45]. As hypothesized earlier[25], one possible mechanism of nuclear entry could be that as polyplexes are released from endosomes, they retain a portion of phospholipid coated membrane electrostatically bound to them. This membrane fragment could fuse with the nuclear membrane and facilitate entry of bound polyplexes into the nucleus. This argument is supported by the observation that naked DNA, which could not bind to the phospholipids, was never detected inside the nucleus. Nevertheless, large fluorescence of labeled DNA in the perinuclear region even 10hr post-transfection does suggest that nuclear import of polyplexes is the primary rate-limiting step in pentablock copolymers mediated gene transfection and should be of focus in their further development. Adding nuclear localization signals (NLS) to the reactive ends of pentablock copolymers can possibly aid their nuclear import[46]. Further, previous studies with PEI and PLL have shown that nuclei of cells directly microinjected with their polyplexes did show transgene expression[39]. This suggests that even if pentablock copolymers were bound to DNA inside

the nucleus, the nuclear machinery is capable of releasing the plasmid bound to cationic copolymers and transcribe it for protein expression.

Although detailed mechanism of nuclear entry and endosomal escape still needs to be elucidated, this work confirms that cationic pentablock copolymers reported here do deliver the DNA into the nucleus. The trafficking involves uptake by endosomes, diffusion in the cytoplasm, escape from endosomes, accumulation in the perinuclear region and final uptake by cell nucleus.

3.3 Transfection efficiency

The final confirmation of delivery of the exogenous gene ferried by copolymers into the nucleus of the cells was made by observing the expression of green fluorescence protein (GFP) encoded by the pEGFP plasmid. Fig. 12a and b show fluorescent images of GFP expressed in SKVO3 cells 48 hr post-transfection with pentablock copolymer/DNA complexes. The intracellular GFP can be seen to fill the cytoplasm of the cells. The intensity of fluorescence in the cells varied from low to high, suggesting amount of GFP expression differed among the cells. One possible reason explaining this could be that different amount of DNA was delivered to the nuclei of different cells. The number of SKOV3 cells expressing GFP protein[19], and total amount of reporter luciferase protein expressed in the cells after transfecting with a luciferase expressing plasmid has been reported in previous reports[19,20]. The transfection efficiency of the pentablock copolymers was found to be comparable to ExGen.

4   Conclusions

The novel cationic amphiphilic pentablock copolymers exist as spherical micelles in aqueous solutions and efficiently condense plasmid DNA into linear or ring shaped thread like structures of 100 to 150 nm diameter. At lower pH, or at higher concentration of copolymer, the condensates were more compact. Lower amount of copolymer was required to condense DNA at acidic pH. Intracellular trafficking studies revealed that the copolymer/DNA complexes were efficiently taken up the cells all along their perimeter, mainly via endocytosis. The buffering capacity of copolymer at low pH aids in the release of

polyplexes from endosomal vesicles possibly via proton sponge hypothesis. Polyplexes could escape the endosomes to assemble in the perinuclear region and finally get localized in the nucleus of the cells. Transport of copolymer/DNA complexes was slower than ExGen/DNA complexes in the cytoplasm, indicating involvement of different mechanisms in the trafficking of the two copolymers. The study indicates that nuclear import of polyplexes, and not their diffusion through cytoplasm, is the limiting step in their intra-cellular trafficking and should be of focus in their further development.

Acknowledgement

References

1. Park TG, Jeong JH, Kim SW. Current status of polymeric gene delivery systems. Advanced Drug Delivery Reviews 2006;58(4):467-486.
2. Tan PH, Chan CL, George AJ. Strategies to improve non-viral vectors - potential applications in clinical transplantation. Expert Opinion on Biological Therapy 2006;6(6):619-630.
3. Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nat Rev Drug Discov 2005;4(7):581-593.
4. Dash PR, Read ML, Barrett LB, Wolfert MA, Seymour LW. Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery. Gene Therapy 1999;6(4):643-650.
5. Putnam D. Polymers for gene delivery across length scales. Nature Materials 2006;5:439–451.
6. Hwang SJ, Davis ME. Cationic polymers for gene delivery: designs for overcoming barriers to systemic administration. Curr Opin Mol Ther. 2001;3(2):183-91.
7. Golan R, Pietrasanta LI, Hsieh W, Hansma HG. DNA toroids: Stages in condensation. Biochemistry 1999;38:14069-14076.
8. Widom J, Baldwin RL. Cation-induced toroidal condensation of DNA : Studies with Co3+(NH3)6. Journal of Molecular Biology 1980;144(4):431-453.
9. Liu G, Molas M, Grossmann GA, Pasumarthy M, Perales JC, Cooper MJ, Hanson RW. Biological Properties of Poly-L-lysine-DNA Complexes Generated by Cooperative Binding of the Polycation. J. Biol. Chem. 2001;276(37):34379-34387.

10. Simberg D, Danino D, Talmon Y, Minsky A, Ferrari ME, Wheeler CJ, Barenholz Y. Phase Behavior, DNA Ordering, and Size Instability of Cationic Lipoplexes. RELEVANCE TO OPTIMAL TRANSFECTION ACTIVITY. J. Biol. Chem. 2001;276(50):47453-47459.

11. Gustafsson J, Arvidson G, Karlsson G, Almgren M. Complexes between cationic liposomes and DNA visualized by cryo-TEM. Biochimica et Biophysica Acta (BBA) - Biomembranes 1995;1235(2):305-312.

12. Soga O, van Nostrum CF, Ramzi A, Visser T, Soulimani F, Frederik PM, Bomans PHH, Hennink WE. Physicochemical Characterization of Degradable Thermosensitive Polymeric Micelles. Langmuir 2004;20(21):9388-95.

13. Mortensen K, Talmon Y. Cryo-TEM and SANS Microstructural Study of Pluronic Polymer Solutions. Macromolecules 1995;28:8829-8834.

14. Dubochet J, Adrian M, Dustin I, Furrer P, Stasiak A. Cryoelectron microscopy of DNA molecules in solution. In: Dahlberg DMJLaJE, editor. DNA Structures Part A: Synthesis and Physical Analysis of DNA: Academic Press; 1992. p 507-518.

15. Gustafsson J, Arvidson G, Karlsson G, Almgren M. Complexes between cationic liposomes and DNA visualized by cryo-TEM. Biochimica et Biophysica Acta 1995;1235(2):305-312.

16. Simberg D, Weisman S, Talmon Y, Faerman A, Shoshani T, Barenholz Y. The Role of Organ Vascularization and Lipoplex-Serum Initial Contact in Intravenous Murine Lipofection. J. Biol. Chem. 2003;278(41):39858-39865.

17. Determan MD, Cox JP, Seifert S, Thiyagarajan P, Mallapragada SK. Synthesis and characterization of temperature and pH responsive pentablock copolymers. Polymer 2005;46(18):6933-6946.

18. Determan MD, Chieh-Tsung L, Thiyagarajan P, Mallapragada SK. Self-assembly of Temperature and pH-Responsive Pentablock Copolymers. Polymeric Materials:Science and Engineering 2005;23:808.

19. Agarwal A, Unfer R, Mallapragada SK. Novel cationic pentablock copolymers as non-viral vectors for gene therapy. Journal of Controlled Release 2005;103(1):245-258.

20. Agarwal A, Unfer R, Mallapragada SK. Investigation of in vitro biocompatibility of novel pentablock copolymers for gene delivery. Journal of Biomedical Materials Research Part A 2007;81A(1):24-39.

21. Wangerek LA, Dahl HH, Senden TJ, Carlin JB, Jans DA, Dunstan DE, Ioannou PA, Williamson R, Forrest SM. Atomic force microscopy imaging of DNA-cationic liposome complexes optimised for gene transfection into neuronal cells. Journal of gene medicine 2001;3(1):72-81.

22. Agarwal A, Vilensky R, Unfer RC, Talmon Y, Mallapragada SK. Colloidal stability and transfection efficiency of self-assembling copolymeric gene delivery vectors in the presence of serum. Biomaterials 2007;In Review.

23. Zabner J, Fasbender AJ, Moninger T, Poellinger KA, Welsh MJ. Cellular and Molecular Barriers to Gene Transfer by a Cationic Lipid. J. Biol. Chem. 1995;270(32):18997-19007.

24. Suh J, Wirtz D, Hanes J. Efficient active transport of gene nanocarriers to the cell nucleus. PNAS 2003;100(7):3878-3882.

25. Godbey WT, Wu KK, Mikos AG. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. Proc Natl Acad Sci U S A 1999;96(9):5177-81.

26. Godbey WT, Barry MA, Saggau P, Wu KK, Mikos AG. Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. J Biomed Mater Res 2000;51(3):321-8.

27. Schaffer DV, Fidelman NA, Dan N, Lauffenburger DA. Vector unpacking as a potential barrier for receptor-mediated polyplex gene delivery. Biotechnology and Bioengineering 2000;67(5):598-608.

28. Bolton PH, Kearns DR. Spectroscopic properties of ethidium monoazide: a fluorescent photoaffinity label for nucleic acids. NUCLEIC ACIDS RES. 1978;5(12):4891-4903.

29. Serikawa T, Suzuki N, Kikuchi H, Tanaka K, Kitagawa T. A new cationic liposome for efficient gene delivery with serum into cultured human cells: a quantitative analysis using two independent

fluorescent probes. Biochimica et Biophysica Acta (BBA) - Biomembranes 2000;1467(2):419-430.

30.   Ruponen M, Ronkko S, Honkakoski P, Pelkonen J, Tammi M, Urtti A. Extracellular Glycosaminoglycans Modify Cellular Trafficking of Lipoplexes and Polyplexes. J. Biol. Chem. 2001;276(36):33875-33880.

31.   J. R. Bellare HTD, L. E. Scriven, Y. Talmon,. Controlled environment vitrification system: An improved sample preparation technique. Journal of Electron Microscopy Technique 1988;10(1):87-111.

32.   Talmon Y. Staining and drying-induced artifacts in electron microscopy of surfactant dispersions. Journal of Colloid and Interface Science 1983;93(2):366-382.

33.   Rackstraw BJ, Martin AL, Stolnik S, Roberts CJ, Garnett MC, Davies MC, Tendler SJB. Microscopic Investigations into PEG-Cationic Polymer-Induced DNA Condensation. Langmuir 2001;17(11):3185-3193.

34.   Wangerek LA, Dahl H-HM, Senden TJ, Carlin JB, Jans DA, Dunstan DE, Ioannou PA, Williamson R, Forrest SM. Atomic force microscopy imaging of DNA-cationic liposome complexes optimised for gene transfection into neuronal cells. The Journal of Gene Medicine 2001;3(1):72-81.

35.   Kwoh DY, Coffin CC, Lollo CP, Jovenal J, Banaszczyk MG, Mullen P, Phillips A, Amini A, Fabrycki J, Bartholomew RM. Stabilization of poly--lysine/DNA polyplexes for in vivo gene delivery to the liver. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression 1999;1444(2):171-190.

36.   Nguyen H-K, Lemieux P, Vinogradov SV, Gebhart CL, Guerin N, Paradis G, Bronich TK, Alakhov VY, Kabanov AV. Evaluation of polyether-polyethylenimine graft copolymers as gene transfer agents. 2000;7(2):126-138.

37.   Tang MXS, F. C. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. Gene Therapy 1997;4(8):823-832.

38.   Zuidam NJ, Posthuma G, De Vries ETJ, Crommelin DJA, Hennink WE, Storm G. Effects of physicochemical characteristics of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes on cellular association and internalization. Journal of Drug Targeting 2000;8(1):51-66.

39.   Pollard H, Remy J-S, Loussouarn G, Demolombe S, Behr J-P, Escande D. Polyethylenimine but Not Cationic Lipids Promotes Transgene Delivery to the Nucleus in Mammalian Cells. J. Biol. Chem. 1998;273(13):7507-7511.

40.   Tseng W-C, Haselton FR, Giorgio TD. Transfection by Cationic Liposomes Using Simultaneous Single Cell Measurements of Plasmid Delivery and Transgene Expression. J. Biol. Chem. 1997;272(41):25641-25647.

41.   Radwan Almofti M, Harashima H, Shinohara Y, Almofti A, Baba Y, Kiwada H. Cationic liposome-mediated gene delivery: Biophysical study and mechanism of internalization. Archives of Biochemistry and Biophysics 2003;410(2):246-253.

42.   Kabanov AV, Alakhov VY. Pluronic block copolymers in drug delivery: from micellar nanocontainers to biological response modifiers. Crit Rev Ther Drug Carrier Syst. 2002;19(1):1-72.

43.   Astafieva I, Maksimova I, Lukanidin E, Alakhov V, Kabanov A. Enhancement of the polycation-mediated DNA uptake and cell transfection with Pluronic P85 block copolymer. FEBS Letters 1996;389(3):278-280.

44.   Xu Y, Szoka FC. Mechanism of DNA Release from Cationic Liposome/DNA Complexes Used in Cell Transfection. Biochemistry 1996;35(18):5616-5623.

45.   Oku N, Shibamoto S, Ito F, Gondo H, Nango M. Low pH induced membrane fusion of lipid vesicles containing proton-sensitive polymer. Biochemistry 1987;26(25):8145-50.

46.   Hebert E. Improvement of exogenous DNA nuclear importation by nuclear localization signal-bearing vectors: a promising way for non-viral gene therapy? Biology of the Cell 2003;95:59-68.
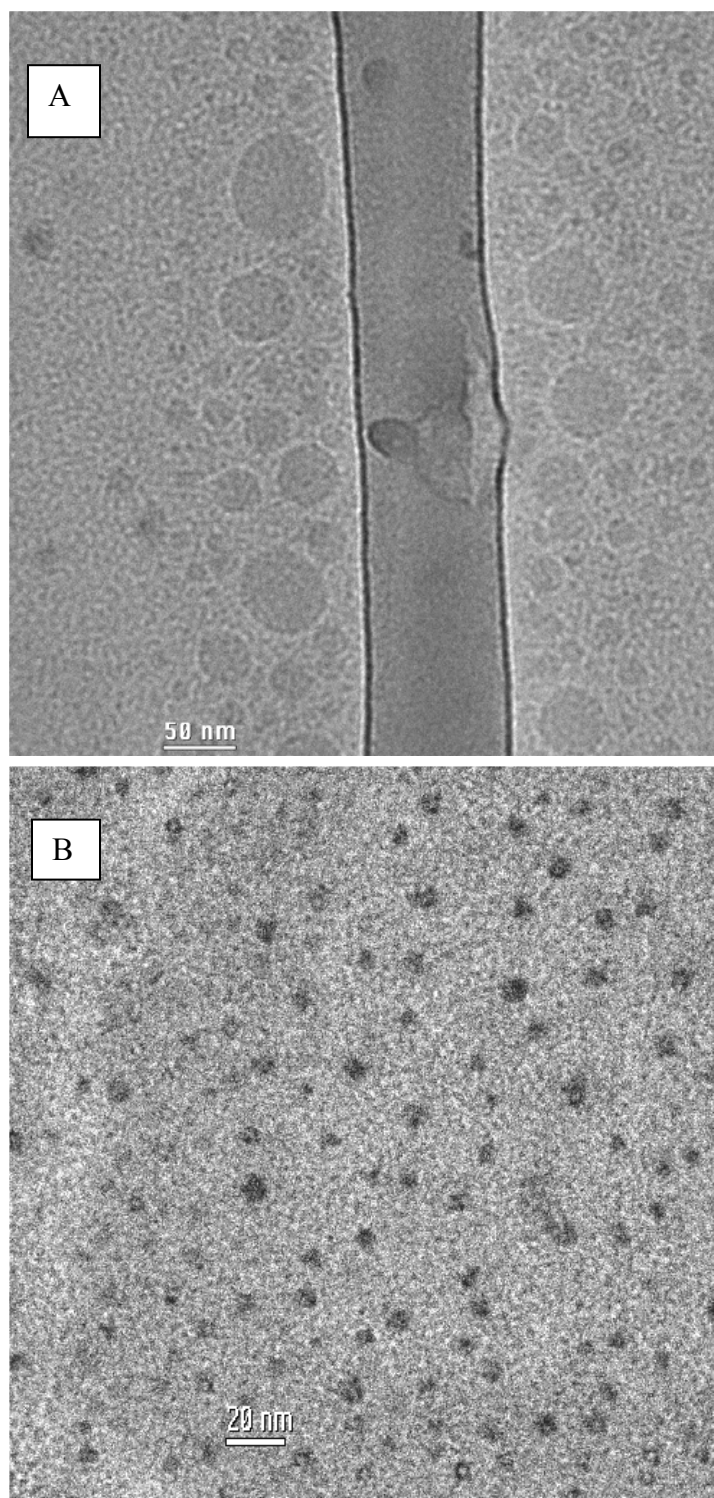
List of Figures

Fig. 1: Micellar structures of pentablock copolymers formed in aqueous buffers at (A) pH 7.4, and (B) pH 4.7.
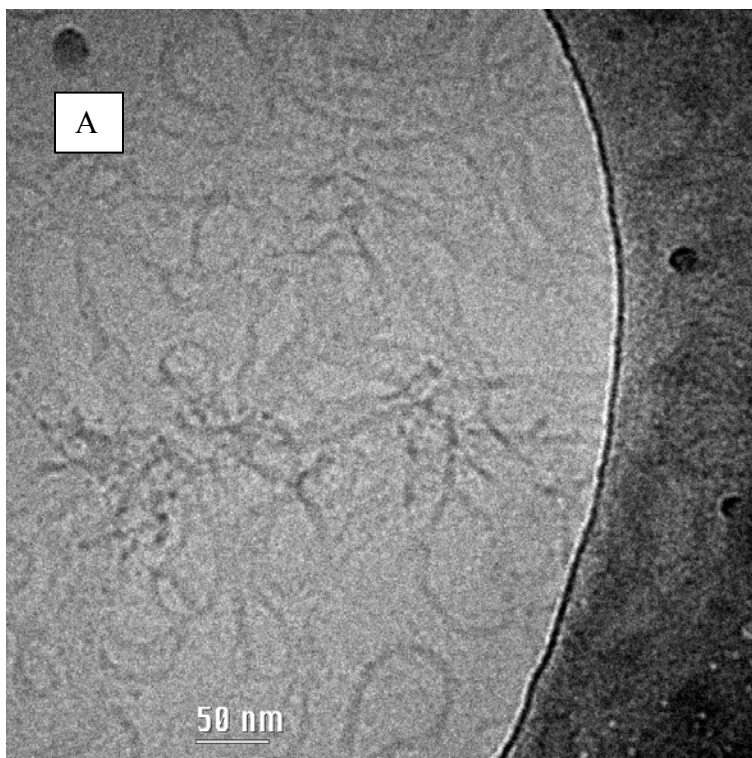
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Fig. 12: Expression of green fluorescent protein in SKOV3 cells 48hr post-transfection with pentablock copolymer/pEGFP DNA complexes at (a) N/P 5, and (b) N/P 13.

