Responsive and *in situ*-forming chitosan scaffolds for bone tissue engineering applications: an overview of the last decade

Ana M. Martins,[abc] Catarina M. Alves,[ab] F. Kurtis Kasper,[c] Antonios G. Mikos[c] and Rui L. Reis*[ab]

Received 6th August 2009, Accepted 21st October 2009
First published as an Advance Article on the web 24th November 2009
DOI: 10.1039/b916259n

The use of bioabsorbable polymeric scaffolds is being investigated for use in bone tissue engineering applications, as their properties can be tailored to allow them to degrade and integrate at optimal rates as bone remodelling is completed. The main goal of this review is to highlight the "intelligent" properties exhibited by chitosan scaffolds and their use in the bone tissue engineering field. To complement the fast evolution of the bone tissue engineering field, it is important to propose the use of responsive scaffolds and take advantage of bioinspired materials and their properties as emerging technologies. There is a growing interest and need for new biomaterials, such as "smart"/responsive materials with the capability to respond to changes in the *in vivo* environment. This review will provide an overview of strategies that can modulate bone tissue regeneration by using *in situ*-forming scaffolds.

1. Introduction

Bone is a highly vascular, living and dynamic tissue remarkable for its combination of mechanical properties and regenerative capacity. Bone possesses a self-regeneration capacity. However, there is a limit to the size of bone fractures and defects that can be self-repaired. This limit is designated as the "critical size defect"[1–3] and will not heal during the lifetime of the patient. For large defects, medical intervention is often necessary to repair the bone. A new field of research that proposes the regeneration of the tissue instead of its substitution is defined as tissue engineering: "an interdisciplinary field of research that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain or improve tissue function".[4] Tissue engineering strategies involving scaffolds include two general categories: (1) the use of acellular matrices (artificial scaffolds or decellularized tissues), which depend upon the natural ability of the body to regenerate for proper orientation and direction of new tissue growth; and (2) the use of scaffolds with cells.[4,5] The most classical paradigm of tissue engineering for tissue regeneration implies the use of a degradable support or scaffold material, bioactive factors and cells.[4,6,7] Several characteristics and properties have been described[8,9] as *sine qua non* requirements for a suitable scaffold to be used in bone-tissue engineering that will be further discussed on the

[a]*3B's Research Group—Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, 4806-909 Caldas das Taipas, Guimarães, Portugal. E-mail: rgreis@dep.uminho.pt; Fax: +351-253-510909; Tel: +351-253-510902*
[b]*IBB—Institute for Biotechnology and Bioengineering, PT Government Associated Laboratory, Guimarães, Portugal*
[c]*Department of Bioengineering, Rice University, 6100 Main Street, Houston, Texas, 77005-1892, USA*

Ana M. Martins

Ana M. Martins was born on May 21, 1979, in Braga, Portugal. She received her Bachelor of Science degree in Applied Biology from the University of Minho in 2004. In 2006, she also completed a post-graduation course on Processing and Characterization of Materials. She received her PhD in Biomedical Engineering from the University of Minho, Portugal in collaboration with Rice University, Houston, USA. Her research focuses primarily on the development and characterization of natural based scaffolds, and incorporation of enzymes into biomimetic coatings of novel scaffolds for bone tissue engineering.

Catarina M. Alves

Catarina M. Alves, PhD, is a Postdoctoral Fellow at Harvard University, USA, and University of Minho, Portugal. She received her Bachelor of Science degree in Applied Biology and her PhD in Biomedical Engineering from University of Minho. The latter in collaboration with the University of Liverpool, UK and the University of Texas at San Antonio, USA. Her interests range from natural based materials, in vitro/in vivo protein adsorption and cellular response to biomaterials. Currently, her studies focus on the interaction of immune and cancer cells with biomaterials.

following sections. The main aim behind the different approaches of bone tissue engineering consists of developing a functionalized responsive and bioresorbable scaffold able to stimulate cell adhesion, proliferation and differentiation, with the objective that osteoblasts produce bone extracellular matrix (ECM).

