Poly(ε-caprolactone)-*graft*-poly(2-(*N*, *N*-dimethylamino) ethyl methacrylate) nanoparticles: pH dependent thermo-sensitive multifunctional carriers for gene and drug delivery†

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pH-dependent temperature- sensitive poly(ε-caprolactone)-*graft*-poly(2-(dimethylamino) ethyl methacrylate) (PCL-*g*-PDMAEMA), a kind of degradable, amphiphilic, cationic copolymer, was synthesized. PCL-*g*-PDMAEMA was self-assembled into core-shell nanoparticles with an ultralow critical association concentration at about 8.1×10^{-4} g L^{-1}. It was found that PCL-*g*-PDMAEMA nanoparticles were able to simultaneously entrap hydrophobic paclitaxel and load DNA. Hydrophobic drug paclitaxel, loaded by PCL-*g*-PDMAEMA NPs, could be released faster in an acidic environment than in a neutral environment, and PCL-*g*-PDMAEMA NPs showed a comparable *in vitro* gene transfection efficiency to Lipofectamine 2000. In addition, the gene transfection efficiency was enhanced by the addition of 5% serum. Besides, confocal microscopic measurements indicated that PCL-*g*-PDMAEMA nanoparticles/DNA polyplexes could escape from the endosome and release the payloads effectively in cytoplasm. These results suggest PCL-*g*-PDMAEMA has great potential for achieving the synergistic effect of drug and gene therapies *in vivo*.

Introduction

Multifunctional biomaterials for drug delivery are being developed to fulfill the complex requirements of various disease therapies, administrations, and different kinds of drugs delivery, such as hydrophobic drugs, gene, protein, *etc*.[1,2] As promising platform carriers, nanoparticles (NPs) with a hydrophobic core and a hydrophilic shell have attracted much attention in developing efficient drug delivery systems. As one of the most important carriers for this application, NPs formed from amphiphilic copolymers have been widely investigated in terms of their combined functions of hydrophobic drug loading capacity, stability, targeted distribution and stealth capability, *in vivo*.[3–5]

Numerous amphiphilic copolymers were synthesized with not only a variety of hydrophilic and hydrophobic blocks but also multiple functions, such as active targeted and stimuli-responsive abilities for anticancer drug delivery.[3] In terms of the special growth microenvironment of tumor tissue with lower pH (5.7–7.8) and higher temperature than that of normal tissues, several kinds of thermo-sensitive, pH-sensitive and dual-sensitive copolymer NPs have been prepared by incorporating pH and/or temperature-sensitive components.[6–11] Recently, the co-delivery of hydrophobic drugs and genes by employing amphiphilic cationic copolymers has been developed for the purpose of combining chemical compounds and genes in anticancer therapy.[12–15] Amphiphilic cationic copolymers can self-assemble into stable, size-controlled and dispersive NPs with a hydrophobic core and a cationic shell with a high surface charge density. Thus, the NPs can not only condense negatively charged DNA, but also entrap hydrophobic drugs into the core. The significant works that Yang's group recently reported on self-assembled micellar nanoparticles of amphiphilic cationic copolymer revealed the potential synergistic effect in cancer therapy by loading nucleic acid and hydrophobic anticancer drugs, simultaneously.[12–14] Therefore, clever combination of different functions by designing a suitable molecular structure is crucial for the development of multifunctional nano-delivery platforms aiming to improve the efficacy of disease therapies. Recently, several amphiphilic graft copolymers were reported to hold ultralow critical association concentrations compared with block amphiphilic copolymers.[16,17] This property is expected to be very useful for the stabiliy of nanocarriers *in vivo*.

Our research strategy in this paper was based on the requirement of a multifunctional polymer nanomedical platform for hydrophobic drug and gene delivery. We designed and prepared a kind of multifunctional NP formed from graft-degradable amphiphilic cationic copolymer, poly(ε-caprolactone)-*graft*-poly(2-(*N*,*N*-dimethylamino) ethyl methacrylate) (PCL-*g*-PDMAEMA, structure and corresponding ^{1}H NMR spectra are shown in Fig. 1). This kind of amphiphilic graft copolymer is assumed to hold several advantages. Firstly, it can self-assemble into core-shell nanoparticles with ultralow critical association concentrations. Secondly, cationic core-shell nanoparticles can carry two payloads simultaneously, anionic nucleic acid and

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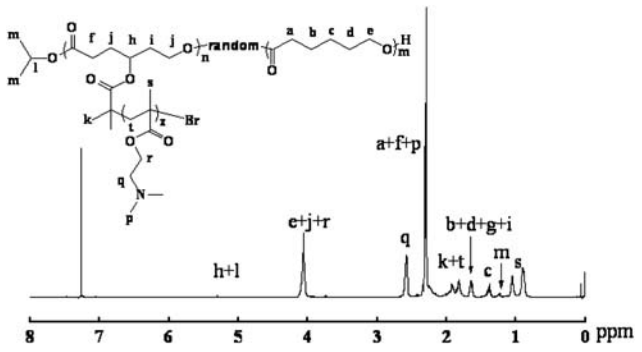