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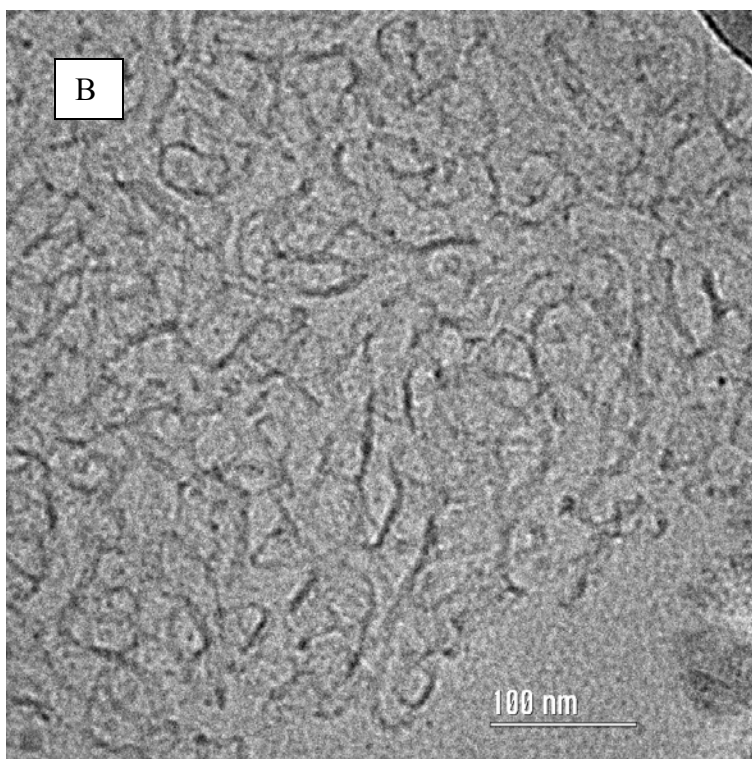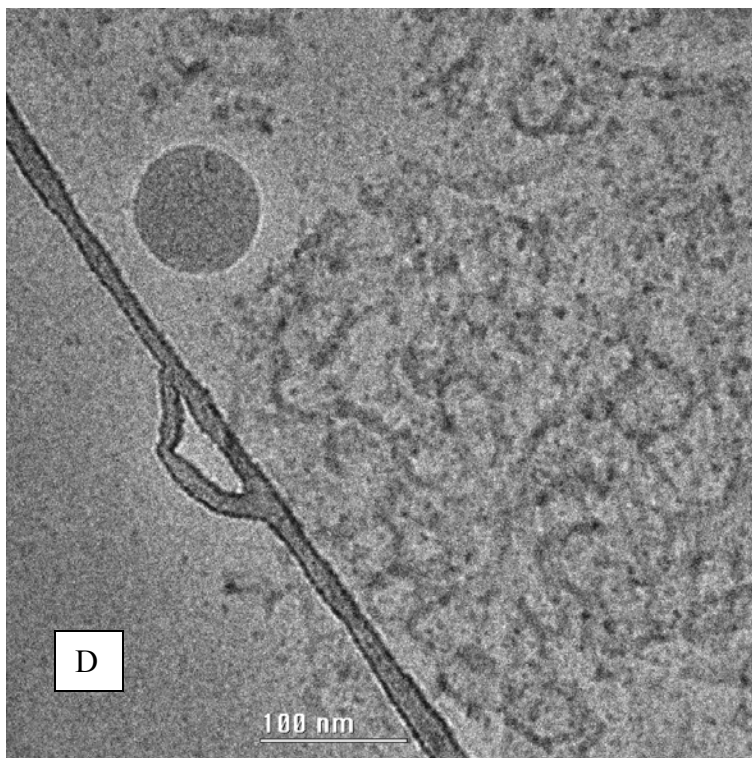
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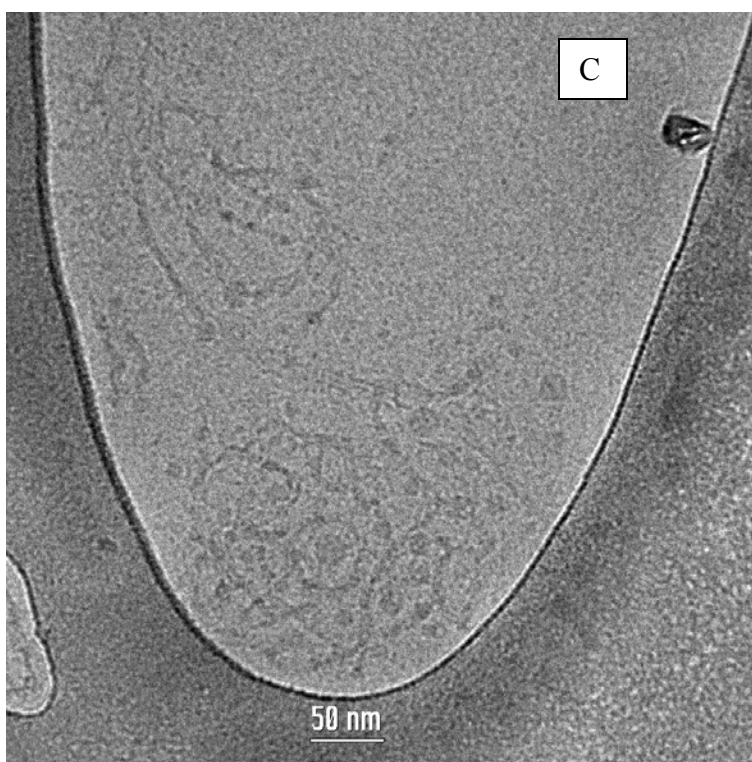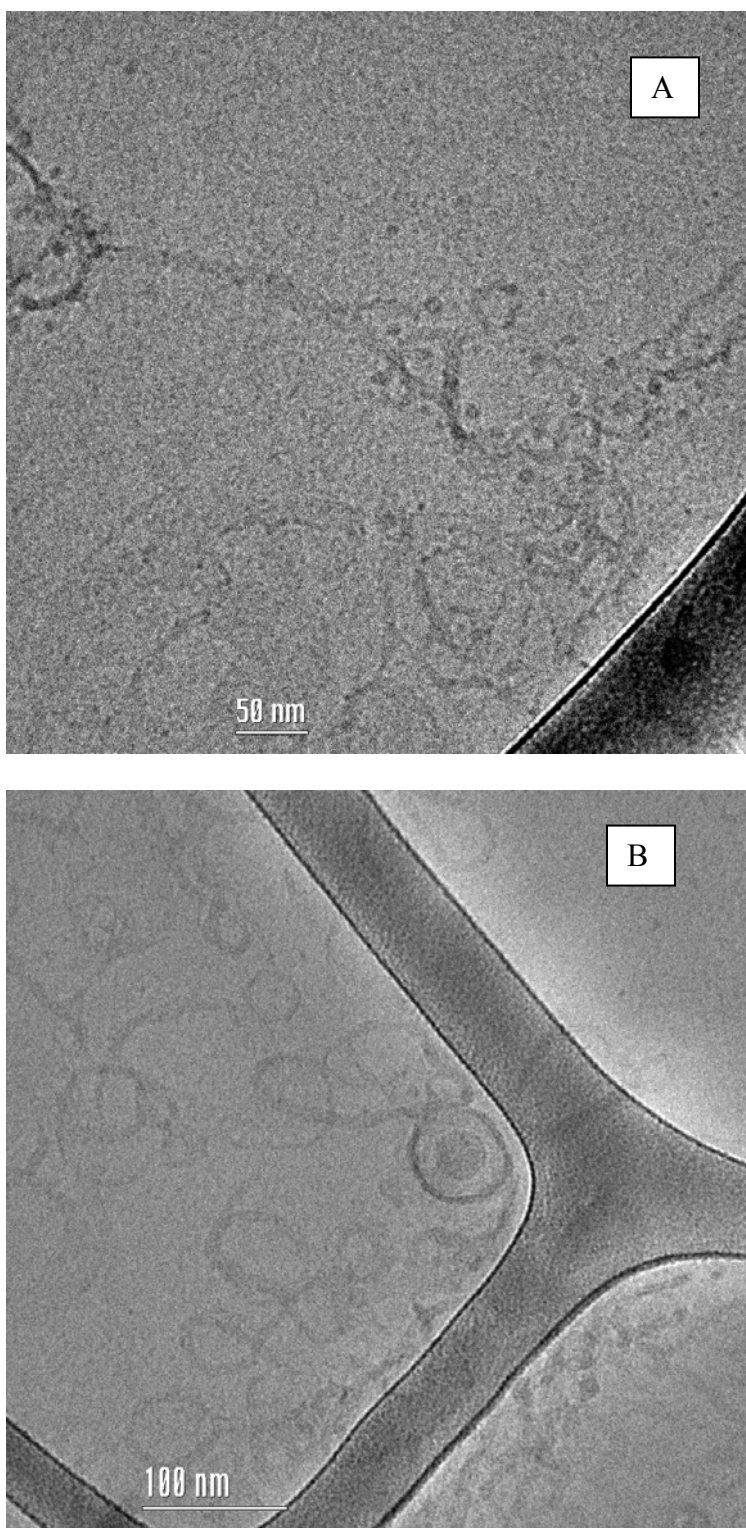
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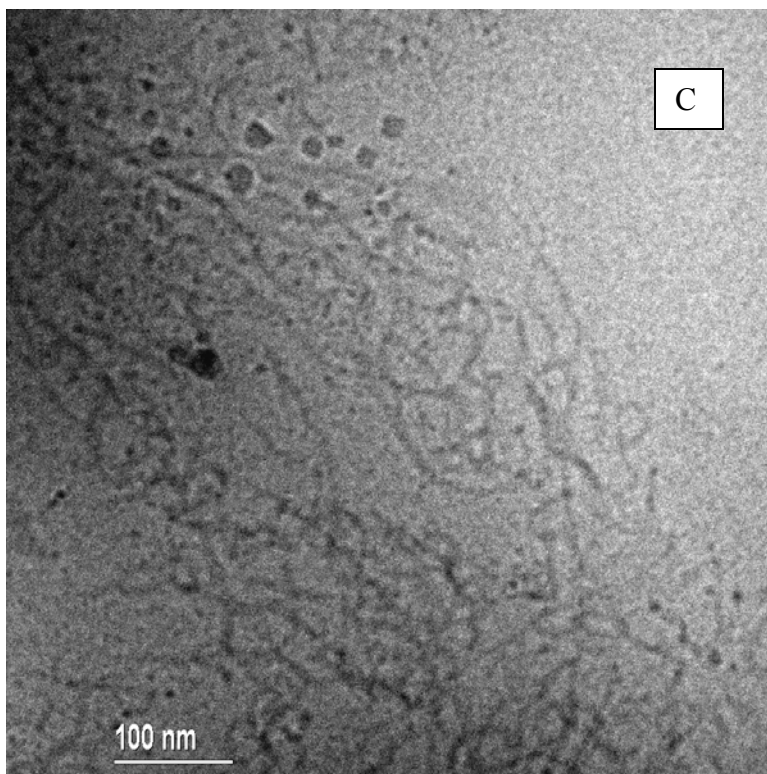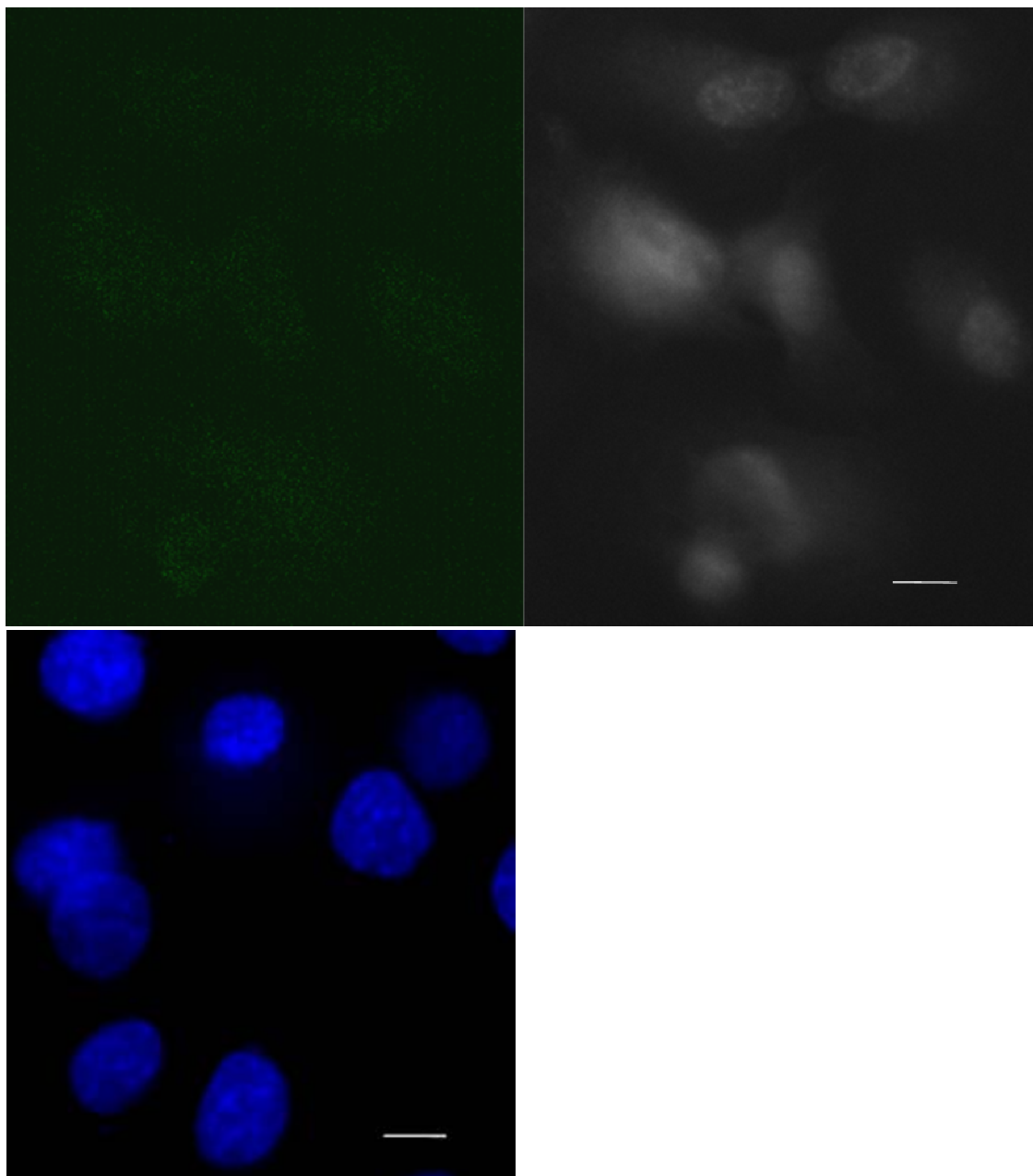
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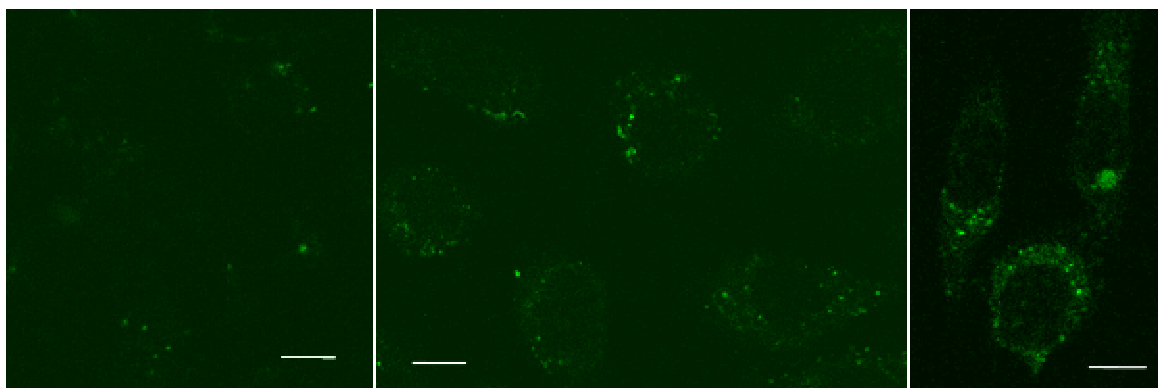
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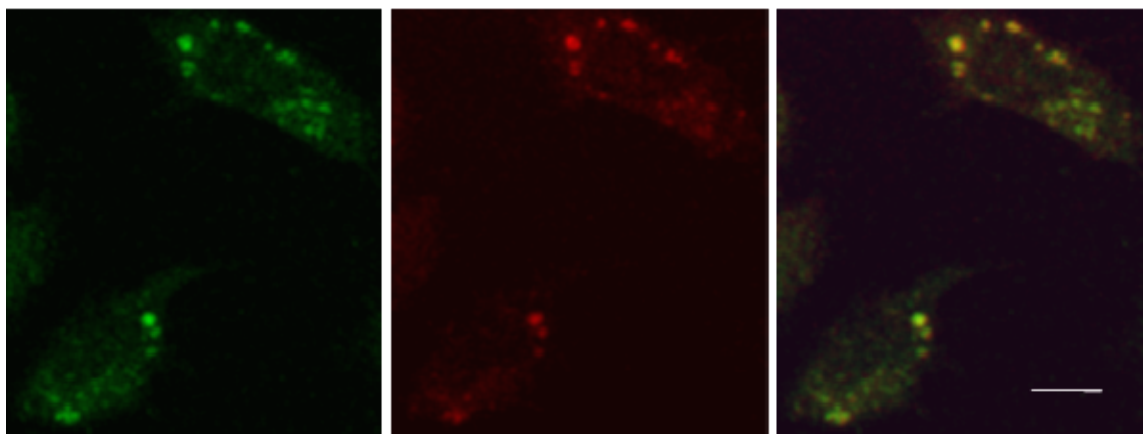
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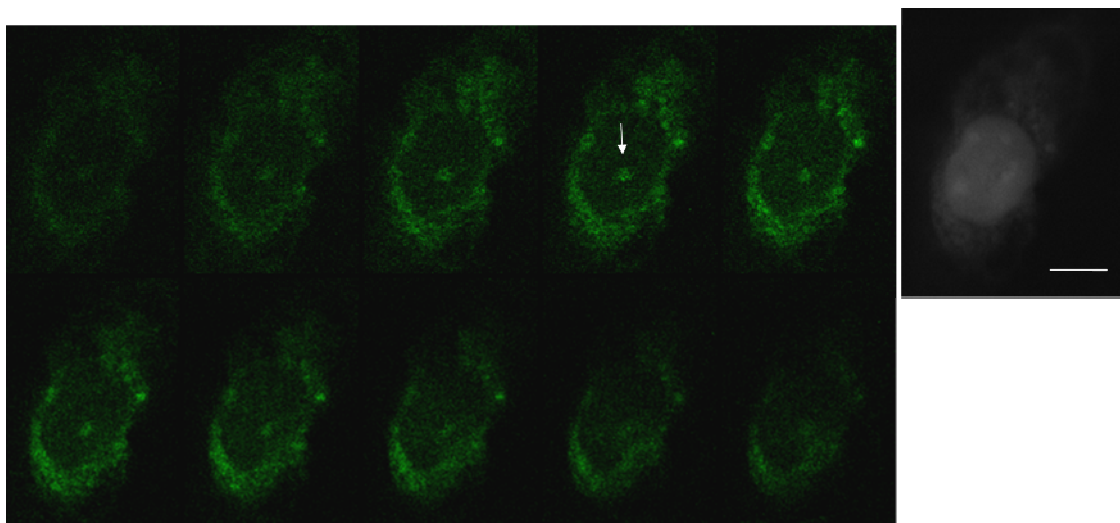
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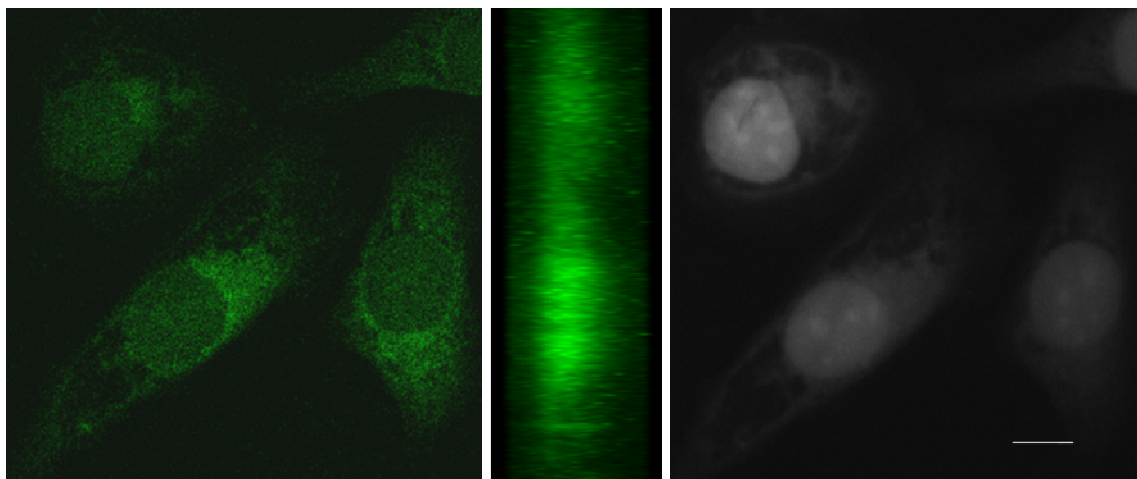
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CHAPTER 6

COLLOIDAL STABILITY AND TRANSFECTION EFFICIENCY OF NOVEL SELF-ASSEMBLING POLYMERIC GENE DELIVERY VECTORS IN SERUM SUPPLEMENTED MEDIA

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AA is primary researcher and author
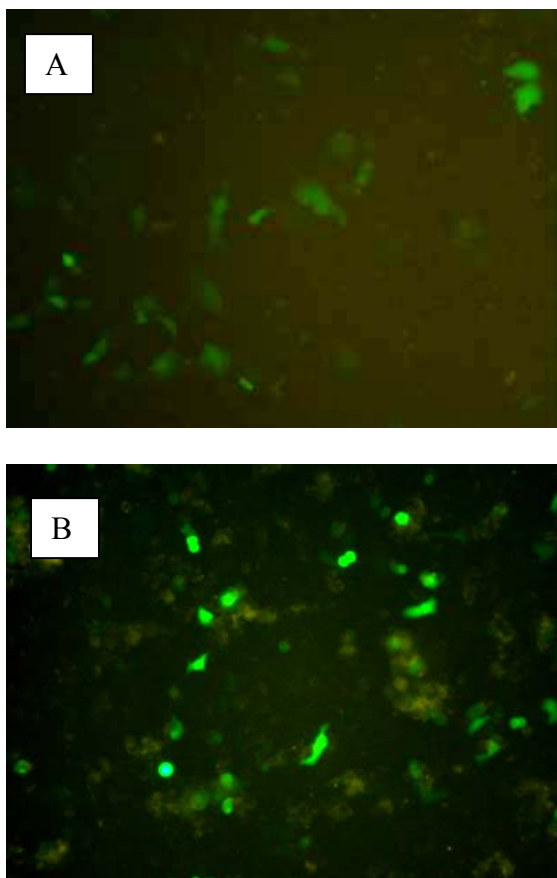
RV performed cryo-TEM

YT, RCU and SKM supervised the work

Abstract

Polymer/DNA complexes of novel pentablock copolymers of poly (diethylamino ethyl methacrylate) (PDEAEM) and Pluronic F127 were investigated from a physiochemical point of view for stability and transfection efficiency in serum-supplemented media. Dynamic light scattering revealed that the copolymers condensed plasmid DNA into polyplexes of 100 to150 nm diameter. The transfection efficiency of the copolymers in SKOV3 cells incubated in OptiMEM I[®] media was comparable to that of commercially available ExGen 500[®]. However, in serum supplemented growth media, the pentablock copolymer based systems formed large aggregates of >600nm dia, drastically reducing their transfection efficiency.

Adding unmodified Pluronic to the formulations stabilized these polyplexes against aggregation with serum proteins by sterically shielding their cationic surface charge, producing polyplexes of ~150-200nm in serum supplemented buffers which gave high levels of transfection. Shielding of cationic surface charges significantly reduced the cytotoxicity of cationic coplymers too, thereby further increasing transgene expression. Cryo-TEM micrographs showed that adding free Pluronic to the polyplex solutions significantly reduced the large number of globules and platelets of serum proteins that were aggregated around the thread like nanostructures of polyplexes. Nuclease resistance studies revealed that pentablock copolymers by themselves were effective enough to protect the condensed plasmid against degradation, and that adding free Pluronic to the formulations had no effect on their nuclease resistance efficiency. Though the total amount of DNA retained by polyplexes of ExGen 500® after nuclease digestion was more than that retained by pentablock copolymers, the amount of plasmid retained in supercoiled form by both the systems was not significantly different. However, the cytotoxicity of pentablock copolymers was significantly less than the ExGen 500® systems. This versatile multi-component micellar system of copolymers provides high transfection efficiency with significantly less cytotoxicity in complete growth media and warrants good performance in systemic applications.

Keywords: Block-copolymers, Gene delivery, Colloidal stability, Transfection, Cytotoxicity

1. Introduction

The delivery of therapeutic genes to the targeted cells using non-viral vectors is widely being explored nowadays as a viable alternative to viral vectors, especially via cationic polymers [1, 2]. More than a dozen new first generation and second-generation polymeric systems have been reported just in the past 5 years [3, 4]. Researchers have increasingly recognized that dangers associated with domesticating viruses for gene delivery such as insertional mutagenesis, potential oncogenesis, immunogenicity and, long-term effect of the

integrated transgene, along with production and packaging problems, can successfully be overcome by designing intelligent synthetic non-viral systems [5, 6].

Cationic polymers electrostatically condense negatively charged DNA into nanoparticles, forming stable polymer/DNA complexes, "polyplexes" [7]. However, though many of these polymeric vectors perform well *in vitro* in reduced serum conditions, they suffer from serious drawbacks when tested *in vivo* [8]. Binding of these polycations to DNA imparts excess positive surface charge to the complexes, which results in non-specific interactions with cellular blood components (erythrocytes), vessel endothelia and plasma proteins in an *in vivo* application [9]. In a systemic application, this leads to their aggregation and accumulation in the "first pass organs" such as lungs (consequently causing pulmonary embolism), liver and spleen, and finally opsonization and clearance by the reticuloendothelial system (RES), limiting their therapeutic applications [10, 11]. Particle size, charge and stability of these polyplexes are key factors in determining their biodistribution, circulation time and transfection efficiency *in vivo* [12]. Different strategies have been developed to improve the *in vivo* stability and efficacy of first-generation polymers [13, 14], resulting in a variety of second generation copolymers made by covalently linking polycations to a non-ionic water soluble polymer, such as poly(ethylene glycol (PEG) [15-19], transferrin [20, 21], or poly(N-(2-hydroxypropyl)methacrylamide (pHPMA) [22, 23], forming a block or graft copolymer architechture. Adding such hydrophilic ligands or grafts to the polycations increases their aqueous solubility, and shields their surface charges, creating a steric barrier against aggregation in blood streams or extra-cellular matrix [23, 24].

Recently we reported the development of novel amphiphilic pentablock copolymers which form thermo-reversible injectable gels, as potential vectors for sustained gene delivery [25]. These copolymers have triblock Pluronic F127 in the center, with cationic PDEAEM poly(diethylaminoethylmethacrylate) groups attached to their ends using an atom-transfer radical polymerization (ATRP) reaction scheme [26]. The copolymers form micelles because of the lower critical solution temperature (LCST) of hydrophobic PPO chains, and retain the thermoreversible gelation properties of the Pluronic. The cationic PDEAEM groups (pK_a~7.3) electrostatically condense DNA into nanoparticles, and provide a good buffering capacity at low pH that aids in the release of entrapped polyplexes from the acidic endosomal

vesciles[27]. As in Pluronics, where the presence of hydrophobic PPO chains provide them with the unique ability to be incorporated into cell membranes [28, 29], the PPO chains in the pentablock copolymers are expected to enhance cell interactions and increase translocation of polyplexes into the cells, with minimal damage to the cell membrane integrity, as compared to the cationic homopolymers PDEAEM or PDMAEM (poly dimethylaminoethylmethacrylate) [30]. These novel pentablock copolymers provide very good transfection efficiency, comparable to ExGen 500[®] (linear PEI), in reduced serum growth media, with minimal cytotoxicity [31], and are expected to retain the biological response modifying properties of the Pluronics [32] as well, making them good candidates to be further investigated for gene therapy.

Here we report the design of formulations of these novel copolymer/DNA complexes to impart serum stability. Since pentablock copolymers are derived from Pluronic F127, it was observed that adding Pluronic F127 to the polyplex formulations added stabilization in serum supplemented media, preventing formation of large aggregates. Both copolymers (pentablocks and Pluronic F127) form micelles in aqueous solutions. It was speculated that when free F127 is added to the polyplex solution, the hydrophobic PPO chains of free Pluronic would bind to the PPO chains of pentablock copolymers on the surface of polyplexes, while the PEO chains of free Pluronic would shield their surface charge. This self-assembly of two copolymers could then sterically stabilize the polyplexes against aggregation with serum proteins. In the present work we have tested the stability and transfection efficiency of this multi-component gene-delivery system in serum supplemented media. The formulations were investigated from a physiochemical point of view by measuring their particle size, zeta-potential, and resistance of incorporated DNA towards nuclease digestion in serum containing buffers at various concentrations of constituent components. The goal was to investigate how efficiently, and at what weight ratios, adding free Pluronic stabilizes the polyplexes, and to assess the role of each component in the overall transfection process.

The knowledge obtained from the current work will be applied toward optimizing the design of this multi-component micellar system for ongoing *in-vivo* gene delivery studies in our labs. These pentablock copolymers are particularly promising toward clinical gene

therapy because they are derived from Pluronics which are known to exhibit biological activity [32], such as sensitizing multi drug resistant (MDR) cancer cells [33], and effects on cell membrane properties [28]. Complete understanding of this copolymer system is further important in its development as a controlled gene delivery system, as injectable aqueous pharmaceutical formulations of these copolymers can form thermo-reversible gels in situ at physiological temperatures [26, 31], a valuable characteristic which can be exploited for sustained delivery of polyplexes to localized tissues.

2   Materials and Methods

2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), OptiMEM I$^®$, fetal bovine serum (FBS), 0.25% trypsin-EDTA solution, Hank's buffered salt solution (HBSS), Ultra-pure^{TM} agarose, EDTA, TAE buffer, Lysotracker Red$^®$ dye and, ethidium bromide were purchased from Invitrogen Inc, CA. HEPES salt, Heparin Sodium salt (cat # H-4784) and XTT (2,3-bis[2methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5- carboxyanilide inner salt) assay kit (Tox-2) were purchased from Sigma, MO. *Renilla* luciferase assay system kit was purchased from Promega Corporation, WI. The Qiagen Maxi Prep kit was purchased from Qiagen, CA. ExGen 500$^®$ (written as ExGen henceforth), GeneRuler^{TM} DNA ladder plus, and 6x TriTrack^{TM} loading dye solution were purchased from Fermentas Life Sciences, MD. DNase I was purchased from Ambion Inc, TX. Ultrapure water with at least 18 megaohm resistivity was used.

2.2 Polymers

Pluronic F127 [(PEO)$_{100}$-b-(PPO)$_{65}$-b-(PEO)$_{100}$] micro pastille surfactant was donated by BASF (Florham Park, NJ, USA) and used without further modification. The pentablock copolymers used for this reported study, PDEAEM$_8$-PEO$_{100}$-PPO$_{65}$-PEO$_{100}$-PDEAEM$_8$, containing 20wt% of PDEAEM, with M_n = 18520 and M_w/M_n = 1.14 as judged by ^{1}H NMR (in deuterated chloroform) and GPC (THF mobile phase, poly(methylmethacrylate) calibration standards) respectively, were synthesized as previously reported [26]. It can be

calculated that 1μg of this copolymer has 1.03 nM of nitrogen residues. The molecular weight of the DEAEM monomer is 185.

2.3 Plasmid DNA

A 4.1 kb plasmid pRL-CMV for encoding *Renilla* luciferase (Promega Corporation, WI), and a 4.7 kb pEGFP-N1 (ClonTech, CA) plasmid encoding for green fluorescence protein (GFP) were used as the reporter genes. DH5α *E. coli* cells were transformed with the plasmid DNA and incubated in selective Luria-Bertani (LB) medium. Amplified plasmid DNA was purified using the Maxi-Prep DNA Purification Kit. The concentration and purity of the resulting DNA in TE buffer, pH 7.4 was determined by measuring the absorbance, A, at 260 nm and 280 nm. All DNA used had a A_{260}/A_{280} ratio of at least 1.80.