Learning from Nature is a concept that implies mimicking Nature to develop novel functional biomaterials such as biomineralized, "smart" or bonelike composite materials.[10–15] The "smart" materials respond to changes of the surrounding environment. "Smart" scaffolds and bioreactors are being developed to enable advanced procedures for delivery of bioactive molecules and mechanical stimuli to cultured cells in order to direct osteogenic differentiation.[13,14,16–19] Increased attention has been devoted to responsive strategies *in vitro*, such as the use of flow perfusion bioreactors. The strategy includes the culture of bone marrow stromal cells onto scaffolds under flow conditions which allow a better distribution of nutrients and oxygen and the necessary mechanical stimuli for cellular differentiation along the osteogenic lineage.[20–22] This approach is an ideal system for the *ex vivo* production of bone constructs.[23–25] The deeply discussed typical bone tissue engineering strategy notwithstanding, which involves the use of a porous scaffold, cells and bioactive molecules, this review will focus on alternative approaches, such as the use of "smart"/responsive acellular scaffolds based on chitosan for bone tissue engineering applications.

The role of naturally derived materials and environmental stimuli in modulating bone regeneration will be discussed. While many excellent biomaterials have been developed in recent years, their translation into clinical practice has been slow. Chitosan was the selected natural polymer to be explored in this review mainly because of its pH responsive properties and biodegradability. The present review intends to provide an overview of the current state of the art of naturally derived responsive scaffolds and *in situ*-forming concepts for bone tissue engineering applications, their aims and limitations.

2. Scaffolds for bone tissue engineering

The simplest way to define a scaffold for tissue engineering is that it should provide mechanical support, shape, and cell-scale architecture for neo-tissue construction *in vitro* or *in vivo* as seeded cells expand and organize.[26] Scaffolds mimic the extracellular matrix and have a crucial role to play in supporting cell growth, differentiation and in delivering growth factors or other bioactive molecules. The extracellular matrix (ECM) is involved in bone formation, remodelling, and repair, and its components include minerals, ions, proteins, and enzymes.

The traditional requirements for a suitable scaffold for bone tissue engineering applications include: biocompatibility, biodegradability into nontoxic products, and adequate resorption rate for the repair of bone;[13,14] adequate surface characteristics for cell adhesion and proliferation; an interconnected porous structure that enable tissue ingrowth, vascularization, exchange of nutrients, oxygen and metabolites;[7,28] and suitable mechanical properties matching those of the native tissue.[4,7,27] The biomaterial must maintain its structural integrity during the first stages of the new bone formation. In this review we will highlight some characteristics of acellular scaffolds, such as mechanical properties and biodegradability.

F. Kurtis Kasper

Dr F. Kurtis Kasper holds an appointment as Faculty Fellow in the Department of Bioengineering at Rice University. He received his bachelor degree in biomedical engineering from Case Western Reserve University and his doctorate in bioengineering from Rice University. His research focuses primarily upon the development and evaluation of novel materials and approaches for the regeneration of orthopaedic tissues.

Antonios G. Mikos

Antonios G. Mikos is the Louis Calder Professor of Bioengineering and Chemical and Biomolecular Engineering at Rice University. His research focuses on the synthesis, processing, and evaluation of new biomaterials for use as scaffolds for tissue engineering, as carriers for controlled drug delivery, and as non-viral vectors for gene therapy. He has been recognized by various awards including the Alpha Chi Sigma Award for Chemical Engineering Research of the American Institute of Chemical Engineers and the Robert A. Pritzker Distinguished Lecturer Award of the Biomedical Engineering Society.

Rui L. Reis

Rui L. Reis PhD, DSc, is the Director of the 3B's Research Group—Biomaterials, Biodegradables and Biomimetics—at the University of Minho in Portugal, and the CEO of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine. He is a member of 12 international research societies, being on the board of the Tissue Engineering & Regenerative Medicine Society, the European Society for Artificial Board and the International Federation for Artificial Organs. He is in the editorial board of some of the major journals in his field of research, being the editor-in-chief of the Journal of Tissue Engineering and Regenerative Medicine.