Fig. 1 ^{1}H NMR spectra of PCL-*g*-PDMAEMA.

hydrophobic anticancer drugs. Thirdly, pH-dependent temperature-sensitivity of PCL-*g*-PDMAEMA NPs could release the drug payload faster in an acidic environment than that in a neutral environment, which is very useful to effectively treat tumors with acidic microenvironments and reduce the side effects of the drug on normal tissues *in vivo*.[18,19] Finally, PCL-*g*-PDMAEMA NPs may serve as a good template for layer-by-layer techniques and their applications.

Experimental

Materials

N,*N*-dimethyl-ethylamine methacrylate, caprolactone and aluminium isopropoxide were purchased from Aldrich Chemicals. γ-(2-Bromo-2-methylpropionate)-ε-caprolactone (BMPCL) was prepared, as reported elsewhere.[20,21] Lipofectamine 2000 (Lipo 2000), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin and 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) were purchased from Invitrogen Corp (Rockville, MD, US). Paclitaxel was provided by Hengrui Co. (Shanghai, China). All other reagents were analytical grade and used without further purification.

Synthesis and characterization of PCL-*g*-PDMAEMA

PCL-*g*-PDMAEMA was designed and prepared by ring-opening polymerization (ROP) and atom transfer radical polymerization (ATRP) methods. Firstly, macroinnitiator, poly(caprolactone-*co*-γ-(2-bromo-2-methylpropionate)-ε-caprolactone) (P(CL-*co*-BMPCL)), was synthesized by copolymerization of CL and BMPCL using aluminium isopropoxide as initiator in toluene solution at room temperature. The molecular weight of P(CL-*co*-BMPCL) was 1.11 × 10^4 g mol^{-1} and P(CL-*co*-BMPCL) had 6 BMPCL units. Then, PCL-*g*-PDMAEMA was synthesized by ATRP of DMAEMA using P(CL-*co*-BMPCL) as macro-initiator. The bulk polymerization was carried out at 60 °C for 14 h and then the product was dissolved in THF. The polymer solution was purified by dialysis into double-distilled water for 24 h, and then freeze-dried to obtain the product.

^{1}H NMR characterization

The ^{1}H NMR spectra were recorded on a Varian INOVA 500MHZ nuclear magnetic resonance instrument using $CDCl_3$ as the solvent and tetramethylsilane (TMS) as the internal standard.

GPC measurement

A gel permeation chromatography system (GPC, Agilent 1100) was used to measure the molecular weight and molecular weight distribution. THF was used as the eluting solvent (1 ml min^{-1}) and polystyrene was used as molecular weight standard.

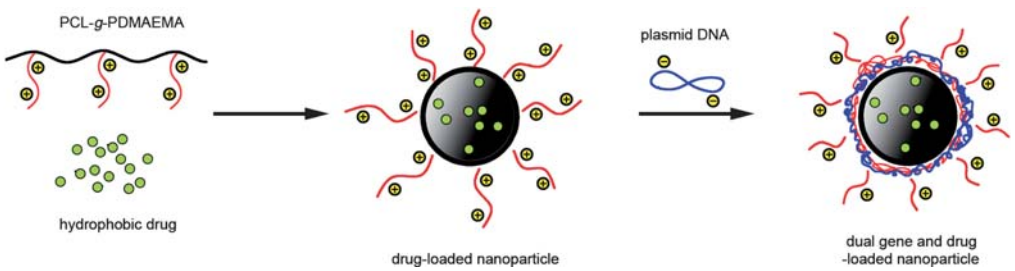
Preparation of NPs and drug-loaded NPs

Polymeric nanoparticles were prepared as follows: firstly, the copolymer solution was prepared by dissolving PCL-*g*-PDMAEMAs (20 mg) in THF (1.5 mL), a good solvent for both PCL and PDMAEMA segments. Then, the polymer solution was added to 10 mL double distilled water drop-wise and under magnetic stirring. The nanoparticles were formed immediately and THF was removed through 4 h evaporation at room temperature. Modification of the pH was carried out by adding a few drops of NaOH (0.1 M) or HCl (0.1 M) solution under the control of a pH meter.

A similar protocol was used to prepare the drug-loaded NPs, as shown in Scheme 1, with addition of paclitaxel. During the self-assembly process of the polymer, the hydrophobic drug, paclitaxel, gathered with hydrophobic PCL segments and was entrapped by hydrophilic PDMAEMA segments. After THF was removed, the aqueous solution was centrifuged (4500 rpm, 15 min) to remove the aggregates.