2.4 Polyplex formulation

Copolymer to DNA ratios are expressed as molar ratio of nitrogen (N) in the pentablock copolymer to phosphate (P) in DNA, and written as N:P. The average molecular weight of a nucleotide is approximately 308. Using the fact that 1μg of DNA contains 3nM of phosphates, the amount of polymer required for corresponding N:P ratios was calculated. Polyplexes were formed by following the precise order of mixing DNA, copolymers and Pluronic F127. Pentablock copolymers were first dissolved in 0.5x HBS (Hepes buffer saline- 20mM of HEPES with 145mM NaCl), pH 7.0 at 4°C. For luciferase transfection and cytotoxicity experiments in 96-well plates, polyplexes were prepared with 2.4 μg pRL-CMV in a final volume of 800 μl, and were then divided into four equal parts of 200 μl for four wells in the plate, such that each well received 0.6 μg of plasmid. First, an aliquot of DNA (1μg/μL) in TE buffer pH 7.4 was taken into a 1.5 ml Eppendorf tube, and was made up to 100μL using 0.5x HBS buffer pH 7.0. Pentablock copolymer solution (2mg/ml) in 0.5x HBS, pH 7.0 was then added to DNA in the required amount to obtain the desired N:P ratio. The tube was vortexed gently, and left to incubate for 20 min at room temperature. If required, Pluronic F127 solution (25mg/ml) in 0.5x HBS, pH 7.0 was then added to the polyplex solution in the tube in the desired wt ratio of F127 to pentablock copolymer. The tube was vortexed again gently and incubated for another 10 mins, before making up the final volume

to 800 µL using desired growth media, OptiMEM I® or DMEM containing 10% FBS. For transfecting cells in 12-well plates with pEGFP, 3µg of DNA per well was used, and samples were prepared separately for each well using the above stated procedure, except that they were made up to final volume of 1ml. For light scattering experiments, samples were prepared in 0.5x HBS buffer with 1µg of DNA, and were made up to a final volume of 1ml with plain buffer or serum supplemented buffer, with final solute concentration between 0.3 to 0.8mg/ml.

2.5 Cell line

The SKOV3 human ovarian carcinoma cell line (from Iowa Cancer Research Foundation) was used for all cytotoxicity and transfection experiments. Cell cultures were maintained in a humidified environment with 5% CO_2 at 37°C and passaged regularly to allow them to remain sub-confluent. Cells were fed with DMEM supplemented with 10% fetal bovine serum (FBS) and 1µM L-glutamine, unless otherwise stated. Neither antibiotics nor antimycotics were used to avoid the possibility of artificial membrane permeabilization effects from these agents.

2.6 DNA condensation, nucleases resistance, serum stabilization

Agarose gel electrophoresis was performed on polyplexes made at different N:P ratios. A total of 0.125 µg of DNA per lane was used. Samples were prepared using an aliquot of 1 µg of pEGFP (0.1 µg/µL) in an Eppendorf tube. Buffer (0.5x HBS, pH 7.0) was added to the tube to make final polyplex solution volume of 100 µL. Then polymer solution (1mg/ml) was added to obtain the desired N:P ratio. Tubes were vortexed gently and incubated for 20 min at room temperature. For samples needing F127, required amount (wt. ratio 5:1 or 10:1 to the pentablock copolymer) was added from a 25mg/ml stock solution in 0.5x HBS buffer, pH 7.0, and samples were incubated for another 10 min. The final 100 µL sample was divided into 4 equal parts of 25 µL each for electrophoresis.

To evaluate resistance to nuclease digestion imparted by polymers, 25 µL polyplex solutions, containing 0.25 µg plasmid DNA, were incubated with 3 µL of 10x DNaseI buffer and 1 µL of DNase1 (2 IU/µL), giving 2 IU/µg DNA, for 15 mins at 37°C. Immediately

following incubation, 5 µL of 0.5 M EDTA was added, and samples were placed in an ice bath for 15 min to inactivate DNaseI. To examine the stability of polyplexes in the presence of serum proteins, 25 µL polyplex solutions were incubated with 25 µL 0.5x HBS buffer containing 20% FBS for 30min in a 37°C incubator. Immediately following incubation, 5 µL of 0.5M EDTA was added, and the samples were placed in an ice bath for 15 min to inactivate any nucleases in the serum.

To asses the integrity of plasmid DNA inside polyplexes, 100 mg/mL heparin solution, an anionic glycosaminoglycan (GAG), was added to the polyplex solutions to the final concentration of 1%w/v and incuabated for 30min, ensuring complete dissociation of DNA from the polymers. After adding 5-7 µL of 6x loading buffer, samples were loaded on a 1% agarose gel stained with ethidium bromide (0.25 µg/mL). The gel was run in TAE buffer at 50V for 2.5 hrs. Visualization and image capture was accomplished using a UV-transilluminator under a Kodak EDAS 290 digital imaging suite (Fisher Scientific; Pittsburg, USA). A 1kb+ DNA ladder and pEGFP-N1 DNA served as controls. All the experiments were repeated atleast 4 times to ensure reproducibility, and obtain error bars on band densitometry data.

2.7 Particle size and zeta(ζ) potential

Z-average diameter and polydispersity of the copolymers and polymer/DNA complexes were measured in Malvern disposable polystyrene cuvettes DTS 0012 at 37°C by a Malvern Zetasizer Nano-ZS90 system, equipped with 4 mW 633 nm He-Ne laser (Malvern Instruments, Southborough, MA, USA). Data analysis was carried out by the Dispersion Techonology Software (DTS) version 4.2. The instrument was calibrated with an aqueous polystyrene dispersion of 100 nm particles, using viscosity and refractive index of pure water at 37 °C. All water and buffers were double filtered using a 0.2 µm polyethersulfone (PES) sterile syringe filters (Fisher Scientific, MO) to remove any dust particles. PES has both, very low protein binding and, very low extractables, and is recommended for filtering cell culture media. The test solutions were vortexed gently and incubated for 30 min at 37°C before measurements. The ζ-potential of the polyplexes was measured at 37 °C in Malvern zeta

potential cuvettes DTS 1060. The instrument was calibrated using a polystyrene dispersion with a known ζ-potential.

2.8 Cryo-TEM

Vitrified specimens of the polymer/DNA complexes were prepared for transmission electron microscopy (TEM) in a controlled environment vitrification system (CEVS) at 25°C and 100% relative humidity, as previously described [34]. Briefly, a drop of the solution was applied onto a perforated holey-carbon film, supported on an electron microscopy 200-mesh copper grid, and held by tweezers in the vitrification system chamber. The sample was blotted with a filter paper, and immediately plunged into liquid ethane at its freezing point (−183°C). The vitrification process captured the copolymers and their DNA condensates in the sample in a state as close as possible to the native state without the need for artifact-inducing staining-and-drying [35]. Samples were examined in a Philips CM120 or an FEI T12 G^2 cryo-dedicated transmission electron microscopes (Eindhoven, The Netherlands), operated at 120 kV, using either an Oxford CT-3500 (for the CM120; Oxford Istruments, Abingdon, England) or a Gatan 626 (for the T12; Gatan, Pleasanton, CA) cooling holders and transfer stations. Specimens were equilibrated in the microscopes below −178°C, examined in the low-dose imaging mode to minimize electron beam radiation damage, and recorded at a nominal underfocus of about 2μm to enhance phase-contrast. Images were acquired digitally by a MultiScan 791 (CM120) or a US1000 (T12) cooled charge-coupled-device cameras (Gatan, Pleasanton, CA), using the Digital Micrograph software.

2.9 Transfection and cytotoxicity

To determine the total protein expressed by a luciferase reporter gene in the transfected cells, a luciferase assay was employed. Cells grown in a 96-well plate up to 70% confluency were transfected with various polyplex solutions in 200 μL media using 0.6 μg of DNA per well. After 3 hrs incubation, the polymer solutions were aspirated; cells were washed with HBSS buffer and incubated for another 45 hrs in complete growth media. For luciferase assay, cells were then lysed by incubating with 40 μL lysis buffer (*Renilla* Luciferase Assay

Lysis Buffer, Promega) for half an hour at room temperature, and the luminescence of the expressed reporter protein was measured on an automated Veritas™ Microplate Luminometer using the Promega *Renilla* Luciferase Assay System (Madison, USA). Cells incubated with naked DNA (without polymer) were used as negative controls.

Luciferase activity (RLU) in each well was not normalized by the total amount of protein (mg) as that gives artificially high values (RLU/mg) in the samples where total protein level has been reduced by the cell death. Instead, since all experiments were performed with same initial number of cells per well (~1.2 x 10^4) in a 96-well plate, luciferase expression is reported as RLU/well for each case, along with percentage cell viability found in each well using XTT assay. For XTT assay (Tox-2, Sigma), 40 µL of XTT stock solution prepared in phosphate buffer saline (PBS) pH 7.4 was added to each well of 96-well plate containing 200 µL of growth media, giving a final XTT concentration of 0.2 mg/mL. Plates were shaken on a gyratory shaker to enhance the dispersion of XTT, and were returned to incubator for another 4 hrs. The concentration of formazan crystals formed by the cleavage of tetrazolium ring of XTT by the mitochondrial dehydrogenases of viable cells [36] was found by measuring the absorbance at 450 nm using a BioTek EL-340 plate reader (Winooski, Vermont, USA). Background absorbance measured at 630nm was subtracted from the main readings. Viability was calculated relative to control cells not exposed to the polymers.

To measure the transfection efficiency of polymers in terms of the percentage of cells transfected in a population, SKOV3 cells seeded in 12-well plates were transfected with the pEGFP plasmid following procedures similar to those used in 96-well plates. Cells were harvested 48 hr after transfection and flow-cytometry was performed using a Beckman-Coulter Epics ALTRA Fluorescence-activated cell sorter (Fullerton, USA), as described in an earlier report[25].

2.10   Statistics

Where appropriate, the data is presented as mean and standard deviation (SD). Four samples were used for each case in all the experiments; mean and SD were calculated over them. Significant differences between two groups were evaluated by Students' t-test, and

between more than two groups by one-way ANOVA analysis of variance, followed by Tukey's test. The level of significance was set at $p<0.05$, unless otherwise stated.

3   Results and discussion

3.1 Colloidal stability

The size distribution of the polymers and polyplexes in buffer solutions at pH 7.0 and 37°C was measured in the absence and presence of serum, with different concentrations of unmodified free Pluronic F127 added to the formluations. The size and stability of polyplexes formed at N:P ratios from 5 to 30 investigated. For most of the samples, single narrow peaks of the scattered light intensity were obtained, while for samples with aggregates, the intensity of scattered light peaked at two different particle sizes, shown as unimodal or multimodal distribution of particles diameter in Fig. 1. Pentablock copolymers and Pluronic F127 had average cumulative micelle sizes of 33nm and 23 nm diameter, respectively, while serum particle sizes were around 9 nm, all with PDI less than 0.1. A 2 mg/ml solution of pentablock copolymers and F127 (5:1 w/w) together had an average micelle size of 25±2 nm. All other results are presented in Fig. 1. All samples were investigated for up to five hours repeatedly to confirm dispersion stability over time. Fig. 1a shows that the pentablock copolymer condensed DNA to form stable polyplexes above N:P ratio 5, with diameter less than 175nm and low PDI. The size of the condensates decreased systematically on increasing the N:P ratios from 5 to 20. This decrease in diameter of polyplexes reflects the process of DNA condensation induced by cationic polymers, and has been explained in several studies by the coil-globule transition of plasmid DNA molecules upon complexing to take the compact conformation [19, 37]. However, when these polyplexes were incubated with buffer containing 10% FBS, the peaks became wider (high polydispersity), and secondary peaks denoting large aggregates appeared at higher N:P ratios (Fig. 1b).

The effect of adding free Pluronic to the formulation was evaluated by first measuring the resulting polyplex sizes in buffers without serum. Fig. 1c shows that at wt ratio of 5:1 (F127 / pentablock), stable polyplexes of 145±11 nm diameter with low polydispersity were

formed at all investigated N:P ratios. Small secondary peaks at 25-30 nm in the figure denote free particles (micelles) of excess pentablock and Pluronic self-assembled together. At higher N:P ratios, when polyplexes are actually smaller in size (notice N:P 20 Fig. 1a), but have higher surface charge, more F127 micelles attach to them to neutralize their surface charge. Thus particle size apparently remained constant at all N:P ratios in the presence of Pluronic.

Fig. 1d shows the fate of these Pluronic stabilized polyplexes in buffers supplemented with 10% (v/v) FBS. Stable polyplexes of around 175nm in diameter, with no other aggregates in solution, were observed up to N:P 15. However, small distinct peaks of large aggregates of ~700±200 nm could be noticed at higher N:P ratios (Fig. 1d). Small peaks observed at ~8 nm and ~30 nm represent serum particles, and free pentablock/ Pluronic micelles, respectively, as had been noticed independently. These peaks however have been omitted in Fig. 1b and 1e to make the peaks of DNA condensates look more legible.

Adding F127 in w/w ratio of 10 to the formulations stabilized polyplexes in serum supplemented buffers at even higher N:P ratios. As shown in Fig. 1e, polyplexes of ~200nm diameter were formed up to N:P 20. At N:P 30, average particle size increased to 337±90 nm. These results again indicate that more F127 is needed to stabilize the particles with larger surface charge density at higher cationic copolymer concentrations. It should be noted that since at higher N:P ratios the size of polyplexes is larger in formulations containing free Pluronic than the ones without it (compare Figs. 1c and 1a), it would take fewer such polyplexes to make same size aggregates (700+ nm), suggesting that smaller amount of plasmid is lost to the aggregates.

Zeta potential of the particles in different formulations was measured in plain buffers containing no serum proteins. The measurements confirmed that pentablock copolymers and their polyplexes have excessive cationic surface charge. Pentablock copolymers by themselves gave zeta potentials of +6.0±1.3mV (with peak width of 10mV), and serum particles showed -6 mV (with peak width of 12 mV). After DNA condensation, the zeta potential of copolymer/DNA complexes (at N:P 20) was still around +2.2±0.23 (with peak width of 12 mV). However, when free F127 was added to the polyplex formulations in wt ratio 5:1 to pentablock copolymer, the zeta-potential was reduced to almost zero (0.037±0.5 mV, with peak width of 11 mV).

Cryo-TEM images of the polplexes were obtained to demonstrate the effect of F127 on their microscopic structure and aggregation in a 10% FBS supplemented buffer. Fig. 2a shows that polyplexes in formulations not containing F127 formed large masses of aggregates with serum proteins. Short thread like structures of polyplexes entangled with a large number of platelets and globules of serum proteins were observed. However, as shown in Fig. 2b, in formulations containing 5:1 w/w F127/pentablock copolymer fine extended thread like structures of polyplexes were observed, with significantly fewer numbers of globular molecules of the serum proteins around them. The representative micrographs shown here suggest that charged polyplexes attract more serum proteins to them and result in the formation of large masses of aggregates. However, when surface charges of polyplexes is sterically shielded by unmodified Pluronic, fewer globules of serum proteins accumulate on the polyplexes, and their long fine thread-like structure remains intact.

3.2 DNA integrity and protection

To serve as an efficient gene delivery vector, the copolymers should preserve the integrity of DNA while condensing it and ferrying to the nucleus of the targeted cells. One important factor in the activity of plasmid DNA is the conformation in which it exists in the solution. A plasmid can be in one of the three states: linear (after getting nicked), open circular (with only one strand nicked), and supercoiled. A varied degree of supercoiling might also exist depending upon the writhes in the plasmid. Fig. 3a shows the pEGFP plasmid in lanes 3-8 released from the polyplexes formed with pentablock copolymers at N:P ratios of 10 and 20, with either 10:1 (w/w), 5:1 (w/w), or no F127 added to the formulation. Lane 2 has plasmid released from the polyplexes of ExGen. As control, lane 1 contains naked plasmid incubated with 1% w/v heparin. Comparing plasmid in these two lanes with that released from polyplexes in other lanes, it can be observed very clearly that there is no difference in the plasmid conformation and band intensity in each conformation. The band intensities were measured in arbitrary units using KODAK 1D image analysis software (data not shown here). There is no presence of a band representing linear DNA, confirming that the plasmid is not cut by the condensation with polymer, and its integrity is maintained in

respective formulations. As expected, no effect of adding free Pluronic in the formulations is observed on the integrity of condensed plasmid.

Polyplexes were also investigated for their resistance towards plasmid degradation by nucleases. DNA released from the polyplexes post incubation with DNase1 was run on the agarose gels to examine its remaining integrity and topology. Fig. 3b shows that copolymers and ExGen provide partial DNase1 protection to the condensed DNA. Lane1 contains native plasmid, which exist in two conformations- open circular, and supercoiled, most of it in the latter form. Lanes 2-8 have DNA bands on the top of the lanes, confirming that there is some plasmid left after nucleases digestion, and that it is still condensed by the polymers. Lane 9 had naked DNA that was completely digested by DNase1, confirming the activity of nucleases. To examine the amount and integrity of DNA inside polyplexes in lanes 2-8, it was released using heparin salt, and run in lanes 10-16. Plasmid in lane 10 was condensed using ExGen at N:P 6. As can be seen, in contrast to naked DNA in lane 1, little amount of plasmid is in the supercoiled form, and most of it is in the open circular state. There is also a light DNA band between supercoiled and open circular DNA bands. This might be a linear plasmid, or can be a relaxed supercoiled form of plasmid with relatively less number of writhes. Comparing this to the plasmid released from polyplexes of pentablock copolymers in lanes 11-16, two points can be inferred. First, the total amount of DNA retained in ExGen polyplexes is greater than that in polyplexes of pentablock copolymer. Second, the total amount of DNA retained in supercoiled form is almost same with both cationic polymers. Another point to be noted is that nuclease resistance provided by pentablock copolymers is good enough at N:P 10, and does not improve on increasing the N:P ratio to 20, and that there is no significant improvement of adding free Pluronic in either ratio 5:1 or 10:1 to the formulations on the nuclease resistance efficiency. Densitometry data obtained over four similar experiments on this nuclease resistance efficiency of polymers is plotted in Fig. 3c, and clearly reiterates above stated inferences.

Fig. 3d shows the stability of polyplexes after incubation with serum proteins. Lanes 1 and 2 contain native plasmid before and after incubation with serum proteins. It can clearly be noted that plasmid is partially degraded in lane 2, and all the supercoiled plasmid is converted into circular form, indicating all the molecules of plasmid got nicked by nucleases

in serum proteins. Lanes 3, 4 and 5 contain plasmid released from polyplexes of ExGen (N:P 6), pentablock copolymer (N:P 20) with no free Pluronic and pentablock copolymer (N:P 20) with 5:1 free Pluronic, respectively, and lanes 6, 7, 8 contain plasmid released from these polyplexes post incubation with serum proteins, respectively. As can be seen, the integrity and topology of plasmid DNA remains intact in all the polyplexes after incubation with serum proteins, and is similar to that before incubation with serum proteins, and is similar to the naked DNA. These results confirm that both cationic polymers provide almost complete protection to condensed DNA against serum proteins, and that there is no significant observable effect of adding free Pluronic to copolymer systems on the stability of polyplexes in serum.

3.3 Transfection and cytotoxicity

DNA condensates of pentablock copolymers showed remarkably high transfection efficiency in the reduced serum media OptiMEM I$^®$. As shown in Figure 4a, transgene expression of luciferase obtained with pentablock copolymers at N:P 10 and above was only one order less than that obtained with ExGen. The luciferase expression increased on increasing the N:P ratios, but it was concomitant with a reduction in the viability of the cells (Fig. 4b). Thus, a increase in transfection efficiency of copolymers at higher N:P ratios was offset by an increase in toxicity, displaying an overall reduced protein expression per well. However, when F127 was added to the polyplex solutions in F127/pentablock w/w 5:1, there was a significant decrease in the toxicity of the copolymers. This in turn increased the total luciferase expression per well at higher N:P ratios, and an overall higher expression was obtained with such a formulation at N:P 15. It should be noted that though ExGen gave one order of magnitude higher protein expression than the copolymers at N:P 12, the cell viability in the presence of ExGen was reduced to 70% as compared to 90% with the pentablock copolymers.

Fig. 5 shows the transfection efficiency and cytotoxicity of the copolymers in complete growth media, containing 10% FBS. As expected from the light scattering study above, little transfection was obtained by incubating cells with only pentablock-copolymer/DNA complexes, as most of these charged complexes bind to serum proteins in the media to form

large aggregates that are unable to get across the cell membrane. This is also evident from the increased cell viability (Fig. 5b) in complete growth media with polyplexes at N:P ratios 10, 15, and 20, as compared to that in OptiMEM I$^®$ (Fig. 4b). Since most of the charged complexes formed neutralized aggregates with serum proteins, few cationic complexes were left in the complete media to interact with the cells, thus decreasing the observed cytoxicity. However, when F127 was added to the formulation F127/pentablock wt ratio 5:1, significantly higher transfection was obtained at all N:P ratios, with total luciferase expression, at N:P 15 and higher, as good as that obtained with pentablock copolymers in the serum-free media. This confirms that most of the polyplexes were prevented from aggregating with serum proteins, and could get across the cell membrane to deliver their DNA payload to the nucleus. This charge shielding effect of F127 was also evident from the reduced toxicity of the complexes on addition of F127 (Fig. 5b) leading to lower cationic surface charge, less cell membrane damage, and increased cell metabolic viability.

Different concentrations of free Pluronic were added to the polyplex solutions to shield their surface charge and to find the optimum formulation. Results with F127/pentablock wt ratio 10:1 are also presented here for comparison. As shown in Fig. 5, though there is no significant effect of higher F127 concentration at lower N:P ratios, a significant increase in transfection efficiency and cell viability is observed at N:P 25 and 30. This can be explained by the fact that at higher N:P ratios a larger amount of unbound cationic copolymer is present in the media, and polyplexes have higher surface charge density. Thus, there is an increase in both the total charged surface area to be shielded (because of increased free copolymer micelles), and total surface charge to be shielded. Hence, an increased concentration of free F127 is required to form stabilized dispersions in serum-supplemented media. This also suggests that for an *in vivo* formulation, where large doses of DNA will be injected in a single dose, higher amount of free F127 should be used to shield higher concentration of copolymers in the solution.

SKOV3 cells were also transfected with a pEGFP gene in the presence and absence of serum to evaluate transfection efficiency of polymers in terms of percentage of cells expressing the transgene product. Pentablock copolymer/ DNA complexes with F127/pentablock wt ratio 5:1 were used. As reported in Fig. 6, similar to results obtained

with luciferase transfection, the transfection efficiency of copolymers increased with the N:P ratio. The pentablock copolymers transfected upto 21% cells in reduced serum media, and upto 17% cells in complete growth media, which is similar to or better than the efficiency obtained with ExGen.

4   General discussion

This work reports the critical evaluation of the design and stability of polycation-DNA complexes based on novel pentablock copolymers. These pentablock copolymers were chosen for further investigation because they had previously been shown to efficiently deliver condensed DNA to the targeted cells in low serum OptiMEM I$^{®}$ media, and had exhibited significantly low cytotoxicity. Furthermore, these copolymers self-assemble to form thermo-reversible hydrogels at physiological temperatures, a characteristic property that can be used to deliver polyplexes in a sustained fashion when injected intramuscularly or in a localized tumor. To exploit these advantages of these novel copolymers as a gene delivery vector in an *in vivo* application, we have tried to improve and evaluate their colloidal stability in serum supplemented media.

At physiological pH 7.4, tertiary amines of PDEAEM blocks (pKa~7.3) in the pentablock copolymers are partially protonated. Thus the cationic copolymers condense DNA via electrostatic interactions into nanoparticles. It was observed that the size of these condensates decreased, as the amount of copolymer added to the DNA increased. However, the extra cationic copolymer used results in excess positive surface charge on the polyplexes. Thus they tend to form large aggregates with anionic serum proteins; such aggregates cannot cross cell membranes to deliver the ferried DNA to the nucleus. Thus all DNA is lost to the aggregates and little transfection is obtained.

Because the pentablock copolymers discussed here have a Pluronic core in their architecture, it was hypothesized that adding unmodified Pluronic to a solution of pentablock copolymer/DNA complexes will lead to formation of polyplexes with a more hydrophilic corona that will sterically shield the cationic surface charge. Pluronics had previously been shown to enhance the uptake of plasmid DNA and cell transfection when added along with cationic polymers [38]. When poly(*N*-ethyl-4-vinylpyridinium bromide) (pEVP-br) and DNA complexes were mixed with 1% Pluronic P85, the DNA uptake in the cells as well as the

transgene expression were significantly increased compared to the cells treated with the pEVP-br and DNA complex alone[39]. Another recent study showed that when free Pluronic was added to the complexes of P123-*g*-PEI(2K) and DNA, they formed more hydrophilic stable dispersions in the presence of serum proteins, and showed enhanced transfection efficiency, P123:P123-g-PEI(2k) (9:1) [40]. Gebhart et al suggested in that study that free Pluronic sterically stabilized the polyplexes by self-assembling with polycations in such a fashion that it masked the hydrophobic PPO chains of the P123 grafted on PEI. However, that system exhibited low level of DNA protection against DNaseI, and gave much lower transfection efficiency as compared to ExGen.

The first objective of this work was to understand the process of polyplex formation, and the mechanism of their complexation with serum proteins. Dynamic light scattering revealed that fine polyplexes of ~100 to 150 nm, formed by pentablock copolymers in serum free buffers, aggregated to yield big particles of ~700 – 1000 nm in the presence of serum. However, when F127 was added to these formulations in F127/pentablock wt ratio 5:1, polyplexes of ~150 nm were formed, that formed stable dispersions of ~150 – 200 nm even in serum supplemented buffers. Though some aggregates of ~ 400-700 nm radii were also observed at N:P 20 and above, their formation was also avoided when F127 was increased to wt ratio 10:1 in the formulations. These observations confirm that adding F127 to the charged polyplex solutions sterically stabilizes the cationic polyplexes against aggregation with serum proteins. Cryo-TEM micrographs further confirmed that adding F127 to the polyplexes reduced the number of globules of serum proteins attached to them, preventing formation of large aggregates.