Table 1 Mechanical properties of cancellous and cortical bone.[7,28,33]

	Compressive strength/MPa	Young's modulus/GPa
Cancellous bone	2–12	0.02–0.5
Cortical bone	100–230	2–30

2.1. Mechanical properties

The mechanical properties of bulk materials represent an important group of characteristics to consider in three-dimensional (3D) artificial ECM (scaffold) design.[29] Bulk materials are fundamental contributors to the mechanical integrity of the scaffold. This property is especially important for tissue engineering of structural tissues. Thus, the scaffolds should have mechanical properties resembling those of healthy tissue during the period of tissue regeneration.[7,27] Mechanical strength is needed for the creation of a 3D structure that will retain its structure after implantation, particularly in the reconstruction of hard and load-bearing bone tissues.[28]

The mechanical properties of the implanted scaffold should ideally match those of living bone.[7,27,30] This demanding balance represents one limitation of scaffolds intended for bone regeneration and generally leads to mechanical biomaterial failure. Low mechanical strength of porous scaffolds may not be suitable for the repair of load-bearing tissues in some clinical applications.[31] Depending upon the application, mechanical properties should be studied keeping in mind the surrounding environment of the scaffold once placed in an *in vivo* system. Besides providing appropriate support in the early stages of healing, the long-term success of the biomaterial will depend on the efficacious graded load transfer needed in the later stages of the remodelling process.[32] The mechanical properties of the two types of bone, *i.e.*, the cortical (compact) and cancellous (spongy) bone, are listed in Table 1. Highly porous structures with interconnected pores may fail in these objectives due to poor mechanical properties.

2.2. Mechanisms of polymer biodegradation

Controlling the degradation of scaffolds to match the rate of bone growth, to create space for the new bone formation until full regeneration is reached, remains a major challenge in scaffold design. The ideal degradable material degrades during its intended application or immediately after it. The process of polymer degradation describes the mechanisms through which polymer chains are cleaved to form oligomers and finally monomers.[34] The process of erosion designates the loss of material owing to monomers and oligomers leaving the polymer.[34,35] All biodegradable polymers have hydrolysable bonds. Their most important degradation mechanism is enzymatic hydrolysis. The latter effect is designated as biodegradation meaning that the degradation is mediated by a biological process.[34] Several factors influence the kinetics of degradation: the type of chemical bonds, pH, polymer composition, crystallinity, molecular weight, porosity, water uptake and location of the implant.[34] Hydrophilic polymers absorb large quantities of water and increase degradation rates. Resorbable biomaterials have the ability to resorb over time. This behaviour is necessary to support the gradual ingrowth of cells and complete replacement of a regenerated matrix by normal tissue, and to avoid risk of complications that can be associated with the long-term presence of foreign material.[36] Scaffold evaluation also includes studying the appropriate degradation rate, which is important because as the scaffold degrades it is replaced by natural tissue. Biodegradation is generally required for a tissue engineering scaffold material, and the degradation rate also needs to match the neo tissue formation rate to ideally serve the template purpose.[27,28,37] Bone tissue engineering generally requires an artificial extracellular matrix (scaffold) to regenerate tissue at the site of implantation. The degradation rate of the scaffolds must be tuned appropriately with the growth rate of the new tissue, by the time the injury site is totally regenerated the scaffold should be totally degraded[2,13,14] If the degradation is faster than the tissue regeneration, the scaffolds will loose its support function for tissue growth. On the other hand, if the degradation is too slow compared to tissue formation, the scaffold will compromise the regeneration of the tissue. This scaffold should disappear through absorption into the body as the new tissue is regenerated.[37]

3. "Smart" and responsive scaffolds for bone tissue engineering applications

Stimuli-responsive, "smart", "intelligent" or environmentally-sensitive polymers respond with large property changes to small chemical, physical or biochemical stimuli (Table 2). The concept of "smart" polymers derived from the development of biomaterials that show large conformational changes in response to small environmental stimuli such as temperature, ionic strength, pH, or light.[26,38] The materials that respond to changes in their surrounding environment are very attractive because these changes, mainly *in vivo*, can be exploited to control parameters such as drug delivery, cell adhesion, mechanical properties, and permeability, among others.[39] The responses of the polymer may include precipitation or gelation, reversible adsorption on a surface, collapse of a hydrogel or surface graft, and alternation between hydrophilic and hydrophobic states.[26,40] Natural polymers may present a more appropriate biological environment to the cells, since they usually contain domains that can send important signals to guide the cells at various stages of development.[41] Collagen and fibrin, natural ECM molecules, have been used as scaffolds for tissue engineering. They have interesting biological properties for tissue engineering research.[42–45] However, their low mechanical properties, instability and deterioration that follow long-term implantation were reported limit the clinical applications of these natural biomaterials.[37]