Preparation of NPs/pDNA polyplexes

2 μg of pDNA and a corresponding quantity of polymer solution based on different N/P ratios (molar ratio of nitrogen atom content in polymer to phosphorous atom content in DNA) were diluted in 50 μL PBS respectively. After 5 min incubation, the DNA and



Scheme 1 The preparation of PCL-*g*-PDMAEMA NPs with payloads of hydrophobic drugs and plasmid DNA.

polymer solutions were mixed and vortexed for 5 s. It is essential to incubate the polyplexes for another 30 min at room temperature to enhance their stability before any further operation.

Agarose gel electrophoresis shift assay

The agarose gel retardation assay was performed as follows: 20 μL well-incubated NPs/pDNA polyplexes was mixed with 4 μL 6 x loading buffer (Takara Biotechnology, Dalian, Liaoning Province, China), and then 20 μL of the mixture was loaded onto 0.8% agarose gel containing 0.5 μg mL^{-1} ethidium bromide. Electrophoresis was carried out at a voltage of 120 V for 40 min in 1 x TAE running buffer. Finally, the results were recorded under UV light (wavelength 254 nm) by a Typhoon Trio (GE Healthcare, Salt Lake City, UT).

TEM observation, size distribution and zeta potential measurements

The transmission electron microscopy (TEM) specimens for polymeric nanoparticles were observed under a JEM-100CX II instrument. The samples were prepared by adding a drop of polymeric nanoparticles solution on the Formvar-coated copper TEM grid, and then dyed by phosphatotungstic acid.

The hydrodynamic diameter of nanoparticles was determined by DLS at 90° using a Spectra-Physics Stabilite 2017 LASER (532 nm, 200 mW, 30 A) and a Brookhaven 9863 collimator. The particle sizes and size distribution were calculated using CONTIN algorithms. For the dynamic light scattering (DLS) analysis, the presented data is intensity-averaged diameter. Each sample was measured for four sub runs with a cumulative time of 2 min. The errors of micellar sizes from the DLS diagrams are within 5% of the mean value.

The zeta potential was determined on a Nano-ZS (Malvern, Worcestershire, UK) zetasizer at a scattering angle of 173° and analyzed using the DTS (Nano) program. Three measurements (each with 20 sub-runs) were performed for each sample.

Fluorescence spectroscopic measurements and determination of critical association concentrations (CAC)

Sample solutions for fluorescence investigation were prepared as follows. Briefly, a known amount of pyrene in THF was added to each of a series of 10 mL vials and the THF was removed by evaporation. The final concentration of pyrene was 6.0×10^{-7} M. A total of 5 mL of various concentrations of aqueous polymer solutions were added to each vial and then heated at 45 °C for 3 h to equilibrate pyrene, and left to cool for 3 h at room temperature.

Fluorescence spectra were recorded with a LS45/55 luminescence spectrometer (Perkin-Elmer). The excitation wavelength was set at 339 nm, and for excitation spectra, the emission wavelength was 390 nm. Excitation and emission bandwidths were 5 nm and 10 nm, respectively. All experiments were carried out at room temperature.

Drug release assays of nanoparticles

The paclitaxel-loaded PCL-*g*-PDMAEMA NPs were prepared by nanoprecipitation techniques, as mentioned above. Paclitaxel-loaded PCL-*g*-PDMAEMA NPs (10 mg) were suspended into phosphate-buffered saline (5 mL) of particular pH values (pH = 5.0, 6.0, 6.8 and 7.4). The solutions were then poured into dialysis bags (MWCO 3500) and immersed into 45 mL of PBS at 37 °C for drug release. Aliquots of 20 mL were periodically withdrawn from the solution to containers. The solution volume was kept constant by adding 20 mL fresh PBS after each sampling. Paclitaxel concentrations in buffer solutions were determined by a high-performance liquid chromatography (HPLC) (Agillent1100, USA) using Krcmafsis (250 × 4 mm, 5 μm) C18 column. A mixture of 65% acetonitrile, 35% water at a flow rate of 1 ml min^{-1} was used as the eluent and UV detection was performed at 254 nm. The accumulated release was calculated as follows:

$$E_r = \frac{V_e \sum_{1}^{n-1} C_i + V_0 C_n}{m_{drug}}$$

where E_r is the accumulated release (%), V_e is the sampling volume (20 mL), V_0 is the initial volume (50 mL), C_i and C_n are the paclitaxel concentrations (μg mL^{-1}), i and n are the sampling times, and m_{drug} is the mass of paclitaxel in PCL-*g*-PDMAEMA NPs (μg).

The drug release measurement was carried out three times and the average value of the three measurements was taken.