The mechanism of the process can most likely be explained as follows, and is sketched in Fig. 7. F127 has same hydrophobic PPO core as pentablock copolymers discussed in this report. On adding F127 to polyplex solutions, hydrophobic PPO blocks of F127 are attracted to the PPO blocks of the extra pentablock copolymers on the surface of polyplexes. While the two hydrophobic cores self-assemble, the long PEO chains of F127 sterically shield the cationic PDEAEM groups of the pentablock copolymers. By adding F127 at a w/w ratio of 5:1 (or more) to the pentablock concentration in the solution, several F127 unimers/micelles are available per extra pentablock micelles on the polyplex surface. Thus, nanoscale

polyplexes with narrow polydispersity are formed that have condensed DNA in their core, and a hydrophilic corona formed by PEO chains of F127 and pentablock copolymers. The masking of charged PDEAEM groups by long PEO chains of several Pluronic micelles reduces the zeta potential of particles to zero and prevents their interaction with serum proteins, while the hydrophilic surface with PEO chains prevents the aggregation of particles with each other. Also, if there is any free pentablock copolymer in the solution, the unimers/ micelles of pentablock and F127 arrange themselves in a dynamic equilibrium to form sterically stabilized micelles with no zeta-potential. Further, since both the copolymers have the same amphiphilic architecture, the resulting polyplex solutions still retain the thermo-gelling properties, and form thermo-reversible hydrogels at 37°C.

The order of addition of F127 to the formulation was also investigated by observing its impact on the size of the particles, and their transfection efficiency. Several formulations were investigated, but two were in particular important -- one as reported above, where Pluronic was added to the formulation after 20 min incubation of DNA with the pentablock copolymer, and second, where Pluronic was added to the DNA aliquots before adding pentablock copolymer to the formulation. No significant difference was observed (data not shown), and the first formulation was adopted for the rest of the study. It should be noted though that the polyplexes were formed in serum-free buffers, and serum supplemented media was added only after addition of Pluronic to the system. The formation of large aggregates of polyplexes with serum proteins is a very rapid and irreversible process, and therefore their surface charge needs to be shielded before serum is added to the system.

The next objective of this work was to confirm the stability of the condensed DNA inside these copolymers. Agarose gel electrophoresis results show that plasmid DNA is retained in its supercoiled topology even after condensation by both ExGen and pentablock copolymers. There is no effect of adding free Pluronic to the formulations of copolymer's polyplexes, suggesting it is the cationic PDEAEM blocks of copolymers that are responsible for condensing the DNA. However, after incubation with DNase1, only partial protection is imparted to plasmid by both ExGen and copolymers, with most of the plasmid converted from supercoiled form to open circular or a relatively relaxed supercoiled form. This conversion of topology might suggest that the integrity of the plasmid is compromised.

Another explanation could be that dissociation of plasmid from the polymers after DNase1 incubation might result in the change of twists and writhes of the DNA molecule, such that the supercoiled plasmid becomes relatively relaxed, reducing its mobility. Although the ExGen based polyplexes protected the largest quantity of DNA, the portion of the supercoiled fraction retained was the same as that with pentablock copolymers, which many would agree is the fraction with greatest integrity. This is evident from the good transfection efficiency obtained with pentablock copolymers too. However, the larger quantity of saved DNA can explain the one order of magnitude higher total gene expression obtained with ExGen. There was no effect of adding free Pluronic to the copolymer's polyplex formulations on the quality of protection provided against DNase1.

The pentablock copolymer-based polyplexes were found stable in the presence of serum proteins, retaining both, the total amount of DNA, and complete portion of supercoiled DNA. Again, free Pluronic had no effect on the stability and protective capacity of polyplexes in serum. This means that low transfection obtained by copolymers without free Pluronic in serum-supplemented media is not because of the degradation of polyplexes or the condensed DNA inside, but by the aggregation of polyplexes with serum proteins, leading to big aggregates (as evident by light scattering) that cannot cross the cell membrane. Thus, the sole function of free Pluronic added to the system is to provide colloidal stability to the polyplexes, and prevent aggregation, allowing the copolymers ferry the DNA into the cells.

Finally, the levels of transgene expression obtained with the pentablock copolymers correlated well with the particle size study. Adding free Pluronic prevented aggregate formation between polyplexes and serum proteins, and provided significantly improved plasmid DNA uptake in SKOV3 cells in serum-supplemented growth media. Another advantage of the charge-shielding action of F127 was significant reduction in the cytotoxicity of the polyplexes. This is of significance as it allowed working at higher copolymer concentrations without compromising the cell viability. For transfection in reduced-serum media, a N:P ratio of 10 was found to be optimum, while for transfection in complete growth media, N:P ratio of 20 was optimum, providing good transgene expression, while maintaining cell viability up to 90%. The total luciferase expression in the cells was one order higher with ExGen than that obtained with pentablock copolymers, and that could be

explained by the larger quantity of plasmid retained by ExGen systems after nuclease incubation. However, the ExGen polyplexes were significantly more toxic at these concentrations. The pentablock copolymers used in this study had 20wt% of PDEAEM content. Perhaps using longer cationic chains in the copolymer can increase their nuclease resistance capabilities, and thus improve their transfection efficiency. Furthermore, since the number of cells expressing GFP after transfection with pEGFP using pentablock copolymers was similar to those obtained with ExGen, it indicates that pentablock copolymers are able to successfully deliver the ferried gene to the nucleus of as many cells as ExGen, and their transfection efficiency is limited by the amount of gene delivered.

A final comment worth to be made is that this is a very dynamic gene delivery system and can be easily tailored to specific therapeutic applications. Pluronic F127 was used is this study because first, it has long hydrophilic chains, and second, it is already approved for use in pharmaceutical preparations, which means the toxicological data exists, and can therefore speed the preclinical development of pentablock copolymer formulations. However, there are many other Pluronics that may be used in these formulations as free Pluronic to shield the surface charge of polyplexes, or can be substituted in pentablock copolymers for F127. A detailed review on Pluronics with their structure, characteristics, and drug delivery applications is discussed elsewhere [32]. Also, the wt% of cationic component PDEAEM can be controlled in the pentablock copolymers conferring varying degrees of cytotoxicity, transfection efficiency, and pH-sensitivity to the polyplexes [25, 31]. The increase in PDEAEM content increases the cytotoxicity of copolymers, but decreases the amount of copolymer required to condense the DNA. However, it was observed that copolymers containing 25wt% of PDEAEM required at least 10:1 wt ratio of free Pluronic to form stabilized dispersions with no aggregates in serum supplemented buffer at N:P ratios where they gave good transfection with low cytotoxicity, similar or better than that obtained with the copolymers reported here (data not shown).

5 Conclusions

In summary, we have shown that adding Pluronic F127 to the polyplexes of pentablock copolymers increased the DNA uptake and expression in the cells in complete growth media primarily by stabilizing the size and stability of the polyplexes. The addition of F127 also showed improved biocompatibility of polyplexes due to masking of their cationic surface charge. We determined that pentablock copolymers provide an efficient protection to condensed DNA against nucleases and serum proteins, and adding free F127 to the formulations did not enhance their nuclease protection efficiency. While the total amount of DNA retained after a nuclease digestion was more with polyplexes of ExGen, the amount of supercoiled DNA retained was same by both cationic polymers. Though the transgene expression obtained with ExGen was one order of magnitude higher than with pentablock:Pluronic copolymer system, the latter had significantly higher biocompatibility both in the presence and absence of serum. However, the transfection efficiency of this copolymer system in terms of total number of cells transfected was similar to that of ExGen. Furthermore, these formulations of polyplexes self-assemble at higher concentrations and physiological temperatures to form thermo-reversible gels that can act as a reservoir to release polyplexes in a sustained fashion when injected intramuscularly or into a localized tumor. This quality of these novel copolymer/DNA complexes, together with the serum stability, nuclease resistance, biocompatibility and high transgene expression shown in this report, makes it a versatile multi-component gene delivery system, and warrants good performance in *in vivo* applications.

References

1. D. Putnam, Polymers for gene delivery across length scales, Nature Materials 5 (2006) 439–451.
2. M. Thomas, A.M. Klibanov, Non-viral gene therapy: polycation-mediated DNA delivery, Applied Microbiology and Biotechnology 62 (1) (2003) 27-34.
3. J.K. Vasir, V. Labhasetwar, Polymeric nanoparticles for gene delivery, Expert Opinion on Drug Delivery 3 (3) (2006) 325-344.
4. T.G. Park, J.H. Jeong, S.W. Kim, Current status of polymeric gene delivery systems, Advanced Drug Delivery Reviews 58 (4) (2006) 467-486.
5. C.M. Henry, Gene delivery--without viruses, Chemical & Engineering News 79 (48) (2001) 35-41.
6. A. El-Aneed, An overview of current delivery systems in cancer gene therapy, Journal of Controlled Release 94 (1) (2004) 1-14.
7. P.L. Felgner, Y. Barenholz, J.P. Behr, S.H. Cheng, P. Cullis, L. Huang, J.A. Jessee, L. Seymour, F. Szoka, A.R. Thierry, E. Wagner, G. Wu, Nomenclature for synthetic gene delivery systems, Human Gene Therapy 8 (5) (1997) 511-512.
8. F. Verbaan, I. van Dam, Y. Takakura, M. Hashida, W. Hennink, G. Storm, C. Oussoren, Intravenous fate of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes, European Journal of Pharmaceutical Sciences 20 (4-5) (2003) 419-427.
9. R.I. Mahato, Y. Takakura, M. Hashida, Nonviral vectors for in vivo gene delivery: physicochemical and pharmacokinetic considerations, Crit Rev Ther Drug Carrier Syst 14 (2) (1997) 133-72.
10. M. Ogris, E. Wagner, Targeting tumors with non-viral gene delivery systems, Drug Discovery Today 7 (8) (2002) 479-485.
11. D.B. Fenske, I. MacLachlan, P.R. Cullis, Long-circulating vectors for the systemic delivery of genes, Current Opinion in Molecular Therapeutics 3 (2) (2001) 153-158.
12. P.R. Dash, M.L. Read, L.B. Barrett, M.A. Wolfert, L.W. Seymour, Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery, Gene Therapy 6 (4) (1999) 643-650.
13. U. Lungwitz, M. Breunig, T. Blunk, A. Gopferich, Polyethylenimine-based non-viral gene delivery systems, European Journal of Pharmaceutics and Biopharmaceutics 60 (2) (2005) 247-266.
14. S.J. Hwang, M.E. Davis, Cationic polymers for gene delivery: designs for overcoming barriers to systemic administration, Curr Opin Mol Ther. 3 (2) (2001) 183-91.
15. M. Lee, S.W. Kim, Polyethylene Glycol-Conjugated Copolymers for Plasmid DNA Delivery, Pharmaceutical Research 22 (1) (2005) 1-10.
16. T. Kawano, M. Yamagata, H. Takahashi, Y. Niidome, S. Yamada, Y. Katayama, T. Niidome, Stabilizing of plasmid DNA in vivo by PEG-modified cationic gold nanoparticles and the gene expression assisted with electrical pulses, Journal of Controlled Release 111 (3) (2006) 382-389.
17. Jeong Soon Jang, So Yeon Kim, Sang Bong Lee, Kyung Ok Kim, Joong Soo Han, Young Moo Lee, Poly(ethylene glycol)/poly([epsilon]-caprolactone) diblock copolymeric nanoparticles for non-viral gene delivery: The role of charge group and

molecular weight in particle formation, cytotoxicity and transfection, Journal of Controlled Release 113 (2) (2006) 173-182.

18. A.M. Funhoff, S. Monge, R. Teeuwen, G.A. Koning, N.M.E. Schuurmans-Nieuwenbroek, D.J.A. Crommelin, D.M. Haddleton, W.E. Hennink, C.F. van Nostrum, PEG shielded polymeric double-layered micelles for gene delivery, Journal of Controlled Release 102 (3) (2005) 711-724.

19. K. Itaka, K. Yamauchi, A. Harada, K. Nakamura, H. Kawaguchi, K. Kataoka, Polyion complex micelles from plasmid DNA and poly(ethylene glycol)-poly(-lysine) block copolymer as serum-tolerable polyplex system: physicochemical properties of micelles relevant to gene transfection efficiency, Biomaterials 24 (24) (2003) 4495-4506.

20. M.L. Kyung, S.K. In, B.L. Yong, C.S. Sang, C.L. Kang, J.O. In, Evaluation of transferrin-polyethylenimine conjugate for targeted gene delivery, Archives of Pharmacal Research 28 (6) (2005) 722-729.

21. R. Kircheis, L. Wightman, A. Schreiber, B. Robitza, V. Rossler, M. Kursa, E. Wagner, Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application, Gene Therapy 8 (1) (2001) 28-40.

22. T.E. Robert C. Carlisle, Simon S. Briggs, Jon A. Preece, Karel Ulbrich, Leonard W. Seymour,, Polymer-coated polyethylenimine/DNA complexes designed for triggered activation by intracellular reduction, The Journal of Gene Medicine 6 (3) (2004) 337-344.

23. D. Oupicky, M. Ogris, K.A. Howard, P.R. Dash, K. Ulbrich, L.W. Seymour, Importance of Lateral and Steric Stabilization of Polyelectrolyte Gene Delivery Vectors for Extended Systemic Circulation, Molecular Therapy 5 (4) (2002) 463-472.

24. F.J. Verbaan, C. Oussoren, C.J. Snel, D.J.A. Crommelin, W.E. Hennink, G. Storm, Steric stabilization of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes mediates prolonged circulation and tumor targeting in mice, Journal of Gene Medicine 6 (1) (2004) 64-75.

25. A. Agarwal, R. Unfer, S.K. Mallapragada, Novel cationic pentablock copolymers as non-viral vectors for gene therapy, Journal of Controlled Release 103 (1) (2005) 245-258.

26. M.D. Determan, J.P. Cox, S. Seifert, P. Thiyagarajan, S.K. Mallapragada, Synthesis and characterization of temperature and pH responsive pentablock copolymers, Polymer 46 (18) (2005) 6933-6946.

27. N.J. Zuidam, G. Posthuma, E.T.J. De Vries, D.J.A. Crommelin, W.E. Hennink, G. Storm, Effects of physicochemical characteristics of poly(2-(dimethylamino)ethyl methacrylate)-based polyplexes on cellular association and internalization., Journal of Drug Targeting 8 (1) (2000) 51-66.

28. E.V. Batrakova, S. Li, S.V. Vinogradov, V.Y. Alakhov, D.W. Miller, A.V. Kabanov, Mechanism of pluronic effect on P-glycoprotein efflux system in blood-brain barrier: Contributions of energy depletion and membrane fluidization, Journal of Pharmacology and Experimental Therapeutics 299 (2) (2001) 483-493.

29. N.S. Melik-Nubarov, O.O. Pomaz, T.Y. Dorodnych, G.A. Badun, A.L. Ksenofontov, O.B. Schemchukova, S.A. Arzhakov, Interaction of tumor and normal blood cells

with ethylene oxide and propylene oxide block copolymers, FEBS Letters 446 (1) (1999) 194-198.

30. P. van de Wetering, J.Y. Cherng, H. Talsma, D.J.A. Crommelin, W.E. Hennik, (2-dimethylamino)ethyl-methacrylate based copolymers as gene transfer agents., J control Rel 53 (1998) 145-153.

31. A. Agarwal, R. Unfer, S.K. Mallapragada, Investigation of in vitro biocompatibility of novel pentablock copolymers for gene delivery, Journal of Biomedical Materials Research Part A 9999 (9999) (2006) NA.

32. A.V. Kabanov, V.Y. Alakhov, Pluronic block copolymers in drug delivery: from micellar nanocontainers to biological response modifiers., Crit Rev Ther Drug Carrier Syst. 19 (1) (2002) 1-72.

33. E.V. Batrakova, S. Li, W.F. Elmquist, D.W. Miller, V.Y. Alakhov, A.V. Kabanov, Mechanism of sensitization of MDR cancer cells by Pluronic block copolymers: Selective energy depletion, British Journal of Cancer 85 (12) (2001) 1987-1997.

34. H.T.D. J. R. Bellare, L. E. Scriven, Y. Talmon,, Controlled environment vitrification system: An improved sample preparation technique, Journal of Electron Microscopy Technique 10 (1) (1988) 87-111.

35. Y. Talmon, Staining and drying-induced artifacts in electron microscopy of surfactant dispersions, Journal of Colloid and Interface Science 93 (2) (1983) 366-382.

36. N.W. Roehm, G.H. Rodgers, S.M. Hatfield, A.L. Glasebrook, An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT, Journal of Immunological Methods 142 (2) (1991) 257-265.

37. S.M. Mel'nikov, V.G. Sergeyev, K. Yoshikawa, Discrete coil-globule transition of large DNA induced by cationic surfactant, Journal of the American Chemical Society 117 (9) (1995) 2401-2408.

38. A. Kabanov, J. Zhu, V. Alakhov, *Pluronic Block Copolymers for Gene Delivery - Advances in Genetics*, in *Non-Viral Vectors for Gene Therapy, Part 1*, M.-C.H. Leaf Huang, and Ernst Wagner, Editor. 2005, Academic Press. p. 231-261.

39. I. Astafieva, I. Maksimova, E. Lukanidin, V. Alakhov, A. Kabanov, Enhancement of the polycation-mediated DNA uptake and cell transfection with Pluronic P85 block copolymer, FEBS Letters 389 (3) (1996) 278-280.

40. C.L. Gebhart, S. Sriadibhatla, S. Vinogradov, P. Lemieux, V. Alakhov, A.V. Kabanov, Design and Formulation of Polyplexes Based on Pluronic-Polyethyleneimine Conjugates for Gene Transfer, Bioconjugate Chem. 13 (5) (2002) 937-944.

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Fig. 2: Micrographs of pentablock copolymer/DNA complexes (N:P 10) in a serum supplemented (10% v/v) buffer with, (A) no free Pluronic, or (B) free Pluronic (5:1 w/w Pluronic : pentablock copolymer) added to the formulation.

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Fig. 3d: Integrity of pEGFP plasmid released from the polyplexes before and after incubating with 10% serum. Lane 1: Naked plasmid, Lane 2: Native plasmid after incubation with seurm; lane 3: ExGen at N:P 6; lane 4, 5: pentablock copolymer at N:P 20, with no Pluronic, and with 5:1 Pluronic, respectively; lane 6,7,8: polyplexes in lane 3, 4, and 5 after incubation with serum.

Fig. 4(a): Effect of free Pluronic F127 on transfection efficiency of pentablock copolymers in SKOV3 cells incubated in OptiMEM I® media. + $p<0.05$, * $p<0.1$, ° $p<0.1$, *** $p<0.005$ (n=4 ± SD).

Fig. 4(b): Effect of free Pluronic F127 on cytotoxicity of pentablock copolymers' polyplexes at different N:P ratios in OptiMEM I® media. Polyplexes, containing 0.6ug pRL, were incubated with SKOV3 cells for 3hrs in Opti-MEM I®, and cell viability was measured after another 48hrs of incubation in complete growth media (n=4 ± SD).

Fig. 4(c): Luciferase expression in SKOV3 cells transfected with pgWiz-luc using pentablock copolymers in OptiMEM I® media. Effect of adding free Pluronic to the formulations is shown. Total luciferase expressed in the cells is normalized by the cell viability of the cell population ($n=4 \pm$ SD).