Table 2 Environmental stimuli that responsive, "smart", "intelligent" or to which environmentally-sensitive polymers respond.[40,46]

Physical	Chemical	Biochemical
Temperature		
Ionic strength	pH	Enzyme ligands
Solvents	Specific ions	Biochemical agents
Electrical and magnetic fields	Chemical agents	
Mechanical stress, strain		

4. Chitosan scaffolds as a responsive biomaterial

Polysaccharides are very attractive for tissue engineering applications mainly because of their biodegradability, biocompatibility and resemblance with the environment of the extracellular matrix.[47,48] Examples of anionic naturally derived polymers are alginate, hyaluronic acid, chondroitin sulfate and carragenans.[48] However chitosan is the only cationic polysaccharide found in Nature. In the last decade, significant attention has been given to chitosan-based biomaterials[13,49–55] in the field of bone tissue engineering. This review will focus on chitosan, a polycationic polymer of natural origin produced by the deacetylation of chitin, a natural component of crustacea exoskeletons (*e.g.* shrimp, crab, lobster, *etc.*), cell walls of fungi and cuticles of insects.[54,56] The degree of deacetylation represents the proportion of *N*-acetyl-D-glucosamine units with respect to the total number of units.[57] Chitosan is degraded, depending on degree of deacetylation by enzymes such as lysozyme, *N*-acetyl-D-glucosaminidase and lipases.[58] *In vivo*, chitosan is degraded by enzymatic hydrolysis, primarily by lysozyme which appears to target acetylated residues.[59,60] Degradation kinetics seem to be inversely related to the degree of deacetylation.[59] Lysozyme breaks down the chitosan polymer chain, diminishing its molecular weight until it becomes short enough to be processed by cells. Glucosamines, the final degradation products of chitosan, are nontoxic, nonimmunogenic, and noncarcinogenic.[61] *In vivo*, the final degradation products undergo normal metabolism pathways and may be incorporated into glycoproteins or excreted as carbon dioxide gas during respiration.[62,63] Lysozyme or muramidase is an enzyme that catalyzes the hydrolysis of the peptidoglycan layer of bacterial cell walls.[64] This enzyme is active over a broad pH range from 3 to 8 and is suited to hydrolyze its substrates both inside and outside cells. Lysozyme is ubiquitous in the human body.[65] It is present in lymphocytes and also secreted by monocytes, macrophages, and granulocytes, which account for the largest source.[66,67] Monocytes and macrophages are the dominating contributors to the lysozyme content in serum.[66] Human serum lysozyme is found in concentrations from 7 to 13 mg L^{-1}.[65]

Chitosan is a binary polyheterosaccharide of *N*-acetylglucosamine and glucosamine with a $\beta 1 \rightarrow 4$ linkage. The superior tissue compatibility of chitosan can be partially attributed to its structural similarity to glycosaminoglycans, which are major components of the ECM of bone and cartilage.[54,68] Chitosan exhibits a pH-sensitive behavior due to the large quantities of amino groups on its chains. It is a biocompatible, pH-dependent cationic polymer, which is insoluble in aqueous solutions above pH 7.[55] However, in dilute or weak acids (pH < 6), the protonated free amino groups of glucosamine facilitate solubility of the molecule.[55] Above pH 6.2 chitosan aqueous solutions lead to the formation of a hydrated gel-like precipitate.[69,70] Due to its cationic nature and predictable degradation rate, chitosan-based materials bind growth factors and release them in a controlled manner.[71] Temperature and pH have been extensively studied in the biomedical field because these two parameters can be easily controlled and applicable both *in vitro* and *in vivo*. Dias *et al.*[72] reported the use of chitosan, a natural and pH-responsive polymer, grafted onto a biodegradable bioactive composite and investigated the effect of pH on the biomineralization process. The authors successfully developed "smart" biodegradable surfaces that respond to pH and that could be used to control the biomineralization process. They also found that the formation of biomimetic apatite was dependent on the conformational changes of chitosan across its critical pH and could be controlled by pH switching.