Transfection and cytotoxicity assay of nanoparticles

293T cells were seeded in 24-well plates at 2×10^5 cells/well, respectively and cultured with DMEM supplemented with 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin for 18 h. Before adding 100 μL fully-incubated polyplex, containing 2 μg plasmid DNA into each well, the culture medium were substituted by serum-free DMEM or 5% serum-containing DMEM culture. After 4 h incubation, the incubating solution was aspirated and replaced by fresh DMEM containing 2% FBS. After an additional 48 h incubation, the cells were detected by flow cytometry (BD FACSAria, BD Biosciences, San Jose, CA). All cells were cultured at 37 °C in 5% CO_2 humidified atmosphere in the transfection processes.

We determined the cytotoxicity of the polyplexes by MTT assay. The 293T cells were seeded in 96-well plates at 1×10^4 cells/well and cultured for 18 h before transfection. The detailed transfection protocol was similar to the description above, but adding 50 μL polyplex solution containing 0.2 μg DNA into each well. After 48 h incubation, 20 μL MTT solution (5 mg mL^{-1} in PBS) was added to each well and incubated at 37 °C for another 4 h. Then, we aspirated the medium and added 150 μL DMSO to each well. When formazan crystals were fully dissolved, the absorbance of each well at 490 nm was measured by employing 1420 Multilabel Counter (PerkinElmer, Waltham, MA). The results were shown as cell viability percentage relative to untreated cells. The transfection and cytotoxicity assay was carried out three times and the average value of the three measurements was taken.

Measurement of polyplexes escape from endosome

HIV-PSV plasmid was labeled with cy5 by using the Label IT Nucleic Acid Labeling Kit (Mirus, Madison, WI) according to

the manufacturer's protocol. 293T cells were placed in 6-well plates, with a glass cover slip at the bottom, at 2×10^5 cells per well one day before transfection. After the culture medium was changed to fresh DMEM, well-incubated polyplex solution containing 1 μg cy5-labeled plasmid ($N/P = 10$) was added into each plate. After 4 h incubation at 37 °C in 5% CO_2 humidified atmosphere, the transfection solutions were aspirated and substituted by complete culture medium. After additional 2 h (8 h and 20 h) incubation, the cells were washed with PBS three times before acidic late endosome staining with LysoTracker blue (Molecular Probes, Eugene, OR). Then, the intracellular distribution was observed using Zeiss confocal microscopy (LSM700, Zeiss, Germany).

Results and discussion

In this paper, PCL-*g*-PDMAEMA was prepared by a combination of ROP and ATRP methods. The obtained PCL-*g*-PDMAEMA is constructed as a PCL chain (mean polymerization degree = 83), grafted by 6 PDMAEMA side chains (mean polymerization degree = 56, calculated according to the ^{1}H NMR spectrum in Fig. 1. The molecular weight distribution index is 1.66, as determined by gel permeation chromatography (GPC, Fig. S1†). The weight average and number average molecular weight are 6.42×10^4 and 3.87×10^4 g mol^{-1}, respectively (Fig. S1†).

Amphiphilic copolymer PCL-*g*-PDMAEMA was supposed to be assembled into nanoparticles composed of hydrophobic PCL and hydrophilic PDMAEMA in aqueous solution. The structure of PCL-*g*-PDMAEMA NPs dispersed in aqueous solution was characterized by employing dynamic light scattering (DLS), TEM and a zeta potential analyzer (Fig. 2 and Table S1†). The effective average hydrodynamic diameter of NPs in deionized water is 535 nm with a polydispersity index of 0.144 (Fig. 2A). However, TEM images (Fig. 2B) indicate that the diameter (around 70–100 nm) is much lower than the DLS-derived value. Actually, discrepancy in TEM and DLS results is common and has been reported previously and is due to difference between the nature of the two determination methods.[22,23] DLS figures show the hydrodynamic radius of NPs as being wet and extended, but TEM represents the dry and shrunk configuration of the same NPs. Therefore, it is reasonable that the diameter value of DLS is higher than that of TEM for the same PCL-*g*-PDMAEMA NPs. The zeta potential of PCL-*g*-PDMAEMA NPs is 40.9 ± 0.9 mV in distilled water.

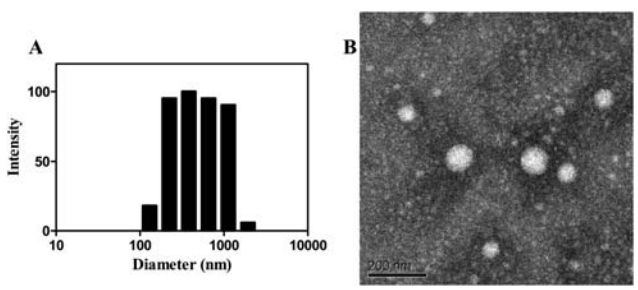


Fig. 2 The characterization of PCL-*g*-PDMAEMA NPs by DLS (A) and TEM (B).