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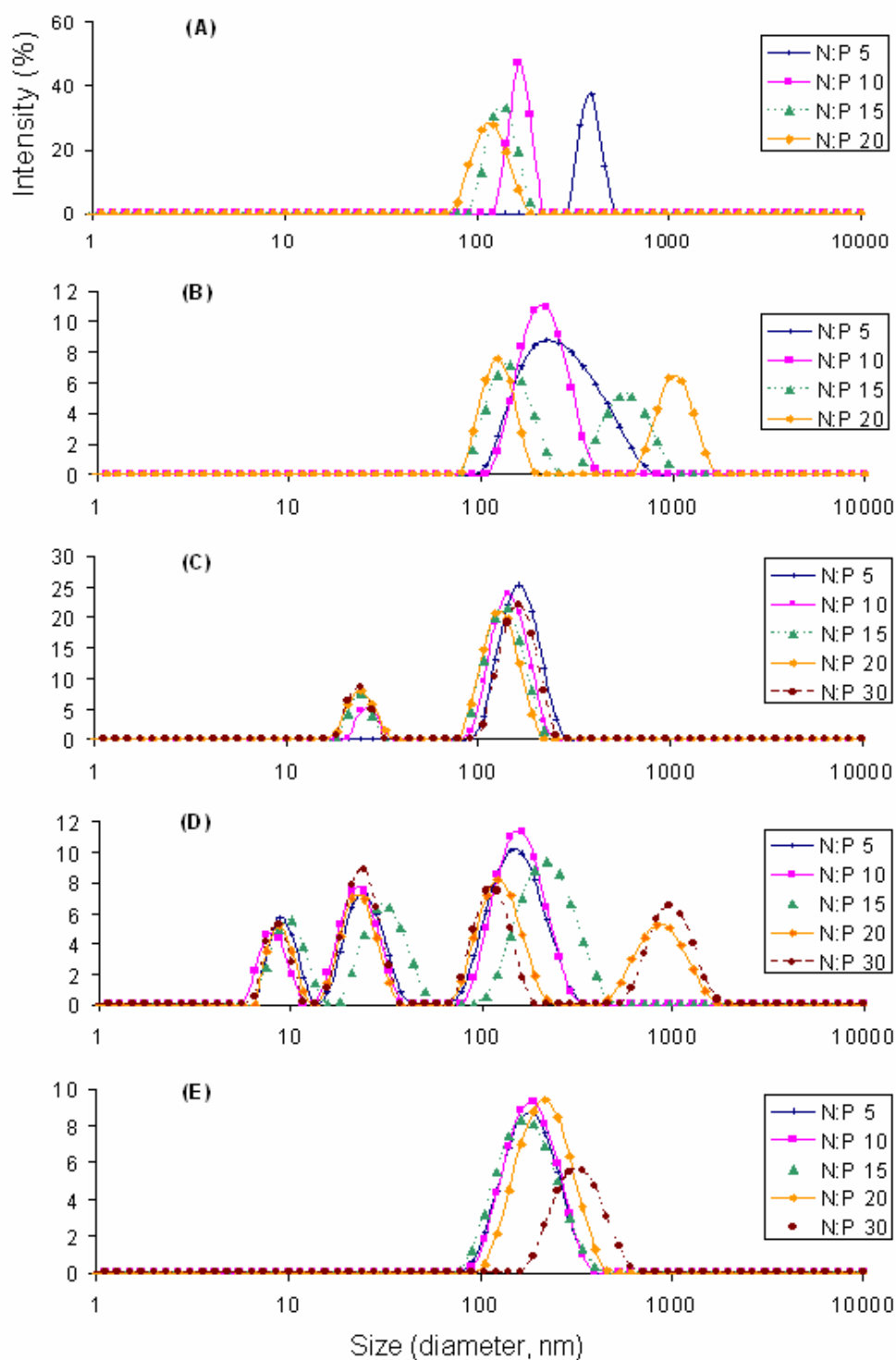
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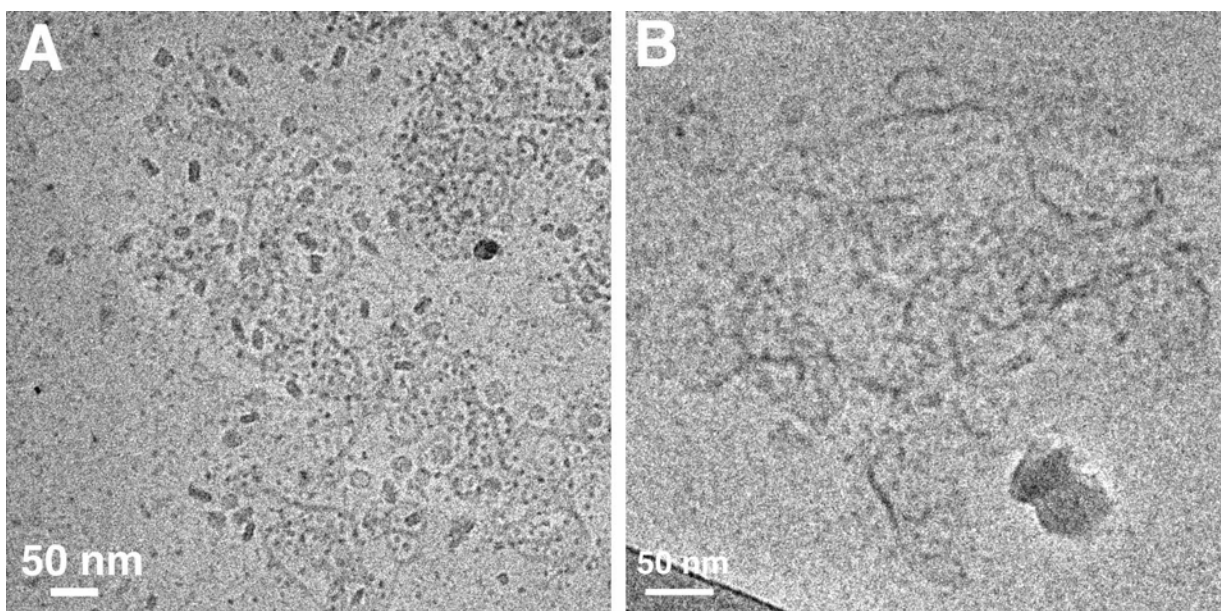
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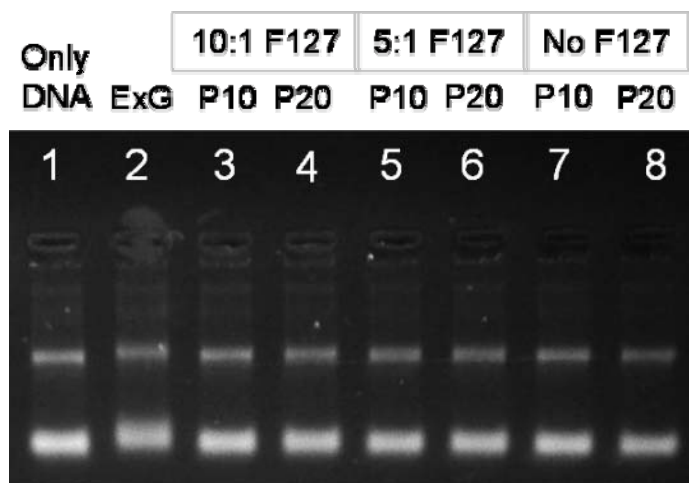
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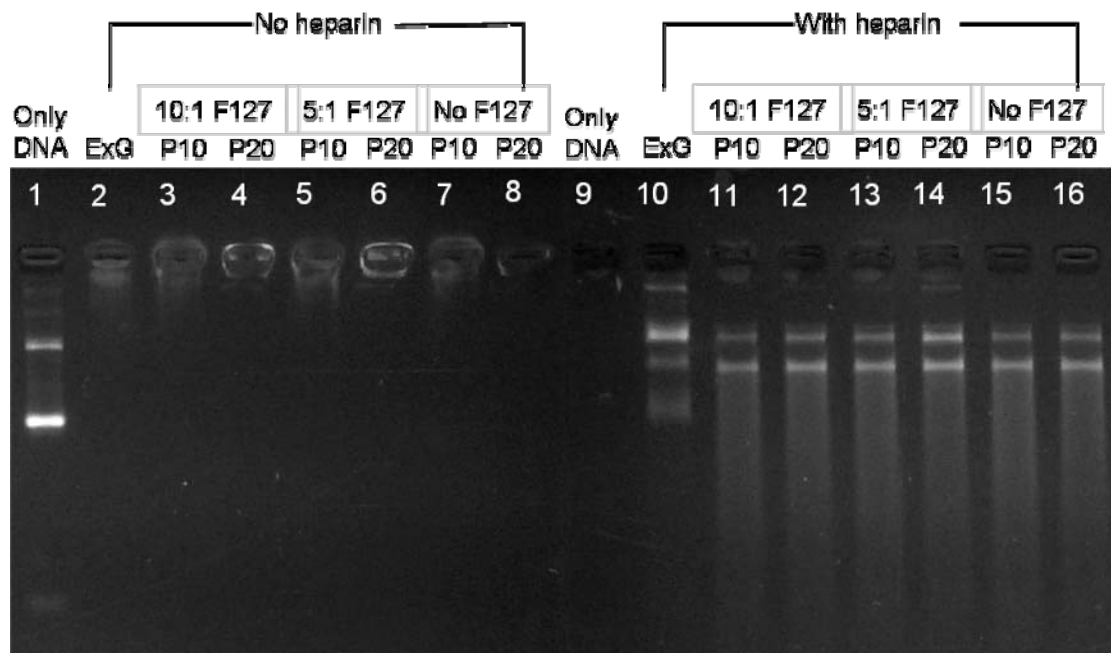
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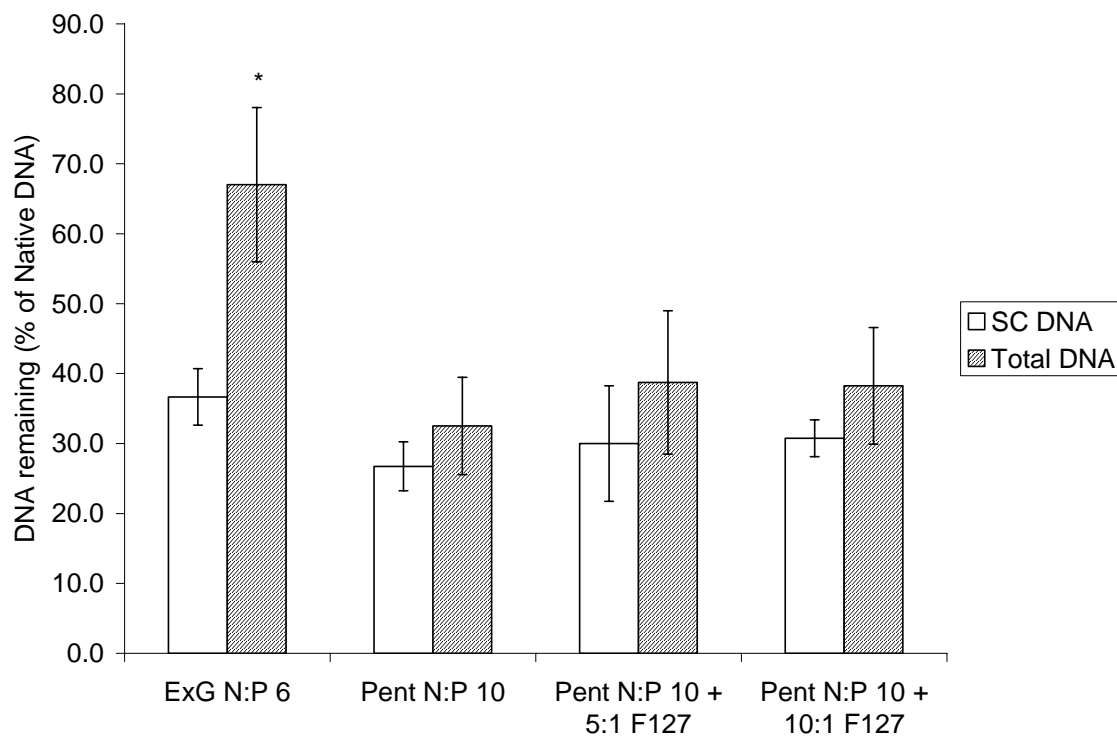
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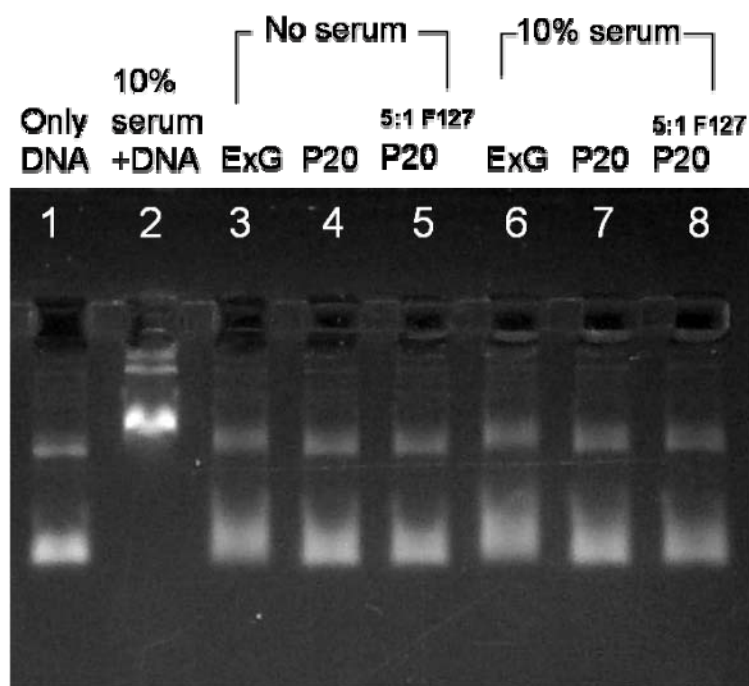
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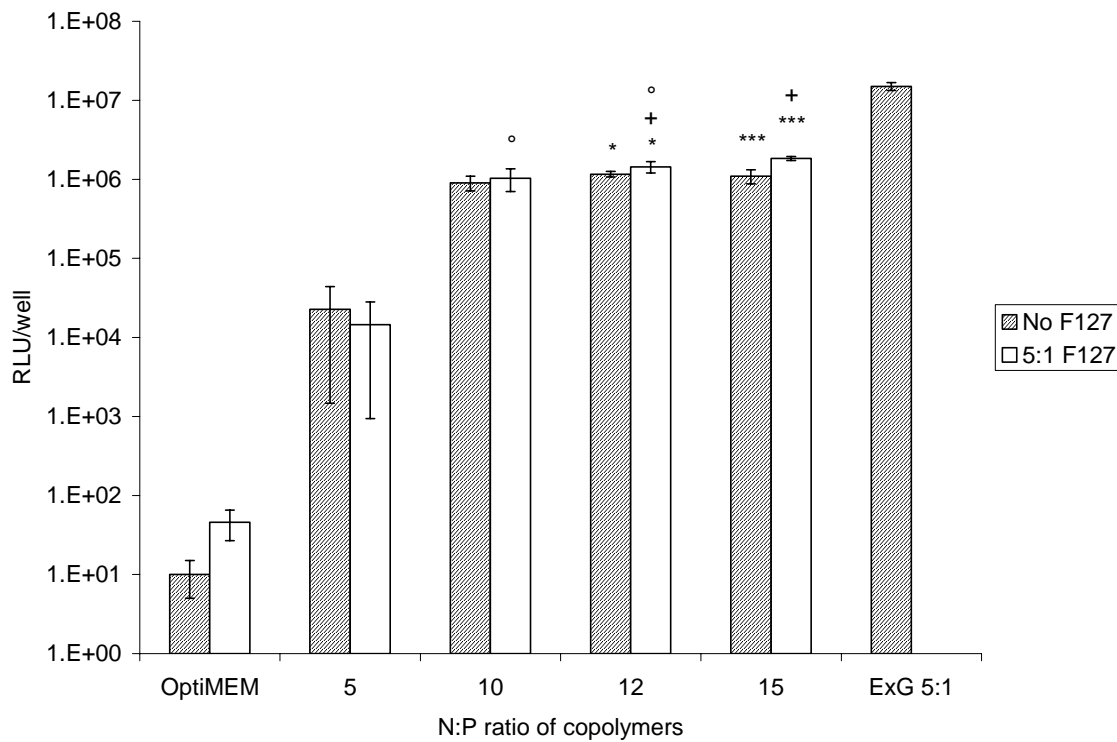
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Fig. 4(b): Effect of free Pluronic F127 on cytotoxicity of pentablock copolymers' polyplexes at different N:P ratios in OptiMEM I® media. Polyplexes, containing 0.6ug pRL, were incubated with SKOV3 cells for 3hrs in Opti-MEM I®, and cell viability was measured after another 48hrs of incubation in complete growth media (n=4 ± SD).

Fig. 4(c): Luciferase expression in SKOV3 cells transfected with pgWiz-luc using pentablock copolymers in OptiMEM I® media. Effect of adding free Pluronic to the formulations is shown. Total luciferase expressed in the cells is normalized by the cell viability of the cell population (n=4 ± SD).

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Fig. 5(a): Effect of free Pluronic F127 on transfection efficiency of pentablock copolymers in SKOV3 cells incubated in complete growth media. + p<0.05, * p<0.1, ** p<0.05 (n=4 ± SD).
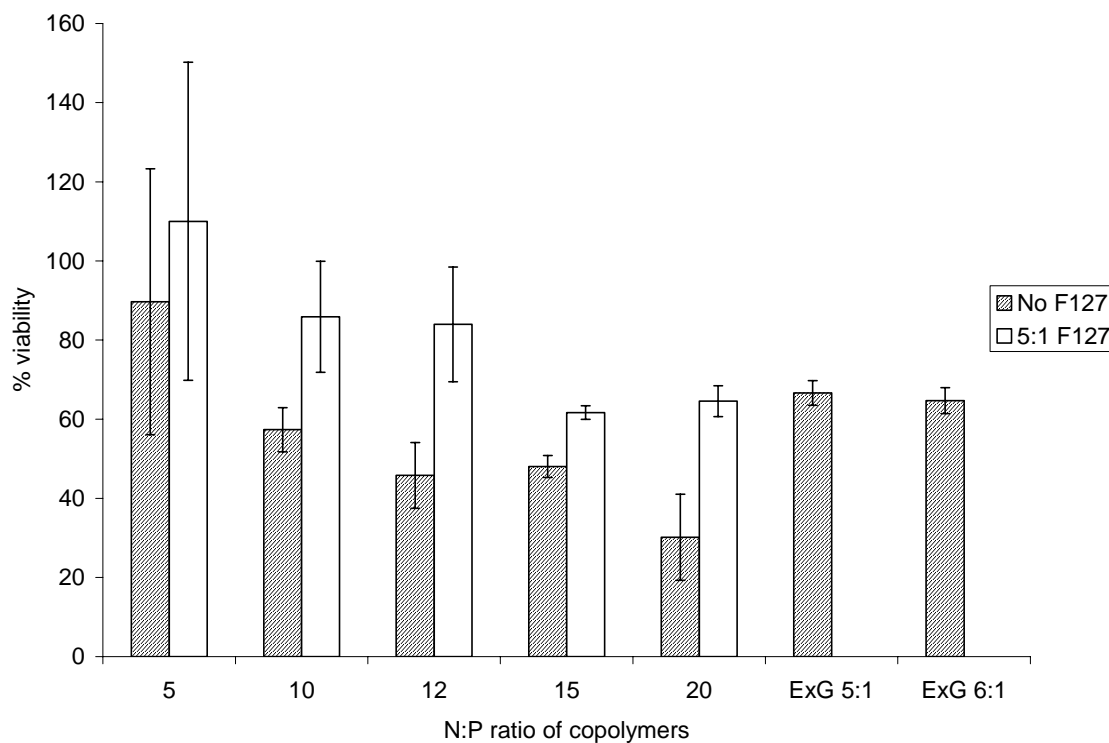
Fig. 5(b): Effect of free Pluronic F127 on cytotoxicity of pentablock copolymers' polyplexes at different N:P ratios in complete growth media. Polyplexes, containing 0.6ug pRL, were incubated with SKOV3 cells for 3hrs in complete growth media, and then replaced with fresh media. Cell viability was measured after another 48hrs (n=4 ± SD).
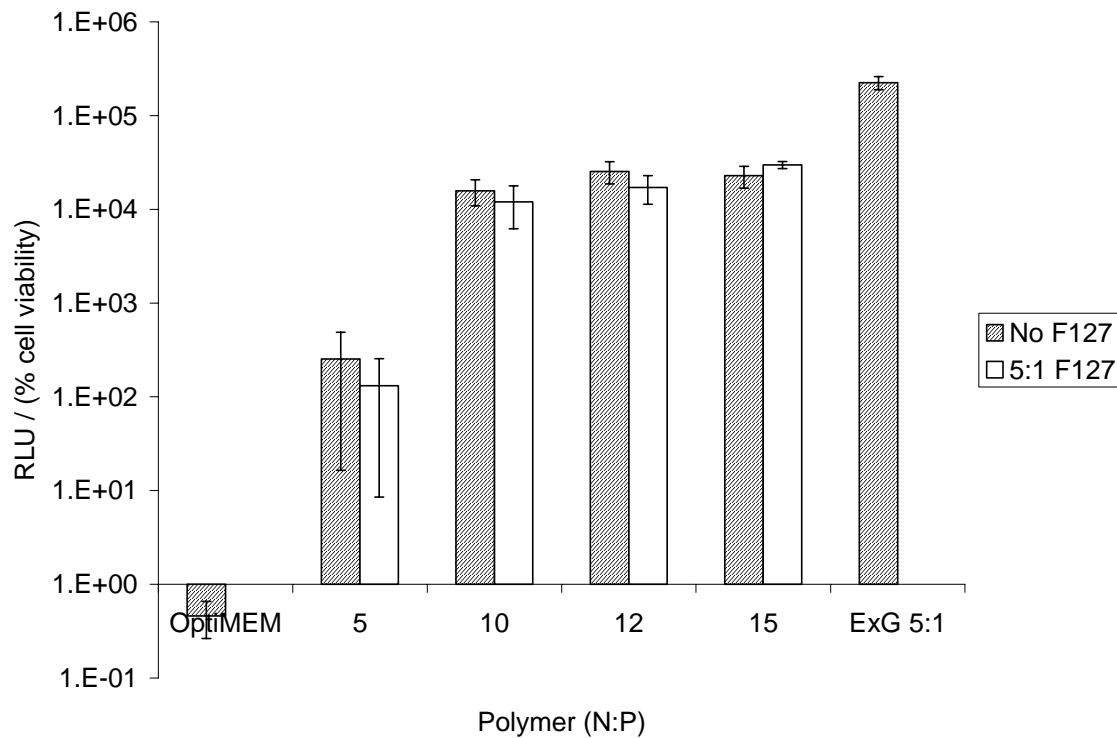
Fig. 5(c): Luciferase expression in SKOV3 cells transfected with pgWiz-luc using pentablock copolymers in complete growth media. Effect of adding free Pluronic to the formulations is shown. Total luciferase expressed in the cells is normalized by the cell viability of the cell population (n=4 ± SD).
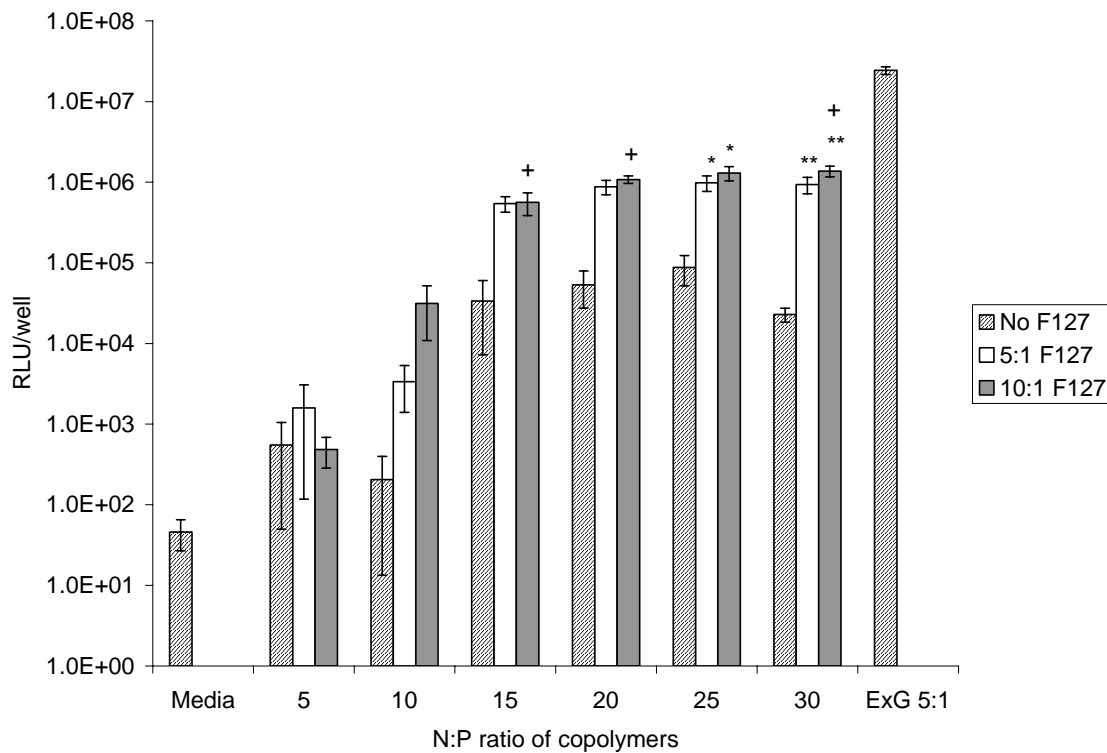
Fig. 6: Percentage of cells expressing green fluorescent protein after transfection with pEGFP using pentablock copolymer/ DNA complexes at different N:P ratios, stabilized with free Pluronic (5:1 wt ratio), in the reduced serum growth media OptiMEM I® or complete growth media supplemented with 10% FBS.

Fig. 7: Schematic showing how adding free Pluronic to a solution of pentablock copolymer/DNA complexes would sterically shield the charged PDEAEM groups (labeled green) on their surface. PEO chains are indicated in red, and PPO chains in blue. Purple core consist of electrostatically neutralized polymer/DNA condensates.

CHAPTER 7

INJECTABLE SELF-ASSEMBLED HYDROGELS OF NOVEL COPOLYMER/DNA COMPLEXES FOR SUSTAINED GENE DELIVERY

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Abstract

Controlled release systems can enhance gene delivery and increase the duration of transgene expression relative to bolus drug delivery in excess buffers. Thermo-reversible hydrogels of novel amphiphilic block copolymers of poly(diethylamino ethyl methacrylate) and Pluronic® have been investigated in this report as sustained gene delivery systems. Aqueous solutions of these thermo-sensitive copolymers self-asse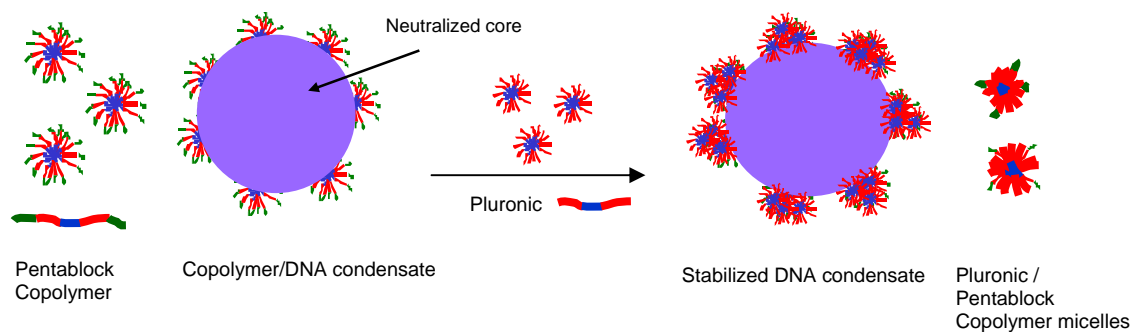mble at physiological temperatures above critical concentrations to form elastic hydrogels. The plasmid DNA can be condensed by cationic copolymers in solution, which when injected into the body at a localized location, would form a hydrogel in situ. The gels can then act as a depot of genetic material, providing a sustained release of DNA protected inside the polymeric nanoparticles. The release of DNA electrostatically bound to copolymers is controlled only by the dissolution profile of the hydrogels, since it cannot freely diffuse out of the polymeric network, preventing initial burst observed with other such controlled release gels/ matrices. While the hydrogels protect the DNA in the extra-cellular matrix, the released DNA is also protected inside the nanoplexes, which further aid in intracellular trafficking and transfection

of the cells. These self-assembled injectable hydrogels have clinical advantages over other chemically cross-linked hydrogels that involve harsh environment, or scaffolds that need to be surgically implanted. The 150 μL gels of copolymers at 15 wt% concentration released condensed DNA up to 7 days *in vitro*, compared to complete release of entrapped naked DNA within few hours by parent Pluronic® gels. The dissolution profile of these hydrogels could be easily modulated by adjusting the concentration of component polymers; by changing the plasmid DNA loading; or by tailoring the PDEAEM content in the pentablock copolymers. These gels had greater mechanical rigidity than parent Pluronic® gels. The nanoplexes released from the gels were colloidally stable, in nanometer diameter size range, and provided efficient transfection in SKOV3 cells significantly greater than naked DNA. Such injectable hydrogels of biocompatible cationic self-assembling copolymers display great potential as sustained gene delivery devices, and have advantages over systems that release naked DNA.

Keywords: Controlled gene delivery, block copolymers, self-assembling, hydrogel, nanoparticles

1. Introduction

Great progress has been made in gene therapy in last two decades, with around 1200 approved clinical trials ongoing at present[1]. Though 70%[1] of these clinical trials use engineered viruses to transfer their genes into the somatic mammalian cells, recent set backs involving severe immune response[2], secondary oncogenesis[3], and transfection of untargeted germ cell lines[4], have re-enforced researchers to develop biologically inactive non-viral methods for gene delivery[5]. However, limited success has been achieved with non-viral gene delivery methods. They give low levels of transfection with only transient expression because of the ultimate loss of the un-integrated plasmid DNA from the transfected cell nuclei. Efficient gene delivery systems that produce enough amount of therapeutic protein in the transfected tissue to give an appreciable physiological response are needed. Injecting gene delivery vectors formulated in large buffer volumes gives limited

bioavailability of the bolus dose. Most of the injected plasmid is lost, or is degraded rapidly in the tissue, or is removed from the tissue by lymphatic system. Delivering vectors in a controlled fashion from sustained gene delivery devices can overcome these extra-cellular barriers of transfecting genes into the cells. Such a device would act as a depot of genetic material, protecting it against degradation in extra-cellular matrix (ECM), and providing a continuous supply of vectors to the targeted cells over a period of time, maintaining an elevated DNA concentration in the cellular microenvironment, increasing the transfection probability, and thus generating prolonged gene expression. Besides a localized injection/implantation of these controlled delivery systems in target tissues can avoid escape of delivered vectors to distant sites which could otherwise lead to toxicity to untargeted cells and unwanted immune responses. Such a system also decreases the amount of genetic material needed for therapy by preventing its rapid loss from the tissue, and circumvents repeated administration of the drug.

Hydrogels are very attractive controlled delivery systems for hydrophilic macromolecules such as DNA because they have high loading efficiency, provide a protective environment and allow easy control of encapsulated gene transport by adjusting cross-linking densities to modulate network structure. Hydrogels of natural polymers like gelatin, chitosan, collagen and agarose, have been used as implantable matrices for sustained gene delivery[6]. The entrapped DNA is released as polymers degrade by the cell-secreted enzymes in the tissue. However, this limits control over the release profiles from these hydrogels, and can reduce the ability to obtain sustain release for longer period of times. Synthetic polymers like polyanhydrides[7], functionalized PEG[8, 9], oligo(poly(ethylene glycol) fumarate) (OPF)[10] can be chemically crosslinked to produce mechanically strong hydrogels that can entrap large amounts of DNA. They offer broader control over the release characteristics by manipulating chemical crosslinking. However, involved gelation conditions, chemical environment, organic solvents and harsh physical forces can damage the DNA. In addition, their acidic degradation products can degrade the released DNA. Physically crosslinked hydrogels that can entrap macromolecular DNA molecules under mild conditions by the simple self-assembly of thermo-sensitive polymers have great advantages over other systems[11]. Particularly interesting polymeric systems are those which allow

preparation of formulations in aqueous solutions and demonstrate in situ hydrogel formation after injection into the body by phase transition, without any chemical reaction or external stimulation[12, 13].