Chitosan hydrogels have been used in a number of gene and drug delivery applications and can deliver growth factors and pharmaceutical agents in a controlled fashion.[58,73,74]

Chitosan possesses interesting characteristics, such as its ability to induce a minimal foreign body reaction, an intrinsic antibacterial nature,[75,76] and the ability to be molded in various shapes, namely porous structures, suitable for cell ingrowth and osteoconduction. The mechanical properties of chitosan scaffolds are dependent on the pore size and pore orientation.[75]

5. Naturally derived *in situ* forming scaffolds

The goal of the *in situ* generated implant strategy is used to engineer biomedical systems at their site of performance using minimally invasive surgeries.[77] Several approaches have been developed[78–80] namely synthetic *in situ* scaffolding materials that could deliver cells or provide a structure for tissue infiltration. Naturally occurring polymers that form thermoreversible gels include gelatine, carrageenan, cellulose derivatives, xyloglucan and chitosan with glycerophosphate.[69] Moreover, several natural biomaterials, including collagen, heparin, hyaluronate, and fibrin have been used for the preparation of injectable *in situ*-forming scaffolds.[81–86] Besides eliminating the need for *ex vivo* implant fabrication, the contact and adhesion between the biomaterial and native bone may be enhanced with a polymer formed directly in the bone defect (*in situ*).[87] Current approaches for implanting medical devices often require complex surgeries. In the past few years, an increasing number of *in situ*-forming systems have been proposed for various biomedical applications, including drug delivery,[88–90] cell encapsulation,[69,91] and tissue repair.[83,92,93] There are several possible mechanisms leading to *in situ* implant formation. It has become increasingly apparent that scaffolds for bone tissue engineering applications should provide more than a temporary 3D structure for developing tissue construct. Due to the pH-sensitive character of chitosan, this polymer has great potential to be used in the fabrication of scaffold and gels that respond to localized conditions of pH in the human body. Hai Bang Lee *et al.*[94] stated that it is difficult to perform *ex vivo* fabrication of certain complex scaffold geometries. As an alternative, they reported the use of *in situ*-forming scaffolds as a promising approach for the fabrication of complicated scaffold geometries. The *in situ*-forming scaffold is based on the idea that if a biomaterial undergoes a simple liquid-to-gel phase transition under physiological conditions, it can be injected as a liquid, and then form the desired gel *in situ*.[94–96] As previously reported, Ruel-Gariepy *et al.*[69,97] demonstrated that a mixture of chitosan and glycerol phosphate disodium salt (β-glycerophosphate) is capable of forming a gel scaffold *in situ.* These formulations possess a neutral pH, remain liquid at or below room temperature, and form monolithic gels at body temperature.[69] The stability of the solution at room temperature and the gelation time increase as the degree of deacetylation decreases.[69,97] Hai Bang Lee *et al.*[94] described the development of

in situ-forming chitosan gels, and their ability to offer a suitable scaffold for rat bone marrow stromal cells (rBMSCs) *in vitro* and *in vivo*. The *in situ*-forming scaffold provides an advantage compared with traditional scaffolds because it is a noninvasive alternative for tissue engineering applications. These results showed that chitosan gel can serve as an *in situ*-forming gel scaffold for entrapped rBMSCs *in vivo*. Studies dealing with the role of stem/progenitor cells in osteogenesis show that chitosan has the ability to promote osteogenic progenitor cell recruitment and attachment, facilitating bone formation.[98]

As cell and molecular biology converge with materials science and biomedical engineering, new applications will benefit from interactive biomaterials that serve to orchestrate cell attachment, growth and differentiation.[26] Recently, a considerable interest has been given to the development of "smart" materials with the ability to instruct the behaviour of cells by releasing bioactive molecules into the local environment.[99,100]

6. *In situ* pore-forming scaffolds

One of the major challenges of bone tissue engineering is the development of scaffolds capable of promoting the differentiation of immature progenitor cells down an osteoblastic lineage (osteoinduction) encouraging the ingrowth of surrounding bone (osteoconduction) and integration into the surrounding tissue.[101,102] The main goal of the *in situ* forming scaffolds strategy is to generate systems at their site of implantation using minimally invasive surgical procedures or eliminate some steps of the common strategies used in bone tissue engineering applications (*i.e.*, cell seeding onto scaffolds *ex vivo*). Acellular scaffolds with properties capable to induce bone regeneration could be an interesting alternative.