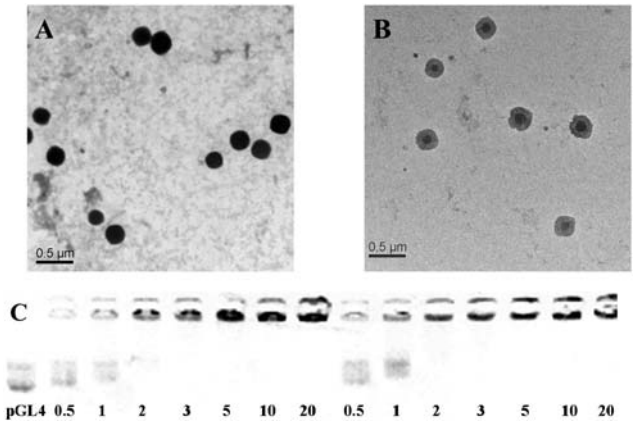


Fig. 3 Characterization of PCL-*g*-PDMAEMA NPs/DNA polyplexes. (A) A TEM image of PCL-*g*-PDMAEMA NPs/DNA polyplexes at a *N*/*P* ratio of 10; (B) paclitaxel-loaded PCL-*g*-PDMAEMA NPs/DNA polyplexes at a *N*/*P* ratio of 10; (C) the electrophoretic mobility shift assay of PCL-*g*-PDMAEMA NPs/DNA (left) and paclitaxel-loaded PCL-*g*-PDMAEMA NPs/DNA polyplexes (right) at various *N*/*P* ratios.

The well-incubated polyplexes formed by plasmid DNA (pGL4.0) and blank or paclitaxel-loaded PCL-*g*-PDMAEMA NPs in PBS (pH = 7.2) were characterized by employing TEM (Fig. 3A and 3B) and agarose gel retardation assays (Fig. 3C). As shown in Fig. 3A and 3B, compact PCL-*g*-PDMAEMA NPs/DNA NPs with sphere morphology were formed. The mobility of the pDNA was decreased when they complexed with blank or paclitaxel-loaded PCL-*g*-PDMAEMA NPs and complete retardations were observed both at a *N*/*P* ratio of 2 (Fig. 3C). These data demonstrate that the PCL-*g*-PDMAEMA NPs have strong DNA-binding capacity, which is not weakened by hydrophobic drug incorporation. In addition, the zeta potential of PCL-*g*-PDMAEMA NPs/DNA ($N/P = 10$) was reduced to 15.6 ± 0.7 mv (Table S1†).

In order to evaluate the co-delivery capacity of PCL-*g*-PDMAEMA NPs, we initially entrapped paclitaxel into the hydrophobic core by a solvent evaporation self-assembly method and then complexed the NPs with DNA (Scheme 1). According to our experimental results, the encapsulation efficiency of paclitaxel determined by HPLC was about 95.7% with 1.9% drug-loading. When the drug-loading reached 12.5%, the encapsulation efficiency was still as high as 71.2%. These data indicated the high hydrophobic drug-loading capacity of PCL-*g*-PDMAEMA NPs. After entrapping 12.5% paclitaxel, the polymer particle size increased to 582 ± 32 nm from 538 ± 15 nm by DLS analysis, and the zeta potential of the NPs decreased to 36.5 ± 1.4 mV from 40.8 ± 0.9 mV, respectively (Table S1†).

The ratio of peak intensities at 338 and 333 nm (I_{338}/I_{333}) of the excitation spectra of pyrene was used to determine the critical association concentration (CAC) of amphiphilic copolymers and the polarity of the microenvironment inside the hydrophobic core. The measured CAC of PCL-*g*-PDMAEMA is about 8.1×10^{-4} g L^{-1} in pH = 7.4 aqueous solution (Fig. S2†), which is 100 times lower than most of the amphiphilic block copolymers.[16,17] It means that PCL-*g*-PDMAEMA NPs may be stable in the bloodstream for a long time. After dedicated design of a new carrier on the base of the stable of PCL-*g*-PDMAEMA

nanoparticles, such as pegylation modification of PCL-*g*-PDMAEMA, the new carrier system may be very useful *in vivo*. Furthermore, we observed that the I_{338}/I_{333} of pyrene in saturated aqueous solution is 1.26 at pH = 7.3. When incorporated into PCL-*g*-PDMAEMA NPs, the value was 1.28, which was almost the same as with DNA binding (Fig. S3†). It is known that a higher ratio of I_{338}/I_{333} indicates a more hydrophobic environment. Therefore, we can conclude that PCL-*g*-PDMAEMA NPs remained integrated structures during the DNA binding process.

The thermo- and pH-sensitivity of the PCL-*g*-PDMAEMA NPs was studied by exploring hydrodynamic diameters and drug release behaviors in this study. As shown in Fig. 4A, with increasing pH, the diameters of the blank and DNA-loaded NPs decrease sharply when the pH value is higher than 7.0. It is observed that the hydrodynamic radius of PCL-*g*-PDMAEMA nanoparticles was sharply changed from pH 6 to pH 8, which was similar to some other reported amphiphilic graft copolymers.[16,24–26] Temperature-dependent variety in particle size can also be observed in the pH range 6.0–8.0 (Fig. 4B).