Amphiphilic multi-block copolymers, with a hydrophobic and a hydrophilic polymer block in their molecular architecture, can display thermo-reversible gelation and have been well investigated for sustained drug delivery[11, 14]. These copolymers self-assemble in aqueous solutions to form micellar structures with a hydrophobic core and a hydrophilic corona. At higher concentrations and above a critical gelation temperature (CGT), these micellar solutions form a lyotropic liquid crystalline phase that results in a transparent hydrogel. As the water diffuses into the gel matrix, solvating a boundary layer of gel and decreasing the polymer concentration below the critical concentration, the gel boundary dissolves, allowing the entrapped drug molecules to be released along with polymer molecules[11, 15]. Though lot of studies have been reported on using such in situ gelling hydrogels for delivering protein therapeutics[16-18] and hydrophobic drugs[19-21], their use for gene delivery has not been explored much.

Thermo-reversible gels of Pluronic block copolymers (PEG-PPO-PEG) [poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO)] have been used for localization and sustained delivery of plasmid DNA and viral vectors[22, 23]. However, Pluronic hydrogels have low mechanical strength, and a loosely cross-linked network structure, which results in rapid release of entrapped DNA molecules through diffusion. In an *in vitro* study, entrapped plasmid DNA was released out very fast in an early incubation stage with subsequent negligible releases after 3 days[24]. Recently we reported a family of novel amphiphilic pentablock copolymers as non-viral vectors for gene delivery[25]. The copolymers were prepared by adding poly(diethylamino ethyl methacrylate) (PDEAEM) blocks to the sides of Pluronic block copolymers using an Atom Transfer Radical Polymerization (ATRP) reaction scheme[26]. While the copolymers show reversible thermo-reversible gelation properties like Pluronics[27, 28], the cationic PDEAEM groups condense the negatively charged DNA and show pH buffering capacity at low pH[25, 26]. The nanoplexes of the copolymers are biocompatible and give DNA transfection efficiency comparable to that of commercially available linear PEI ExGen 500®[29]. Aqueous solutions of these copolymers at

concentrations above 15wt%, sol at room temperature, form self-assembled thermo-reversible hydrogels at physiological temperatures[28]. The solution to hydrogel transition is driven by an increase in volume fraction of copolymer micelles (as in PEG-PPO-PEG) due to hydrophobic interactions between collapsed hydrophobic blocks, resulting in ordered packing of the micelles into a crystalline lattice[11, 28]. In this report we have investigated these hydrogels for long term gene delivery. The cationic copolymers can condense DNA in aqueous solution at room-temperature into nanoplexes. When injected subcutaneously into a tissue, these solutions of nanoplexes containing 15wt% w/w or more copolymer instantly form elastic hydrogel in situ at the site of injection. Under *in vivo* conditions, the ingress of tissue fluid into the hydrogel would result in dissolution of the hydrogel matrix, giving a sustained release of DNA electrostatically bound to copolymers. Thus, while gels can act as a DNA depot, the released DNA is also protected inside nanoplex nanoparticles in both ECM and inside the cells, with copolymers aiding in intracellular localization by endocytosis and release from endosomes after entrapment. Such in situ gelling self-assembled hydrogels display great potential as injectable sustained gene delivery devices, and have advantages over systems that release naked DNA. This report examines the mechanical properties of these hydrogels, their sustained in vitro DNA release profile, and the stability and activity of released condensed DNA.

2. Materials and Methods

2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), OptiMEM I®, fetal bovine serum (FBS), 0.25% trypsin-EDTA solution, Hank's Buffered Salt Solution (HBSS), Ultra-pure™ Agarose, EDTA, TAE buffer, and ethidium bromide were purchased from Invitrogen (Carlsbad, CA). HEPES salt and Heparin Sodium salt (cat # H-4784) were purchased from Sigma (St Lousi, MO). Luciferase assay system kit was purchased from Promega Corporation (Madison, USA). ExGen 500® (written as ExGen henceforth), and 6x TriTrack™ loading dye solution were purchased from Fermentas Life Sciences (Hanover, MD).

2.2 Polymer synthesis

Pluronic F127 [$(PEO)_{100}$-b-$(PPO)_{65}$-b-$(PEO)_{100}$] micro pastille surfactant was donated by BASF (Florham Park, NJ) and used without further modification. Pentablock copolymers of PDEAEM-PEO-PPO-PEO-PDEAEM were synthesized using an Atom Transfer Radical Polymerization (ATRP) reaction scheme as explained in detail elsewhere[26]. Molecular weight and poly-dispersity of the copolymers were measured using H^1 NMR (in deuterated chloroform) and GPC (THF mobile phase, poly(methylmethacrylate) calibration standards). Copolymers reported in this study had 20wt% of PDEAEM, with architecture- $PDEAEM_8$-PEO_{100}-PPO_{65}-PEO_{100}-$PDEAEM_8$, and $M_n = 18520$ and $M_w/M_n = 1.14$ Copolymers were synthesized with a molecular weight less than 20KDa so that they can be removed from the body via renal clearance system after gene delivery in in vivo applications. It can be calculated that 1μg of this copolymer has 1.03 nM of nitrogen residues. The molecular weight of the DEAEM monomer is 185.

2.3 Plasmid DNA

Plasmid DNA encoding for luciferase gene, gWiz-luc, with 6732bp was obtained from GeneTherapy Systmes Inc, CA and was used as the reporter gene. DH5α *E.coli* cells were transformed with the plasmid DNA and incubated in Kanamycin selective Luria-Bertani (LB) medium. Amplified plasmid DNA was purified using the Maxi-Prep DNA Purification Kit from Qiagen (Valencia, USA). The concentration and purity of the resulting DNA in TE buffer, pH 7.4 was determined by measuring the absorbance (A) at 260 nm and 280 nm. All DNA used had a A_{260}/A_{280} ratio of at least 1.80.

2.4 Nanoplex formulation

Copolymer to DNA ratios are expressed as molar ratios of nitrogens (N) in the pentablock copolymer to phosphates (P) in DNA, and written as N:P. The average molecular weight of a nucleotide is approximately 308. Using the fact that 1μg of DNA contains 3nmol of phosphates, the amount of polymer required for corresponding N:P ratios was calculated. Nanoplexes were formed by following the precise order of mixing DNA, pentablock

copolymers and Pluronic F127. Pentablock copolymers were first dissolved in 0.5x HBS (Hepes buffer saline- 20mM of HEPES with 145mM NaCl), pH 7.0 at 4°C. For luciferase transfection in 96-well plates, nanoplexes were prepared with 2.4µg pRL-CMV in a final volume of 800ul and were then divided into four equal parts of 200ul for four wells in the plate such that each well got 0.6µg of plasmid. First, an aliquot of DNA (1 µg/µL) in TE buffer pH 7.4 was taken into a 1.5 mL Eppendorf tube and made up to 100 µL using 0.5x HBS buffer pH 7.0. Pentablock copolymer solution (2 mg/ mL) in 0.5x HBS, pH 7.0 was then added to DNA in the required amount to obtain the desired N:P ratio. The tube was vortexed gently, and allowed to incubate for 20 min at room temperature. If required, Pluronic F127 solution (25 mg/mL) in 0.5x HBS, pH 7.0 was then added to the nanoplex solution to obtain desired wt ratio of F127 to pentablock copolymer. The tube was vortexed again gently and incubated for another 10 mins, before making up the final volume to 800 µL using desired growth media, OptiMEM I® or DMEM containing 10% FBS.

2.5 Hydrogel formulation

15% w/w copolymer/DNA gels were prepared as follows. First, an aliquot of desired amount of plasmid DNA (1 µg/µL) was taken in a 1.5 mL Eppendorf tube in the amount of 0.5x HBS, pH 7.0 buffer that would make final volume of formulation 150 µL. Freshly prepared pentablock copolymer solution (100 mg/mL) was added to DNA to give an N:P ratio of 25. The solution was stirred gently and incubated for 20 minutes at room temperature to allow DNA condensation by the cationic copolymer. Next, an aliquot of free Pluronic solution (200 mg/mL) was added to the mixture to obtain a wt ratio of Pluronic : pentablock copolymer 5:1 or 10:1. The formulation was stirred again and incubated for 10 minutes to allow copolymers to self-assemble. At this step, colloidally stabilized nanoplexes were formed, which have been shown previously to give transfection comparable to that of ExGen in serum supplemented growth media (unpublished data[30]). Extra pentablock copolymer or free Pluronic was added to this formulation in solution or powder form to obtain final polymeric concentration of 15wt% and the desired final wt ratio of Pluronic to pentablock. The Eppendorf tube was incubated on ice for half an hour to let copolymers

dissolve, mix homogenously and self-assemble, and later transferred into a 37°C incubator to allow the formation of hydrogel for in vitro dissolution.

2.6 Dissolution study

Self-assembled hydrogels of polymer/DNA complexes (total volume ~150 μL) contained in 1.5 mL Eppendorf tubes were dissolved in 500 μL of pre-warmed 0.5x HBS, pH 7.4 buffer. The buffer was added from the side of the tubes to avoid any erosion of gel surface by tangential forces of flow. The tubes were transferred to a portable shaking incubator maintained at 100rpm, and 37°C. As the gel dissolved over time, 400ul aliquot of buffer was collected at different time points and was replaced with same amount of fresh pre-warmed buffer. Collected samples were stored at 4°C until further examined for electrophoretic mobility, size, zeta-potential or DNA content. To measure the DNA concentration in the samples, 100 mg/mL heparin solution, an anionic glycosaminoglycan (GAG), was added to the solutions to the final concentration of 1%w/v and incubated for 30min, ensuring complete dissociation of DNA from the polymers. Negatively charged GAG are long unbranched polysaccharides with repeated sulfated or carboxylic disaccharide units. The polyanionic heparin thus competes with DNA to bind the cationic polymer [31], and have been shown to effectively dissociate plasmid DNA from the polymer/DNA complexes without any DNA degradation, making it available for the fluorescent stains to bind with[32]. The DNA concentration was then measured using fluorescence picogreen assay, which detects only double stranded DNA and is less affected by the presence of impurities than conventional spectrophotometer measurements. A BioTek Synergy HT multi-detection microplate reader (Vermont, USA) was used.

2.7 Dynamic Mechanical Analysis

A Rheometric Scientific's rheometer was used to monitor the mechanical properties of the copolymer hydrogels as a function of time and temperature. Dynamic time sweep test was conducted on the Advanced Rheometric Expansion System (ARES) in a shear strain-controlled mode. The principle of the test is that as a rotary actuator (servomotor) applies shear deformation on the sample in the form of strain, the sample in response generates toque which is measured by the transducer. Strain and torque are used to calculate dynamic

mechanical test data such as modulus and viscosity. A parallel-plates type of geometry (test fixture) was used, with plate diameter 25mm, and gap between the plates maintained around 0.5mm during the test. Polymer solutions at 4°C were transferred to the top surface of rheometer plate maintained at room temperature using a needle and syringe, making sure it is spread evenly on the plate with no bubbles. Soon after, the upper plate (probe) was lowered to contact the sample, maintaining a gap of 0.5mm between the plates. An approximately 300ul of the sample was used. The mechanical test was started instantly, where the temperature of the plates was set to 37°C. It took less than a minute for the plates to reach 37°C from room temperature, and thus gave a chance to identify the sol to gel transition point by monitoring changes in storage and loss modulus with time and temperature. Frequency of the actuator was maintained at 10Hz, and strain was fixed at 10%. Minimum applied dynamic force was 1gmf, with maximum auto-tension displacement 3mm, and maximum auto-tension rate 0.01mm/s. Since the sample chamber could not be humidified during the course of the experiment, the gels started dehydrating on prolonged exposure to high temperatures. To avoid this, the measurements were made up to a maximum of 10 minutes.

2.8 Particle size and zeta potential

Z-Average diameter and polydispersity of the freshly prepared copolymers, polymer/DNA complexes solutions, and those released from nanoplex gels were measured in Malvern disposable polystyrene cuvettes DTS 0012 at 37 °C on a Malvern Zetasizer Nano-ZS90 system, equipped with 4 mW 633nm He-Ne laser (Malvern Instruments, Southborough, MA, USA). Data analysis was done by the Dispersion Techonology Software (DTS) version 4.2. The instrument was calibrated with an aqueous polystyrene dispersion of 100 nm particles, using viscosity and refractive index of pure water at 37 °C. All water and buffers used for sample preparation were double filtered using a 0.2μm polyethersulfone (PES) sterile syringe filters (Fisher Scientific, MO, USA). Polymer solutions and buffers containing 10% serum were also filtered using these syringe filters to remove any dust particles. PES has both, very low protein binding and, very low extractables and, is recommended for filtering cell culture media. The test solutions were vortexed gently and incubated for 30min at 37 °C before measuring the size distribution and surface charge

properties of the nanoplexes. The ζ-potential of the nanoplexes was measured at 37 °C in Malvern zeta potential cuvettes DTS 1060. The instrument was calibrated using a poly(styrene) dispersion with a known ζ-potential.

2.9 Agarose gel electrophoresis

Samples released from the nanoplex gels were run on an agarose gel to check if naked DNA or DNA complexed with cationic copolymer was released, based on the mobility of the DNA during electrophoresis. To assess the integrity of plasmid DNA inside nanoplexes, 100 mg/mL heparin solution, an anionic glycosaminoglycan (GAG), was added to the nanoplex solutions to the final concentration of 1%w/v and incubated for 30min, ensuring complete dissociation of DNA from the polymers. After adding 6x loading buffer, samples were loaded on a 1% agarose gel stained with ethidium bromide (0.25 µg/mL). The gel was run in TAE buffer at 50V for 2.5hrs. Visualization and image capture was accomplished using a UV-transilluminator under a Kodak EDAS 290 digital imaging suite (Fisher Scientific; Pittsburg, USA). A 1kb+ DNA ladder and pgWiz-luc DNA served as controls. All the experiments were repeated at least 4 times to ensure reproducibility and get error bars on band densitometry data.

2.10   Cell line

The SKOV3 human ovarian carcinoma cell line, obtained from ATCC^{TM} (Manassas, VA), was used for all experiments. Cell cultures were maintained in a humidified environment with 5% CO_2 at 37°C and passaged regularly to allow them to remain sub-confluent. Cells were fed with DMEM growth media supplemented with 10% FBS and 1µM L-glutamine, unless otherwise stated. Neither antibiotics nor antimycotics were used to avoid the possibility of artificial membrane permeabilization effects from these agents.

2.11   Transfection efficiency

In order to determine the total protein expressed by a reporter gene in the transfected cells, a luciferase assay was employed, using pgWiz-luc as the reporter gene. Cells were seeded in a 96-well plate up to 70% confluency prior to transfection, and were then

transfected with various freshly prepared nanoplex solutions or nanoplexes released from nanoplex gels in 200 µL media using 0.6µg of DNA per well, unless otherwise stated. After 3hrs incubation, the polymer solutions were aspirated; cells were washed with HBSS buffer and incubated for another 45hrs in complete growth media. For luciferase assay, cells were then lysed by incubating with 20 µL lysis buffer (Passive Lysis Buffer, Promega) for half an hour at room temperature, and the luminescence of the expressed reporter protein was measured on an automated Veritas™ Microplate Luminometer using the Promega Luciferase Assay System. Cells incubated with naked DNA (without polymer) were used as controls.

2.12   Statistics

Where appropriate, the data is presented as mean and standard deviation (SD). Four samples were used for each case in all the experiments and, mean and SD were calculated over them. Significant differences between two groups were evaluated by Students' t-test and between more than two groups by one-way ANOVA analysis of variance, followed by Tukey's test. The level of significance was set at $p < 0.05$, unless otherwise stated.

3. Results and Discussion

3.1 Dissolution profile

Self-assembled hydrogels containing DNA condensed by pentablock copolymers were prepared using various formulations to control the DNA release profiles and mechanical strength of the gels. These gels have advantage of allowing pharmaceutical formulation preparation in aqueous solution. The future objective is to use these DNA loaded gels for long-term gene delivery in localized muscle or tumor tissues to generate a localized or systemic sustained protein expression, where a maximum volume of 150 µL solution can be injected at a time in mice. Therefore, for in vitro studies, hydrogels with volumes no greater than 150 µL were prepared and investigated. Previous cytotoxicity studies have indicated using an in vitro model that pentablock copolymer gels of 25% w/w concentration were non-toxic to SKOV3 cells[29]. Since copolymer/DNA solutions form strong elastic hydrogels at as low as 15wt% polymer concentration, the dissolution profile of only these low polymer

concentration gels were further evaluated, decreasing the chances of any toxicity or inflammation in an implanted tissue in vivo. Furthermore, it was observed in previous studies (unpublished data[30]) that nanoplexes of pentablock copolymers had cationic surface charge, and adding free Pluronic to them in a wt ratio of 5:1 to 10:1 (Pluronic/pentablock copolymer) shielded their surface charge, and successfully prevented their aggregation with serum particles. This was proved by showing efficient cell transfection and transgene expression in SKOV3 cells in serum supplemented complete growth media. Therefore, to ensure colloidal stability of the nanoplexes released from the hydrogels, free Pluronic was also added to the formulations in wt ratio of 5:1 to 10:1, while still maintaining total polymeric concentration (pentablock copolymer plus Pluronic) in solution as 15 wt%. This further decreased the amount of pentablock in the hydrogels, making them more biocompatible.

Nanoplex solutions (150 μL) containing 20μg of plasmid DNA and 15 wt% of copolymers were prepared using different amounts of free Pluronic to investigate their effect on the dissolution profiles of their gels. A 150 μL Pluronic gel (15 wt%) dissolved completely within 6 hr[17] releasing all entrapped naked DNA (data not shown). As shown in Fig. 1, nanoplex gels made with pentablock copolymer and Pluronic dissolved up to 5 days, providing a first order sustained release profile of compacted DNA. Release rate of nanoplexes from the hydrogels made with 10:1 Pluronic was similar to those made with 18:1 Pluronic. Since DNA is electrostatically bound to the pentablock copolymers in the hydrogels, its release rate is governed only by the dissolution profile of the hydrogels, and not by diffusion. This also prevents initial burst release of DNA from the hydrogels, as observed in controlled drug delivery devices of inert polymers. Hydrogels made with 25 wt% total polymeric concentrations using excess Pluronic containing nanoplexes made at N:P 25 dissolved for longer times. However, the rate of nanoplex release was similar to other hydrogels. The release rate decreased at the end of dissolution. Hydrogels loaded with higher amount of plasmid DNA provided a more sustained release profile (data not shown). This was in agreement with other investigators who have shown that adding macromolecules like proteins[18], polysaccharides[19], or electrolytes[33] to the Pluronic hydrogels made them more rigid by increasing the self-assembly of polymeric network, and thus decreased their

dissolution rate. Similar decrease in dissolution rate of these pentablock copolymer gels loaded with lysozyme protein was observed in a recently reported study by our group[17].

3.2 Mechanical strength

DMA studies gave the storage modulus of the hydrogels, and also provided the time it took for the sol formulation to form a gel when it was transferred from 4°C to 37°C. As shown in Fig. 2, hydrogels formed with only pentablock copolymers were mechanically stronger, with their curing time much shorter than all other gels. Pluronic gels had the least storage modulus, and they took a very long time to form stiff gels. In fact, with 15wt% Pluronic solutions, the storage modulus, G' never crossed over the loss modulus, G", suggesting they formed only strongly viscous solution but never turned into a gel. Hydrogels containing 5:1 and 10:1 Pluronic to pentablock copolymers by wt had intermediate mechanical strength and viscosity, though there was not much difference between their moduli. The mechanical properties of the gels are summarized in Table 1. A high storage modulus is desired in the hydrogels as it assures that the currents in tissue fluid or blood stream will not distort their shape easily, and they can stay as an assembled depot for longer period of times, with less surface area exposed to fluids to diffuse in and dissolve them. Instant increase in the viscosity of the formulations as they are transferred from 4°C to the parallel plates of rheometer at 37°C is shown in Fig. 3. The quick onset of gelation signifies that the easily injectable aqueous formulations will instantly form a localized gel depot in the tissue when injected subcutaneously, giving very less time for the formulation to run off the tissue and get dissolved in tissue fluid or up-taken by the lymphatic system, but still enough time to fit into the injected body cavity or defect.

3.3 Stability of nanoplexes

The nanoplex hydrogels dissolved to release nanoplexes, and not naked DNA. No DNA band was detected moving down the lanes in agarose gel electrophoresis of the samples collected at different days from dissolving hydrogels (Fig. 4). Lanes 2, 3, and 4 in the agarose gel contained respectively samples released at days 1, 3 and 5 from a nanoplex gel (10:1 Pluronic : pentablock). However, when the samples were incubated with heparin

(1wt%) and run again on agarose gel, the DNA recovered from nanoplexes was found to be as intact as control plasmid DNA, with most of it in supercoiled form that many would argue is the fraction retaining greatest integrity[34, 35]. The results ensure the stability of plasmid DNA in the hydrogels, and in the released nanoplexes that were stored for several days at 4°C. These nanoplexes of pentablock copolymers have been shown previously to provide efficient resistance to DNA degradation by nucleases[25].

The colloidal stability of the nanoplexes in released buffer was examined using light scattering to measure their size (hydrodynamic diameter) and zeta-potential. As shown in Table 2, two ranges of particle size were observed in most of the samples, one between 150 to 300 nm, and other between 400 to 600 nm. We have shown previously[25, 30] that the diameter of a pentablock copolymer/DNA complex stabilized with free Pluronic is between 150 to 300nm. The first size distribution denotes individual nanoplexes in the buffer, and dominates the population in number. Data presented shows the sensitive intensity distribution scattered by particles in solution and, can be converted to volume or number distribution using Mie theory (for $D_H>100nm$, Mass=$f(D_H^6$, RI), where RI is refractive index of the particles). Particles larger in diameter produce much higher scattering, even though they might be very few in number. For example, the total number particles of diameter 562 nm in the day 1 sample in Table 1(a), calculated using DTS software (Malvern Instruments, MA), was <1%. The second size range denotes small aggregates of nanoplexes. Since the nanoplexes released from the hydrogels were not removed instantly during in vitro dissolution in a shaking incubator, they tend to settle down by gravity on the surface of the dissolving hydrogel and aggregate with the freshly released nanoplexes. This is not expected to happen in an in vivo situation where the released nanoplexes will either be instantly up-taken by the cells in the surrounding tissue, or will be cleared by lymphatic system. Besides, since the size of these aggregates is still on nanometer scale, they can be easily up-taken by the cells via endocytosis[36] for subsequent cell transfection.

Zeta-potential of nanoplexes released in buffer from Pluronic/pentablock nanoplex hydrogels was found to be close to zero (0.2±0.5 mV with peak width of 10mV), suggesting that their cationic surface charge was effectively shielded by the free Pluronic added to the formulations. However, released samples from hydrogels made with only pentablock

copolymers (containing stabilized nanoplexes) had partial positive charge of +3.0±1 mV. This observed zeta-potential could also be because of excess cationic pentablock copolymer chains that do not bound to nanoplexes.

3.4 Transfection efficiency

Freshly prepared dilute solutions of nanoplexes of pgWiz-luc and pentablock copolymers, sterically stabilized with Pluronic/pentablock w/w 5:1, gave excellent transfection efficiency when incubated with SKOV3 cells in both – reduced serum media, and serum supplemented complete growth media (Fig. 5a). Nanoplexes released from hydrogels (Pluronic/pentablock w/w 10:1) provided transfection comparable to freshly prepared nanoplexes (Fig. 5b). To ensure the transfection ability of nanoplexes is preserved at different steps of hydrogel preparation, freshly prepared formulations with only 2.4 µg DNA in 150 µL buffer; with 20 µg DNA in 150 µL (concentrated); and with 20 µg DNA in 150 µL solution containing 15wt% copolymer, were tested. All these controls provided efficient transfection with no significant difference. No transfection was observed with naked DNA alone incubated with cells. Though samples collected at later time points (day 5 and later) gave reduced luciferase expression, it was still significantly greater than that obtained with naked plasmid. The reduced transfection could be due to the aggregation of nanoplexes during in vitro dissolution of the gel. The released samples collected at later days had greater time to interact with the hydrogels and other nanoplexes in the released buffer. During in vitro hydrogel dissolution in a shaking incubator, the released nanoplexes actually settle down on the surface of hydrogel by gravity, if not removed instantly. Thus, the freshly released nanoplexes from the hydrogel have less chance to mix into the buffer, and end up aggregating with nanoplexes settled on their surface. Since these large aggregates are still on nanometer scale, they can get enter the cells via endocytosis but might not be able to release the entire DNA condensed inside them, giving a low transgene expression. However, this is not expected to happen in an in vivo situation, where the released nanoplexes will either be instantly uptaken by the cells in the surrounding tissue, or will be cleared by lymphatic system, giving no chance for freshly released nanoplexes to aggregate with the accumulating nanoplexes.

4 General discussion

In this study we have demonstrated a novel approach of sustained gene delivery that involves condensing negatively charged plasmid DNA with cationic block copolymers in aqueous solution and in situ gelation of the nanoplex solution by the self-assembling of the amphiphilic polymeric micelles. This system thus combines advantages of efficient transfection obtained with DNA compacted by polycations, and those of long term gene delivery. Though other systems like scaffolds of non-ionic polymers embedded with DNA compacted with other polycations have been explored, they have problems of DNA loading efficiency, amount of DNA compaction, stability of released DNA in polymer degradation products, aggregation of released factors, and involve chemical environment that can damage the loaded DNA[37-39]. Besides, the scaffolds need to be surgically implanted into the body. The non-invasively injectable hydrogel presented in this study perform address all these issues in a single system, and thus present a more versatile approach for sustained gene delivery.