The pH sensitivity is due to the changes in protonated degree of PDMAEMA segments. The PDMAEMA segments with higher protonation degree at a lower pH present a stretching conformation that can hardly be influenced by temperature. However, if the pH value is increased, the solubility of deprotonated PDMAEMA segments becomes temperature sensitive, *i.e.*, the solubility is decreased with increasing temperature when the temperature is higher than the lower critical soluble temperature (LCST). Therefore, it is rational that the average diameter of PCL-*g*-PDMAEMA NPs shows dual-response to temperature and pH.

It is worth noting that the shell of PCL-*g*-PDMAEMA NPs keep a swollen state with a bigger size at an acidic pH range of 6.0–6.9 at 37 °C or higher, but retract at about pH 7.4 and become even tighter when the temperature is above 37 °C. That is because PCL-*g*-PDMAEMA NPs can respond to the slight differences in pH and temperature between tumor and normal tissues. As expected, the thermo- and pH-sensitivity of PCL-*g*-PDMAEMA NPs could lead to pH-dependent, thermo-sensitive drug release. As shown in Fig. 4C, in aqueous media at 37 °C and pH 7.4, a negligible drug release from PCL-*g*-PDMAEMA NPs is observed. If we decrease the pH value, an obvious increase in the drug release rate can be observed. Thus, PCL-*g*-PDMAEMA NPs can provide an excellent tumor drug delivery character responding to mildly acidic environments. This property of PCL-*g*-PDMAEMA NPs should be very useful to effectively treat tumors with acidic microenvironments and reduce the side effects of the drug on the normal tissues.

Recent reports showed that cationic nanoparticles assembled from amphiphilic cationic copolymers have provided a new route to improve the gene delivery efficacy.[22,27,28] It was expected that PCL-*g*-PDMAEMA NPs with cationic shells and integrated hydrophobic cores could improve the gene transfection efficiency with no significant cytotoxicity. As shown in Fig. 5A, the PCL-*g*-PDMAEMA NPs/DNA complex NPs present lower cell toxicity when compared to commercial gene transfection reagent Lipo 2000 when the *N*/*P* ratio is lower than 10. The *in vitro* transfection experiments were conducted on 293T cells in serum-free and 5% serum-containing culture medium by using EGFP as a reporter gene and are shown in Fig. 5B and 5C. Compared with Lipo 2000, PCL-*g*-PDMAEMA NPs show comparable efficiency on 293T cells at *N*/*P* values of 10 and 15, indicating the gene transfection efficiency strongly depended on the *N*/*P* ratios. Particularly, the gene transfection efficiency by PCL-*g*-PDMAEMA NPs at a *N*/*P* ratio of 10 was enhanced from 26.9% to 36.7% by the addition of 5% serum. Serum, as a kind of anion macromolecule, is very likely to coat the surface of the PCL-*g*-PDMAEMA NPs/plasmid polyplexes, indicating that serum may play an important role in improving the gene transfection efficiency of the PCL-*g*-PDMAEMA NPs system by mediating the internalization of PCL-*g*-PDMAEMA NPs/plasmid polyplexes. It also suggests that the PCL-*g*-PDMAEMA NPs system may be easily modified by simple layer-by-layer technology to bear target ligand and pegylation. All these results indicate that the PCL-*g*-PDMAEMA NPs system is a potential carrier of gene and drug for application *in vitro* and *in vivo*. In addition, Fig. 5D shows fluorescence microscopic measurement of 293T cells transfected with Lipo 2000 and PCL-*g*-PDMAEMA NPs polyplexes at each optimal *N*/*P* ratio. PCL-*g*-PDMAEMA NPs show comparable efficiency on 293T cells at a *N*/*P* value of 10 to Lipo 2000.

It is known that successful escape from endosomes is crucial for gene carriers to improve gene transfection efficiency. To confirm the endosome escape of PCL-*g*-PDMAEMA NPs/plasmid polyplexes, the intracellular distribution of polyplexes was investigated by confocal laser scanning microscopy (CLSM) using cyanine-5-labeled pDNA (Fig. 6). The HIV-PSV plasmid was labeled by cy5 dye and the endosome was stained with

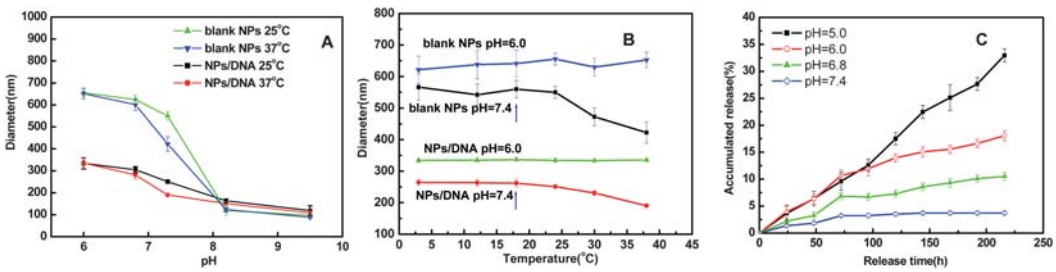


Fig. 4 The pH-dependent thermo-sensitivity of PCL-*g*-PDMAEMA NPs and polyplexes at a *N*/*P* ratio of 10. (A) and (B), the hydrodynamic diameter of blank NPs and polyplexes at different temperatures and pH; (C) the release behaviors of paclitaxel from PCL-*g*-PDMAEMA NPs with 1.9% paclitaxel entrapped in media at different pH, all of the measurements were done with temperature at 37 °C. The experiments were performed in triplicate and the means (±SEM) of one representative experiment ($n = 3$) were plotted.

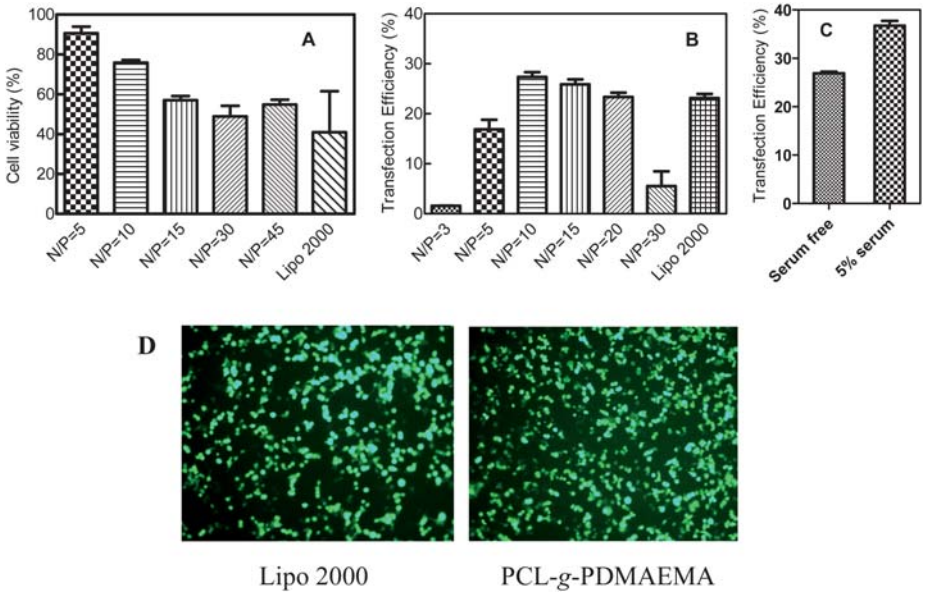


Fig. 5 The *in vitro* cytotoxicity and transfection efficiencies of PCL-*g*-PDMAEMA/DNA polyplexes at different *N*/*P* ratios were evaluated using 293T cells. (A) The cell viabilities of PCL-*g*-PDMAEMA/pEGFP polyplexes determined by MTT assay; (B) and (C) The transfection efficiencies of PCL-*g*-PDMAEMA complexed with EGFP as a reporter gene, determined by flow cytometry in serum-free and serum-containing culture medium ($N/P = 10$), respectively. Experiments were performed in triplicate; values represent the EGFP-positive cells as mean ±SEM of one representative experiment ($n = 3$). (D) Fluorescence microscopy of 293T cells transfected with Lipo 2000 and PCL-*g*-PDMAEMA NPs ($N/P = 10$) polyplexes.