The pentablock copolymers form strong hydrogels, with storage and loss moduli significantly greater than parent Pluronic hydrogels. This is of significance from several perspectives. First, the gels dissolve to provide a more sustained release profile, up to 7 days, compared to few hours given by Pluronic gels[24]. The strong and tighter polymeric network makes the hydrogel more rigid, providing more resistance to the diffusion of water into the gel, and thereby reducing its dissolution rate. Second, high storage modulus helps in reduced deformation of the gel depot by tissue fluid or current of blood in the tissue. Thus reduced surface area is presented to the ingressing fluids, extending their dissolution time. Furthermore, greater mechanical stability can be instrumental in a tissue engineering application where level of gene transfer and expression in surrounding cells is also influenced by the mechanical stimulation of the hydrogel matrix[40], and the matrix has to provide mechanical support to the cells. The dissolution profile of the gels can be controlled by manipulating the content of free Pluronic added to the formulation, or by tuning the PDEAEM content in the pentablock copolymers. It was observed that pentablock copolymers with greater wt% of cationic PDEAEM group in their architecture formed stronger hydrogels and had longer dissolution time period[17].

It was confirmed that these hydrogels release DNA electrostatically bound to pentablock copolymers. This ensures that DNA is not just entrapped inside the hydrogels, but is also condensed by the copolymers. Therefore, there cannot be escape of DNA just by diffusion from the hydrogels. In non-ionic Pluronic gels, or in scaffolds of non-ionic polymers loaded with cationic polymer/DNA complexes, there is an early loss of entrapped DNA by fast diffusion through the loosely bound polymeric network. However, with pentablock hydrogels, DNA is released only along with the dissolution of polymer hydrogel. As the water diffuses into the gel matrix, solvating a boundary layer of gel and decreasing the polymer concentration below the critical concentration, the gel boundary dissolves, releasing DNA bound to the cationic copolymer. This provides a more sustained release profile, and prevents initial burst observed with other controlled gene delivery systems.

The integrity of DNA, with most of it in supercoiled topology, was maintained inside the released nanoplexes. The nanoplexes released were colloidally stable, with a large fraction existing as nanoparticles of diameter 100 to 250nm. Small aggregates of size up to 700nm were also formed. We suggest that these aggregates were formed in *in-vitro* study because of the deposition of released nanoplexes on the surface of dissolving hydrogel. In an *in vivo* situation, where released nanoplexes will be quickly removed from the hydrogel surface, such aggregates are less likely to occur. However, the aggregates are still in nanometer scale, and should be easily uptaken by cells via endocytosis[36]. Hydrogels containing Pluronic in 5:1 or 10:1 wt ratio were shown to provide similar sustained release profiles, much better than only Pluronic gels. The nanoplexes released from these gels were sterically stabilized by free Pluronic, which shield their surface charge, and thus prevent aggregation with serum proteins. It has been reported previously by our group that these sterically stabilized nanoplexes are colloidally stable in serum supplemented buffers, and provided efficient resistance against DNA degradation by nucleases (unpublished data[30]).

Nanoplexes released from hydrogels formed using Pluronic/pentablock copolymers in wt ratio 10:1 were shown to provide transfection as good as freshly prepared nanoplexes. Though samples collected at later time points (day 5 and later) provided reduced reporter transgene expression, it was still significantly greater than that obtained with naked DNA. Besides, this should not deter the efficiency of this gene delivery system. In the treatment of

a lot of localized disease conditions, such as angiogenesis, bone regeneration, restenosis (a vasculo-proliferative condition), and inducing neovasculature in cardiac and limb ischemia, sustained and regulated gene expression is more effective than administrations of doses with high transient expression[41-44].

These controlled release gels provide some fundamental advantages for a clinically feasible sustained gene delivery system. The formulations are easily injectable, and form self-assembled gels within injected tissue soon after administration, preventing any run-off of the dose. The gels are tissue-comfortable, improving the patient's compliance, and can be adherent to a variety of tissues like skin, tumors, muscles, eye, mucosa. The system releases DNA condensed with the polymers, instead of naked DNA as released by other investigated systems. Thus, besides ensuring protection of DNA from nucleases in extra-cellular matrix, the released polymer/DNA complex facilitates easy access across the cell membrane via endocytotosis, protect DNA from nucleases inside the cell, and helps in escaping the low-pH hostile environment of endosomes. The copolymers have a molecular weight of less than 20Kda, and can thus be removed from the body through renal clearance after bioabsorption. These self-assembling thermo-reversible gels have potential advantages over other chemically cross-linked gels. Further, the non-invasive injection is better than surgical implantation and removal of synthetic matrices or devices for controlled gene delivery. From a product design perspective, the formulations are cost-effective and amenable to scale-up. The manufacturing process should be relatively simple, and the final formulation can be lyophilized in a single vial. Another advantage of these hydrogels is the possible co-delivery of bioactive molecules with the genes of interest. For example, additional nucleic acid, proteins, peptides, or small molecule drugs could be potentially delivered slowly over time within same formulation to enhance or modulate protein expression or activity. The micelles of the pentablock copolymers facilitate the encapsulation of less hydrophilic proteins/ drugs in their hydrophobic cores, increasing their bioavailability, and reducing systemic toxicity[17].

5 Conclusions

In summary, we have designed and developed a novel polymeric in situ gelling injectable system for long-term DNA delivery to the localized tissues. Such hydrogels protect the DNA in the matrix and increase its retention time of plasmid DNA in the tissue. The gels have higher mechanical strength than parent Pluronic hydrogels, and release condensed DNA that provides transfection at much higher levels than naked DNA, unlike other controlled gene delivery systems where encapsulation material is inert and do not aid in cell transfection. The release of condensed DNA is controlled only by the dissolution of the hydrogel, and not by the diffusion of DNA through polymeric network. The dissolution profile of hydrogels can be easily modulated by changing the formulation or architecture of the pentablock copolymers. Finally, the hydrogels facilitate simple aqueous pharmaceutical preparation that can be easily injected subcutaneously into the body.

References

1. M. Edeistein, *Vectors used in gene therapy clinical trials http://www.wiley.co.uk/genmed/clinical/*. 2006, John Wiley and Sons Ltd.
2. I.M. Verma, A Tumultuous Year for Gene Therapy, Molecular Therapy 2 (5) (2000) 415-416.
3. E. Check, Gene therapy: A tragic setback, Nature 420 (6912) (2002) 116-118.
4. N. Boyce, Trial halted after gene shows up in semen, Nature 414 (6865) (2001) 677.
5. G.D. Schmidt-Wolf, I.G.H. Schmidt-Wolf, Non-viral and hybrid vectors in human gene therapy: an update, Trends in Molecular Medicine 9 (2) (2003) 67-72.
6. J.M. Dang, K.W. Leong, Natural polymers for gene delivery and tissue engineering, Advanced Drug Delivery Reviews 58 (4) (2006) 487-499.
7. D.J. Quick, K.K. Macdonald, K.S. Anseth, Delivering DNA from photocrosslinked, surface eroding polyanhydrides, Journal of Controlled Release 97 (2) (2004) 333-343.

8. D.J. Quick, K.S. Anseth, DNA delivery from photocrosslinked PEG hydrogels: encapsulation efficiency, release profiles, and DNA quality, Journal of Controlled Release 96 (2) (2004) 341-351.

9. K. Roy, D. Wang, M.L. Hedley, S.P. Barman, Gene delivery with in-situ crosslinking polymer networks generates long-term systemic protein expression, Molecular Therapy 7 (3) (2003) 401-408.

10. F.K. Kasper, S.K. Seidlits, A. Tang, R.S. Crowther, D.H. Carney, M.A. Barry, A.G. Mikos, In vitro release of plasmid DNA from oligo(poly(ethylene glycol) fumarate) hydrogels, Journal of Controlled Release 104 (3) (2005) 521-539.

11. B. Jeong, S.W. Kim, Y.H. Bae, Thermosensitive sol-gel reversible hydrogels, Advanced Drug Delivery Reviews 54 (1) (2002) 37-51.

12. C. Matschke, U. Isele, P. van Hoogevest, A. Fahr, Sustained-release injectables formed in situ and their potential use for veterinary products, Journal of Controlled Release 85 (1-3) (2002) 1-15.

13. E. Ruel-Gariepy, J.-C. Leroux, In situ-forming hydrogels--review of temperature-sensitive systems, European Journal of Pharmaceutics and Biopharmaceutics 58 (2) (2004) 409-426.

14. T. Kissel, Y. Li, F. Unger, ABA-triblock copolymers from biodegradable polyester A-blocks and hydrophilic poly(ethylene oxide) B-blocks as a candidate for in situ forming hydrogel delivery systems for proteins, Advanced Drug Delivery Reviews 54 (1) (2002) 99-134.

15. B.C. Anderson, N.K. Pandit, S.K. Mallapragada, Understanding drug release from poly(ethylene oxide)-b-poly(propylene oxide)-b-poly(ethylene oxide) gels, J Control Release 70 (1-2) (2001) 157-67.

16. J.-Y. Seong, Y.J. Jun, B.M. Kim, Y.M. Park, Y.S. Sohn, Synthesis and characterization of biocompatible poly(organophosphazenes) aiming for local delivery of protein drugs, International Journal of Pharmaceutics 314 (1) (2006) 90-96.

17. M.D. Determan, J.P. Cox, S.K. Mallapragada, Drug release from pH-responsive thermogelling pentablock copolymers, Journal of Biomedical Materials Research Part A 9999 (9999) (2006) NA.

18. A.D. Lewis P. Stratton, Mark C. Manning, John F. Carpenter,, Drug delivery matrix containing native protein precipitates suspended in a poloxamer gel, Journal of Pharmaceutical Sciences 86 (9) (1997) 1006-1010.

19. H.-R. Lin, K.C. Sung, W.-J. Vong, In Situ Gelling of Alginate/Pluronic Solutions for Ophthalmic Delivery of Pilocarpine, Biomacromolecules 5 (6) (2004) 2358-2365.

20. G.D. Kang, S.H. Cheon, S.-C. Song, Controlled release of doxorubicin from thermosensitive poly(organophosphazene) hydrogels, International Journal of Pharmaceutics 319 (1-2) (2006) 29-36.

21. B. Jeong, Y.H. Bae, S.W. Kim, Drug release from biodegradable injectable thermosensitive hydrogel of PEG-PLGA-PEG triblock copolymers, Journal of Controlled Release 63 (1-2) (2000) 155-163.

22. Y.-K.O. Jeong-Sook Park, Ho Yoon, Jung Mogg Kim, Chong-Kook Kim,, In situ gelling and mucoadhesive polymer vehicles for controlled intranasal delivery of plasmid DNA, Journal of Biomedical Materials Research 59 (1) (2002) 144-151.

23. P.M. Strappe, D.W. Hampton, B. Cachon-Gonzalez, J.W. Fawcett, A. Lever, Delivery of a lentiviral vector in a Pluronic F127 gel to cells of the central nervous system, European Journal of Pharmaceutics and Biopharmaceutics 61 (3) (2005) 126-133.

24. K.W. Chun, J.B. Lee, S.H. Kim, T.G. Park, Controlled release of plasmid DNA from photo-cross-linked pluronic hydrogels, Biomaterials 26 (16) (2005) 3319-3326.

25. A. Agarwal, R. Unfer, S.K. Mallapragada, Novel cationic pentablock copolymers as non-viral vectors for gene therapy, Journal of Controlled Release 103 (1) (2005) 245-258.

26. M.D. Determan, J.P. Cox, S. Seifert, P. Thiyagarajan, S.K. Mallapragada, Synthesis and characterization of temperature and pH responsive pentablock copolymers, Polymer 46 (18) (2005) 6933-6946.

27. M.D. Determan, L. Chieh-Tsung, P. Thiyagarajan, S.K. Mallapragada, Self-assembly of Temperature and pH-Responsive Pentablock Copolymers, Polymeric Materials:Science and Engineering 23 (2005) 808.

28. M.D. Determan, L. Guo, P. Thiyagarajan, S.K. Mallapragada, Supramolecular Self-Assembly of Multiblock Copolymers in Aqueous Solution, Langmuir 22 (4) (2006) 1469-1473.

29. A. Agarwal, R. Unfer, S.K. Mallapragada, Investigation of in vitro biocompatibility of novel pentablock copolymers for gene delivery, Journal of Biomedical Materials Research Part A 9999 (9999) (2006) NA.

30. A. Agarwal, R. Vilensky, R.C. Unfer, Y. Talmon, S.K. Mallapragada, Colloidal stability and transfection efficiency of self-assembling copolymeric gene delivery vectors in the presence of serum, Biomaterials In Review (2007).

31. M. Ruponen, S. Yla-Herttuala, A. Urtti, Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies, Biochimica et Biophysica Acta (BBA) - Biomembranes 1415 (2) (1999) 331-341.

32. I. Moret, J. Esteban Peris, V.M. Guillem, M. Benet, F. Revert, F. Dasi, A. Crespo, S.F. Alino, Stability of PEI-DNA and DOTAP-DNA complexes: effect of alkaline pH, heparin and serum, Journal of Controlled Release 76 (1-2) (2001) 169-181.

33. G. Mao, S. Sukumaran, G. Beaucage, M.-L. Saboungi, P. Thiyagarajan, PEO-PPO-PEO Block Copolymer Micelles in Aqueous Electrolyte Solutions: Effect of Carbonate Anions and Temperature on the Micellar Structure and Interaction, Macromolecules 34 (3) (2001) 552-558.

34. D.P. Shuichi Ando, Daniel W. Pack, Robert Langer,, PLGA microspheres containing plasmid DNA: Preservation of supercoiled DNA via cryopreparation and carbohydrate stabilization, Journal of Pharmaceutical Sciences 88 (1) (1999) 126-130.

35. J. Akbuga, C. Aral, S. Ozbas-Turan, L. Kabasakal, M. Keyer-Uysal, Transfection efficiency of chitosan microspheres: Effect of DNA topology, S.T.P. Pharma Sciences 13 (2) (2003) 99-103.

36. R.A. Kristensen, J.P. Clamme, C. Vuilleumier, Y.M. Kurthy, Role of endocytosis in the transfection of L929 fibroblasts by polyethyleneimine/DNA complexes, Biochim, Biophys. Acta 1514 (2001) 21-31.

37. F. Scherer, U. Schillinger, U. Putz, A. Stemberger, C. Plank, Nonviral vector loaded collagen sponges for sustained gene delivery in vitro and in vivo, The Journal Of Gene Medicine 4 (6) (2002) 634-643.

38. G. De Rosa, F. Quaglia, M.I. La Rotonda, M. Appel, H. Alphandary, E. Fattal, Poly(lactide-co-glycolide) microspheres for the controlled release of oligonucleotide/polyethylenimine complexes, Journal Of Pharmaceutical Sciences 91 (3) (2002) 790-799.

39. Y.-C. Huang, M. Connell, Y. Park, D.J. Mooney, K.G. Rice, Fabrication and in vitro testing of polymeric delivery system for condensed DNA, Journal Of Biomedical Materials Research. Part A 67 (4) (2003) 1384-1392.

40. H.J. Kong, J. Liu, K. Riddle, T. Matsumoto, K. Leach, D.J. Mooney, Non-viral gene delivery regulated by stiffness of cell adhesion substrates, Nature Materials 4 (6) (2005) 460-464.

41. T.P. Richardson, M.C. Peters, A.B. Ennett, D.J. Mooney, Polymeric system for dual growth factor delivery, Nature Biotechnology 19 (11) (2001) 1029-1034.

42. J. Bonadio, E. Smiley, P. Patil, S. Goldstein, Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration, Nature Medicine 5 (7) (1999) 753-759.

43. T. Ohno, D. Gordon, H. San, V.J. Pompili, M.J. Imperiale, G.J. Nabel, E.G. Nabel, Gene therapy for vascular smooth muscle cell proliferation after arterial injury, Science 265 (5173) (1994) 781-784.

44. W.S. Lee, M.K. Jain, B.M. Arkonac, D. Zhang, S.Y. Shaw, S. Kashiki, K. Maemura, S.L. Lee, N.K. Hollenberg, a. Lee et, Thy-1, a novel marker for angiogenesis upregulated by inflammatory cytokines, Circulation Research 82 (8) (1998) 845-851.

Table 1: Mechanical properties of the self-assembled hydrogels (15 wt%) made with pentablock copolymers and free Pluronic.

15wt% hydrogel	Time(s) to form gel (G'/G'' crossover)	Modulus (Pa) at gelation point	Max G' (Pa)	Max G'' (Pa)	Max Viscosity, η (Pa-s)
Pentablock	98.4	93.04	5553	966	897
Pluronic/Pent 5:1	125.6	135.6	3362	2273	635
Pluronic/Pent 10:1	157.7	202.2	3564	2420	672
Pluronic	-	-	1749	2117	436

Table 2: Hydrodynamic diameter of released polyplexes in samples collected at different days from the dissolving 15wt% hydrogels containing 20μg plasmid DNA, made with different ratios of free Pluronic and pentablock copolymer (n≥3, SD- Standard Deviation).

(a) Hydrogel with Pluronic/Pentablock copolymer in wt ratio 10:1.

Day	Size (nm)	SD	% Intensity
1	123	58	14.4
	592	167	67.4
3	151	56	41.6
	674	187	30.7
5	185	81	55
	537	149	37

(b) Hydrogel with Pluronic : Pentablock copolymer in wt ratio 5:1.

Day	Size (nm)	SD	% Intensity
1	155	54	23.5
	562	232	66.8
3	165	19	85.7
5	198	74	51.4
	613	212	44

(c) Hydrogel made with only pentablock copolymer.

Day	Size (nm)	SD	% Intensity
1	164	84	16.2
	686	149	71.9
3	231	40	39.4
	421	187	18
5	172	114	82.4

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Fig. 4: Mobility of polymer/DNA complexes released from hydrogels at different time points. Lane 2, 3, 4 contain polyplexes released on day 1, 3, 5 respectively, and lanes 5,6,7 contain DNA recovered from those polyplexes after incubating them with heparin. Lane 1 contains naked pgWiz-luc plasmid.

Fig. 5: Total luciferase expression in SKOV3 cells transfected with (A) nanoplexes prepared at different N:P ratios with 2.4 μg DNA in 800μL buffer, displaying stability in serum supplemented growth media; (B) nanoplexes at N:P 25 prepared with either 2.4 μg DNA in 150μL buffer (fresh), or with 20 μg DNA in 150 uL (concentrated), or with 20 μg DNA in 150 μL solution containing 15wt% copolymer, or those released from 15wt% copolymer hydrogels at different time points.
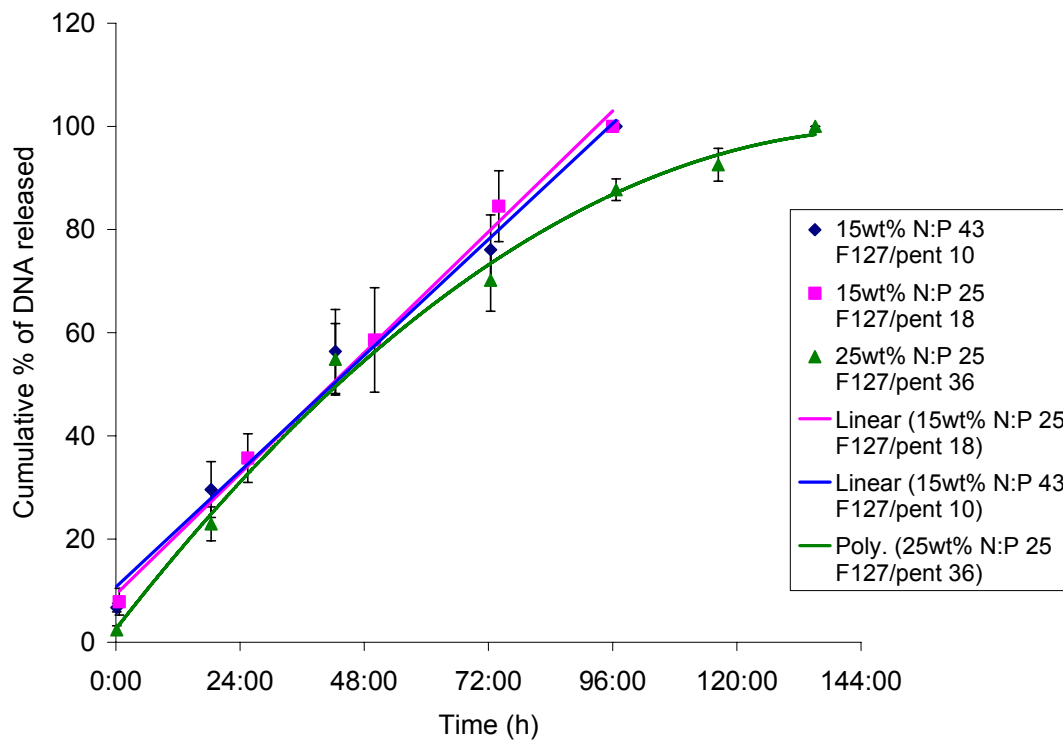
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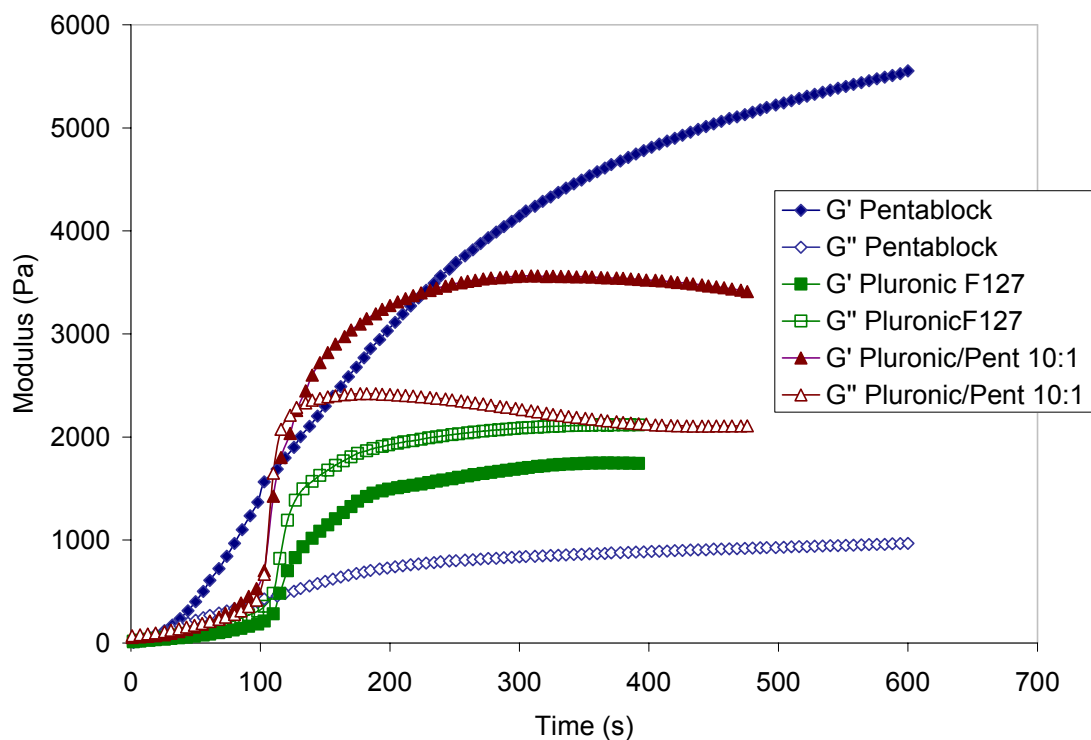
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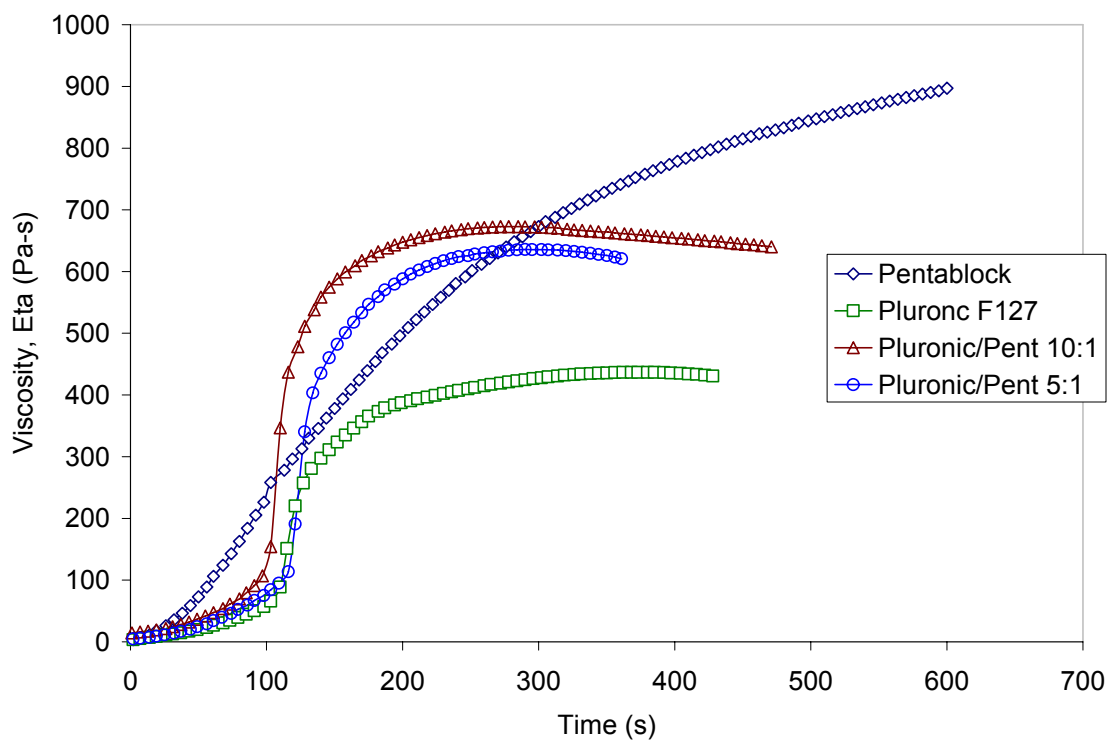
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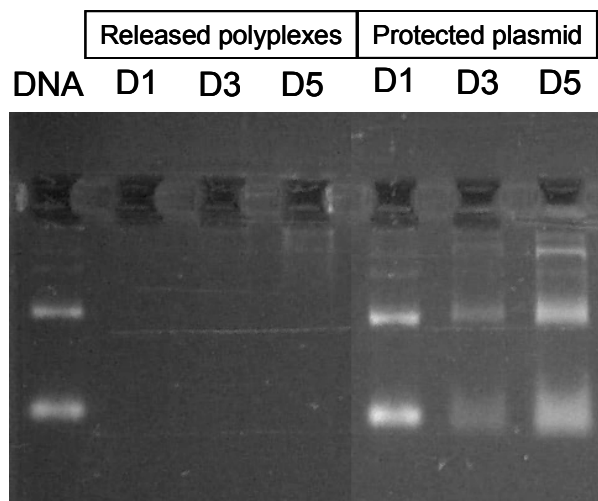
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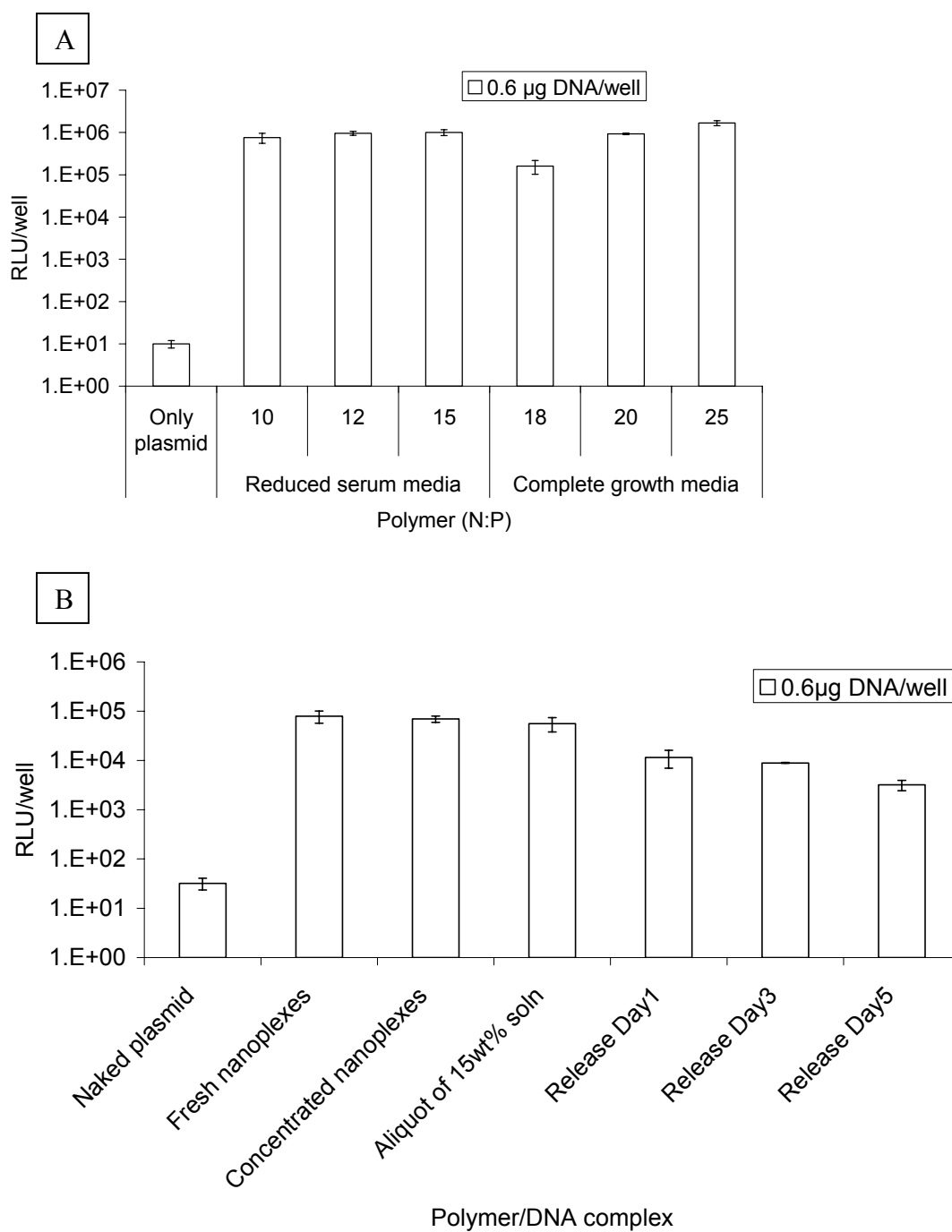
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CHAPTER 8
CONCLUSIONS