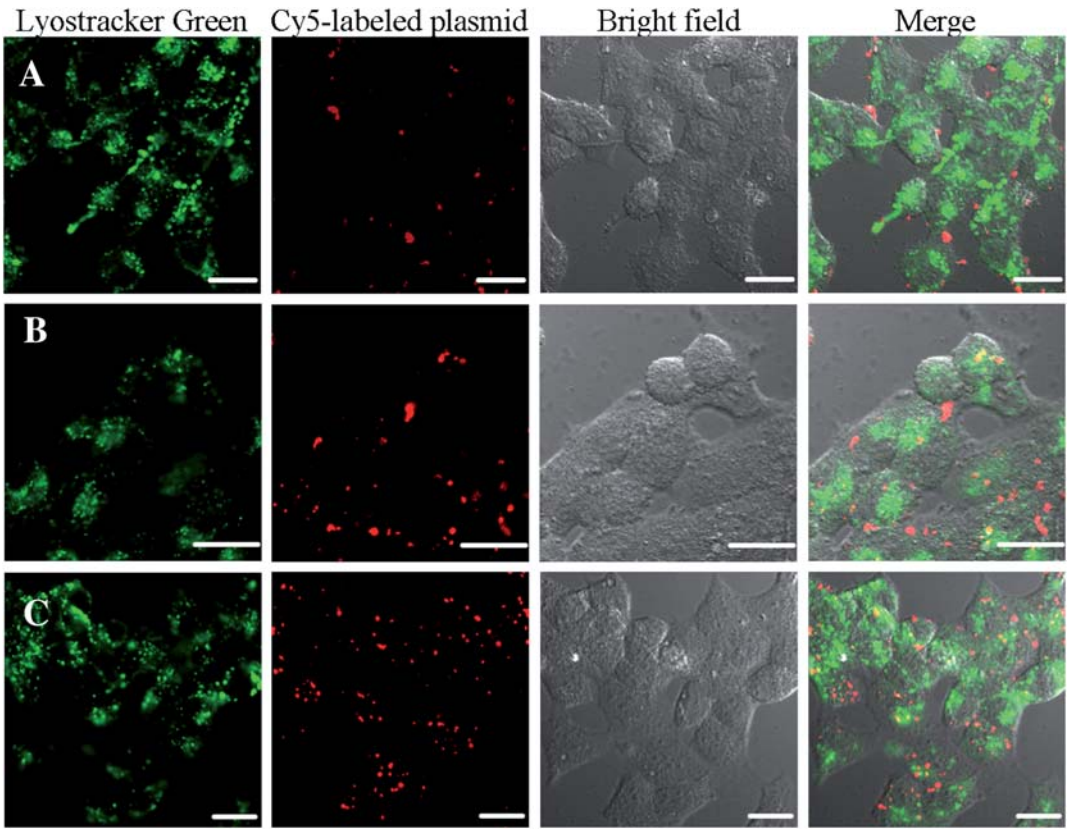


Fig. 6 Confocal images of PCL-*g*-PDMAEMA/DNA polyplexes at N/P ratio of 10 *in vitro.* Confocal images of 293T transfected with PCL-*g*-PDMAEMA NPs/DNA polyplexes were taken after transfection for 6 h (A), 12 h (B) and 24 h (C), respectively. The late endosome and lysosome network were stained with LysoTracker green and plasmid DNA was labeled with Cy5. Each scale bar represents 20 μm.

LysoTracker green after transfection to identify the localization of polyplexes. For the transfection experiment, cells were incubated with PCL-*g*-PDMAEMA NPs/DNA polyplexes for 4 h. As shown in Fig. 6, we find that only a little of the polyplexes were internalized into the cells and some polyplexes were adherent to the cell membrane at the initial 6 h incubation. After 12 h incubation with polyplexes, more polyplexes entered into the cells and they were partially distributed in the endosome, which was demonstrated as yellow spots. With 24 h incubation, it was clearly shown that many PCL-*g*-PDMAEMA NPs/DNA polyplexes were visible in the cells and released into cytoplasm, while not in the endosome. We believe that the high escape capacity of PCL-*g*-PDMAEMA NPs/DNA polyplexes is very helpful to improve the gene transfection efficiency. Because of the amphiphilic nature of PCL-*g*-PDMAEMA NPs, the enhanced hydrophobic property of the polyplexes may help polyplexes disrupt the membranes of cells easily. The exact mechanism is still under investigation.

Conclusions

In summary, the multiple functions of a new kind of core-shell nanoparticle, self-assembled from degradable amphiphilic cationic copolymer, poly(ε-caprolactone)-*graft*-poly(2-(*N*,*N*-dimethylamino) ethyl methacrylate) (PCL-*g*-PDMAEMA), has been demonstrated. PCL-*g*-PDMAEMA NPs with am ultralow critical association concentration can simultaneously entrap a hydrophobic drug in the core and load DNA on the shell for application in co-delivery of hydrophobic drugs and gene *in vitro* and *in vivo*. An intelligent drug release in response to the weakly acidic pH in pathological tissues is another function of the PCL-*g*-PDMAEMA nanoparticles, which may provide a promising anticancer drug delivery system to effectively regress tumors and reduce the side effects of the drug on normal tissues. Furthermore, PCL-*g*-PDMAEMA NPs show excellent gene transfection efficiencies both in serum-free and serum-containing culture media. The transfection efficiency of cationic PCL-*g*-PDMAEMA NPs enhanced by polyanionic serum suggests that PCL-*g*-PDMAEMA NPs may serve as a template for layer-by-layer technology to improve PCL-*g*-PDMAEMA NP systems. As a promising cancer therapy system, the combination effect of therapeutic plasmid and paclitaxel carried by PCL-*g*-PDMAEMA NPs to treat tumors was evaluated, *in vivo*, in our laboratory.

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