The overall objective of the presented research work was to develop and characterize novel polymeric gene delivery vectors based on copolymers of Pluronic and poly(diethylamino ethyl methacrylate) (PDEAEM); and develop their injectable *in situ* forming self-assembled hydrogels for long-term sustained gene delivery. In order to achieve this objective, the research was divided into three specific goals and the main findings are summarized below.

The specific goals of the project were:

SG1

To characterize the physiochemical properties of pentablock copolymers pertaining to plasmid DNA compaction and protection against nucleases; and, hydrodynamic size, surface charge, and morphology of polymer/DNA complexes in aqueous solutions.

SG2

To tailor the copolymer design and, improve the colloidal stability of their DNA complexes with optimized formulations for maximum gene transfection in cells with minimum cytotoxicity, and investigate their intracellular trafficking pathway to identify steps that limit their transfection efficiency.

SG3

To develop injectable self-assembled in situ forming hydrogels of pentablock copolymer/ DNA complexes for long-term sustained gene delivery- modulate their *in vitro* dissolution profile, and improve the formulations for maximum gene stability and transfection efficiency of released polymer/DNA complexes.

Physiochemical properties of the pentablock copolymers and their complexes (polyplexes) with plasmid DNA molecules were characterized using Multi-Angle Laser Light Scattering (MALLS), Dynamic Light Scattering (DLS), and Zeta-sizer. Copolymer itself was found to exist as micelles above critical concentrations in aqueous solutions with hydrodynamic diameter of ~25nm and each micelle containing approximately 132 polymer chains. Cationic copolymers efficiently condensed negatively charged DNA into nanoparticles of 100-150nm in diameter at appropriate N/P ratio (molar ratio of nitrogen in polymer to the phosphates in plasmid DNA), and provided efficient protection to the condensed plasmid against degradation by nuclease enzymes (DNase I). The polyplexes had positive surface charge, which depended on the amount of copolymer (N/P ratio) used to condense the DNA. The results suggest that copolymer/DNA complexes are small enough to be up taken by cells via endocytosis, while the positive surface can help these polyplexes to fuse with negatively charged plasma membranes.

Real-space imaging of nanostructures in their native state in aqueous conditions by cryo-TEM revealed that copolymer exist as spherical micelles, and condense plasmid DNA in extended linear thread like structures that tend to bend into rings. DNA condensates were found to be more compact at higher N/P ratios, as also suggested by DLS. Similar compact condensates were obtained at lower N/P ratios in low pH buffers, indicating copolymers have higher cationic surface due to increased protonation at low pH. This pH sensitive quality of copolymers to absorb protons at low pH is instrumental in the escape of polyplexes from acidic endosomal vesicles after cellular uptake.

Intra-cellular trafficking studies of the copolymer/DNA complexes using fluorescent labeling and confocal microscopy confirmed that cationic polyplexes do fuse with the cell membrane all along its perimeter, and are uptaken predominantly by endocytosis. The labeled plasmid was found to be entrapped into the endosomes, and later in matured lysosomes, up to 7 hrs post-transfection, and was finally localized in the nuclei of transfected cells after 10 hrs post-transfection. The study confirmed that cationic pentablock copolymers do deliver the condensed DNA into the cell nuclei, and the trafficking involves entrapping

into the endosomes, and final disruption of vesicles arguably with the aid of pH buffering capacity of copolymers.

A detailed biocompatibility study of pentablock copolymers and their polyplexes using several different cell based assays revealed that copolymers enter the cells with very little damage/ leakage to the cell membrane, apposed to other cationic polymers like ExGen 500® (linear PEI) where cell death was found to be accompanied with an early loss of cell membrane integrity. The coopolymers were found to be significantly less toxic than ExGen, both in terms of cell membrane leakage, and in terms of metabolic activity retained by incubated cells. It was confirmed that pentablock copolymers did not either induce apoptosis into the cells. Polyplexes of copolymers were less toxic than copolymers alone, suggesting shielding the positive charge of copolymers decreases their cytotoxicity. It was illustrated that the cytotoxicity of the copolymers can be tuned by tailoring their molecular weight or cationic content. Optimized formulations with ideal cell incubation periods to provide maximum transgene expression of a reporter gene, with minimum cytotoxicity, were shown to be obtained by varying the DNA dose, polymer concentration and N/P ratio.

The transfection of efficiency of the pentablock copolymers, the ultimate test to their gene delivery efficiency, was conducted on several cancer cell lines all along the research work using two different reporter genes- one encoding for GFP to account for the percentage of cells expressing the transfected reporter genes, and the another encoding for luciferase protein to provide the total amount of transgene expression in a population of the transfected cells. Commercially available polymeric transfection reagent, ExGen 500® was used as positive control. Pentablock copolymers were found to transfect as many cells as ExGen, confirming their efficient capability to deliver genes to competitive number of cell nuclei. The total transgene expression in cells was also comparable, but one order less than that obtained with ExGen. Detailed nuclease resistance studies revealed that the total amount of DNA retained by polyplexes of ExGen after nuclease digestion was more than that retained by pentablock copolymers, accounting for the greater transgene expression of ExGen. However, the amount of plasmid retained in supercoiled form by both the systems was not

significantly different, which many would argue is the fraction with greatest integrity. Besides, the observation that pentablock copolymers have significantly higher biocompatibility than ExGen makes them a more favorable gene delivery vector.

The colloidal stability of pentablock copolymer polyplexes in serum supplemented buffers was drastically improved using a novel strategy of adding non-ionic copolymer Pluronic F127 to the formulation. Since pentablock copolymers are derived form Pluronic F127, they have same hydrophobic core. It was hypothesized that while the hydrophobic cores of two copolymers would tend self-assemble together, the long PEO chains of F127 would sterically shield the cationic PDEAEM groups of the pentablock micelles. It was confirmed using DLS and cryo-TEM that such stabilized copolymer/DNA complexes did not aggregate with globular serum proteins; had a neutralized zeta-potential, and formed nanostructures of hydrodynamic diameter 150-200nm in serum supplemented buffers. The formulations provided increase uptake of plasmid DNA and expression in the cells in complete growth media, and displayed improved biocompatibility due to masking of cationic surface charge on polyplexes. The results warrant good performance of this multi-component gene delivery system in systemic applications *in vivo*.

Finally, sustained release of polymer/DNA complexes was investigated from their thermo-reversible hydrogels. At higher concentrations and above a critical gelation temperature (CGT), micellar solutions of pentablock copolymers with hydrophobic cores and hydrophilic coronas form a lyotropic liquid crystalline phase that results in a transparent hydrogel. Similar hydrogels were formed by polymer/DNA complexes at higher copolymer concentrations and physiological temperatures that provided sustained release of these complexes when dissolved in excess buffers. Hydrogels formed with 150 μl formulations, maximum injectable volume in mice muscles, gave sustained release of DNA up to 7 days. The free flowing formulations at low temperatures containing 15wt% copolymers instantly formed elastic hydrogels when transferred from 4°C to 37°C rheometer plates using 1ml syringes equipped with 27G needles (ones used for normal insulin injections in body). Different formulations were investigated to obtain tailored release profiles, and improve gene stability. The hydrogels had greater storage mechanical strength than parent Pluronic

copolymers, with a faster curing time to gel. The release of DNA electrostatically bound to copolymers is controlled only by the dissolution profile of the hydrogels, since it cannot freely diffuse out of the polymeric network, preventing initial burst observed with other such controlled release gels/ matrices. Polyplexes released from these hydrogels were colloidally stable, in nanometer size range, and provided transfection efficiency comparable to the freshly prepared polyplexes.

In summary, the dissertation provides the development and complete *in vitro* evaluation of a novel polymeric gene delivery vector. The biocompatible copolymers provide efficient gene transfection in cells with little or no cytotoxicity *in vitro* compared to other commercially available transfection reagents, and warrant good performance in systemic applications *in vivo*. At higher concentrations, the copolymer/ DNA complexes form non-invasively injectable *in situ* forming self-assembled hydrogels that provide long-term sustained DNA release, and have distinct clinical advantages over other chemically cross-linked hydrogels that involve harsh environment, or matrices that need to be surgically implanted. The presented work lays a firm foundation for a multitude of gene therapy studies that can be performed with these systems.

CHAPTER 9
FUTURE DIRECTIONS

A comprehensive systematic evaluation of the novel polymers introduced in this thesis was provided based on their physiochemical properties, biocompatibility, colloidal stability and transfection efficiency *in vitro*. One of the immediate extensions of this work is to use this analysis, and optimized formulations of copolymers, to deliver reporter genes in localized tissues in a mice model after direct subcutaneous injections. Formulations with lower polymer concentrations should be first evaluated for transfection efficiency of the copolymers and any inflammation caused by them. Later, the formulations with higher polymer concentrations that would form hydrogels in situ after injection should be tested for long-term sustained systemic or localized reporter protein expression. Preliminary studies were performed to deliver luciferase reporter genes to the localized tumors of human prostrate cancer cell (DU 145) grown on the flanks of nude mice using sol formulations (that do not form gel *in situ*). The study was used to optimize the *in vivo* formulations and their preparation for improved gene delivery, but was too small to deliver statistically significant results. However it did confirm that polymers were able to deliver the genes to the tumor cells, as evident by transgene expression shown in Fig. 1, and that the copolymers caused mild to moderate inflammation (Fig. 2), as evident by little infiltration of leucocytes in the tumor tissue. Mild inflammation was found along the needle path even in tumors injected with only plain buffers, and in tumors with no injections because of the invasive growth of tumors. Since the growth of tumors varied in mice, it accounted for another variable for observed differences in evaluating gene delivery efficiency of copolymers. To overcome this, we have a new protocol for *in vivo* mice studies approved by animal care committee at Iowa State University where polymer/DNA complexes will be injected in the femoral muscles of mice, and reporter gene expressing an extra-cellullar protein will be employed. Thus, level of systemically expressed protein can be measured at different time points by simply taking a blood sample from the same mice each time, a great facility for long-term gene delivery study using copolymer gels as it would limit the number of test mice needed.
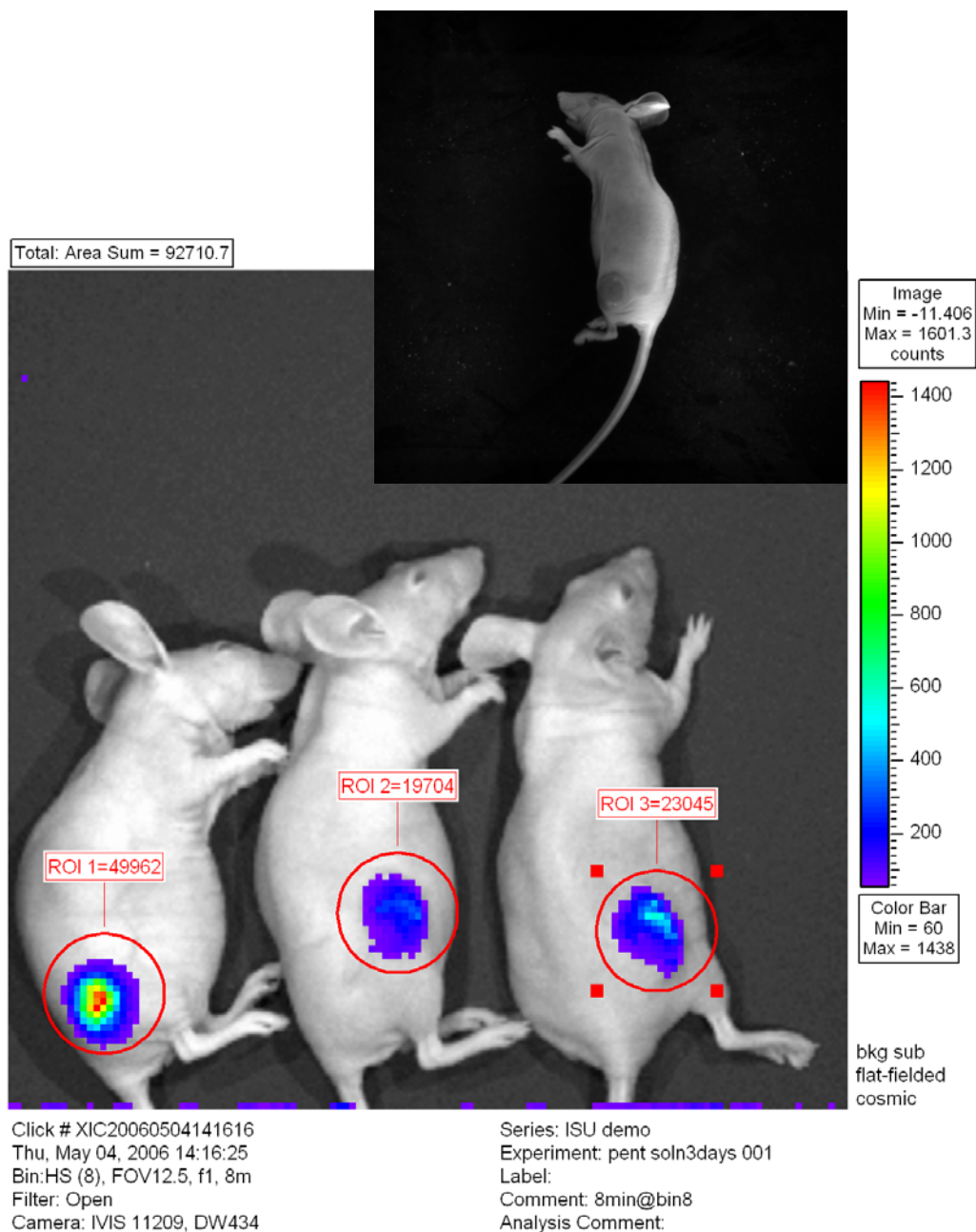
Fig. 1: Emission of photons from the tumors growing on the flanks of mice during the reaction of luciferin substrate, injected intra-peritoneally, with the luciferase enzyme expressed in the tumor cells after their successful transfection with a plasmid encoding it using pentablock copolymers. The image gives both, the total amount of gene expressed, and its 2-D distribution, inside the tumor. The image was obtained using Xenogen's (MA, USA) *in vivo* bio-photonic imaging station. Inset shows a mouse with a tumor grown on its flank.
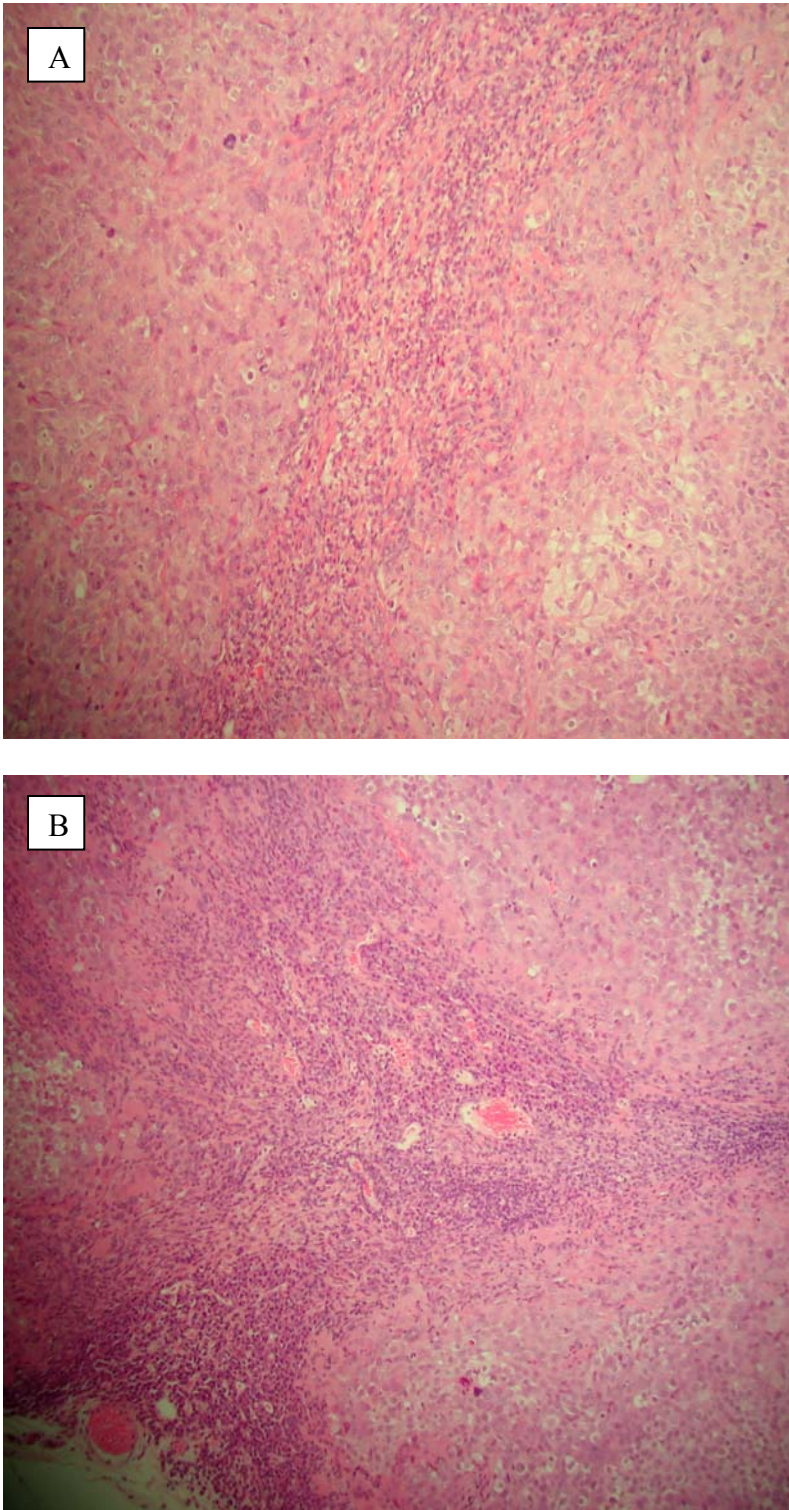
Fig. 2: H&E stained slices (5-10µm) of paraffin embedded tumors, showing infiltration of leukocytes in the tumor tissue two days after injection with (a) pentablock copolymer/ DNA complexes, and (b) ExGen/DNA complexes.

Though the morphology of polymer/DNA complexes has been well studied using cryo-TEM in this thesis, a detailed study to investigate the interactions of polymer molecules with plasmid DNA is need. Molecular architecture and macroscopic self-assembly of polymer micelles and their DNA condensates can be deciphered accurately using Small Angle Neutron Scattering (SANS). Detailed structure studies can be done with SANS using contrast variation technique where desired molecules can be made opaque to incident neutron beam by making the scattering length density of solvent equal to that of the molecule. This tool can be used to preferentially visualize the DNA or polymer molecules in the polyplex solution, by making one of them opaque to the neutrons. This feature is really interesting as it can provide the exact location of polymer and DNA in a polymer/DNA complex, and can thus help identify how to improve the protection of DNA condensed inside polyplexes against degradation by nucleases.

Finally, the pentablock copolymers have reactive ends in their architecture that facilitate covalent attachment of cell-specific ligands for target recognition, or nuclear localization signals (NLS) for improving nuclei translocation, or fluorophores for intra-cellular trafficking of polymers using fluorescence confocal microscopy. In particular, attaching cell binding ligands such as Epidermal Growth Factor (EGF) to the polymers will preferentially increase their uptake in the cells over expressing these receptors (like most of the cancer cell lines, in particular A431 human epidermoid carcinoma) by receptor mediated endocytosis, and reduce their uptake by non-targeted cells. These ligands can also aid in shielding the surface charge of the polymer/DNA complexes, preventing their aggregation with serum proteins, and reducing their opsonisation by the immune system.

Once a safe and efficient gene delivery system using these pentablock copolymers is developed, it can be used to ferry suicide genes like Herpes simplex type 1 thymidine kinase gene (HSV1-TK) or *Escherichia coli* Cytosine Deaminase (CD) gene to selectively kill the targeted cancer cells *in vivo*.

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   A Agarwal, R C Unfer, S K Mallapragada, Novel self-assembling copolymers for sustained gene delivery in the presence of serum, Polym Mater Sci Eng Proceed, 94, 88-89, 2006.
   A Agarwal, R C Unfer, S K Mallapragada, Novel cationic pentablock copolymers as non-viral vectors for gene therapy, J of Controlled Release, 103 (1), 245-258, 2005.
   A Agarwal, R C Unfer, S K Mallapragada, Colloidal stability and transfection efficiency of novel self-assembling polymeric gene delivery vectors in serum supplemented media, J of Controlled Release: Special issue: Nanomedicine Symp, In Review, 2007.

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   Novel amphiphilic block copolymers and their self-assembled injectable hydrogels for sustained gene delivery, Polymers (West) Gordon Research Symp, Ventura, CA, 2007.