One innovative approach was described by the group of Robert Langer[103] for the first time in 2003 proposing *in situ* pore formation in a polymer matrix by differential degradation. The common approaches have been developed to introduce porosity in a polymer matrix *ex vivo* and thus facilitate cell seeding either *in vitro* or *in vivo*. In that paper, the authors stated that controlling pore formation *in vivo* with specific tissue ingrowth could be beneficial for bone tissue engineering and presented a new paradigm for the formation of pores in a polymer matrix *in situ*.[103] For that, polymer microspheres were used as a pore-forming agent (porogen).[103]

It has been demonstrated that hydrophilic polymers such as chitosan, depending on the processing method and shape present suitable mechanical properties for bone tissue engineering applications.[13]

The inclusion of enzymatically degradable phases in biomaterials seems to be a very promising approach to obtain scaffolds with adequate mechanical properties in the initial stage of implantation and with a gradual *in vivo* pore-forming ability. Martins *et al.*[13] described the development of a biodegradable matrix, based on chitosan and starch with the ability of forming a porous structure *in situ* due to the attack by specific enzymes present in the human body, taking advantage of the inflammatory response. One of the critical factors that can control bone tissue regeneration is the degradation rate of the scaffold, as previously discussed. Tailoring the degradation rate of scaffolds can facilitate scaffold remodelling and replacement by cells

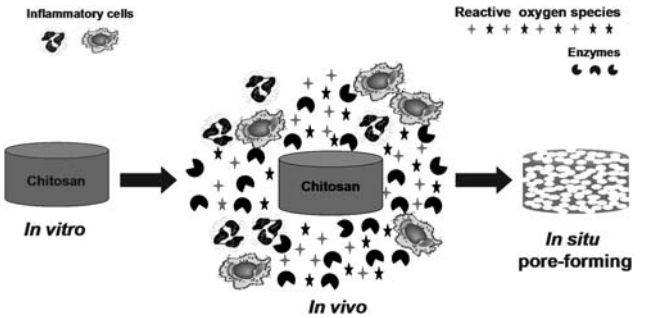

Fig. 1 Schematic representation of *in situ* pore forming concept.

in situ, enhancing bone tissue regeneration. Inflammation is often viewed as a negative event, but it is fundamental for tissue regeneration processes.[104] Consequently, modulating the response of implanted material by harnessing characteristics of the inflammatory response is a powerful tool for driving tissue regeneration *in situ*. In this section alternative strategies will be discussed, such as the potential of using responsive scaffolds to take advantage of the host inflammatory response and thus obtain a beneficial outcome for bone tissue engineering applications. When a material is implanted, an acute inflammation is initiated involving several cell types.[105–107] During inflammation, neutrophils, monocytes and macrophages are present. These cells release lysosomal enzymes, such as lysozyme, into the surrounding tissue.[108] It has been suggested that macrophages and neutrophils produce superoxide, hydrogen peroxide and hydroxyl free radicals which contribute to the biodegradation of implanted materials.[108] The results of the compressive tests showed that these materials, based on chitosan and starch, exhibited very interesting mechanical properties in the dry and even in the wet state.[13] The mechanical properties exhibited by the scaffolds in the wet state fall in the normal ranges of strength and modulus for trabecular bone.[7,28] This approach seems to be a promising strategy to produce an *in vivo* responsive scaffold, the properties of which may be regulated by the bone regeneration process, with gradual formation of pores *in situ* and consequent resorption. Using this innovative methodology, authors aimed at developing a biodegradable matrix based on chitosan and starch that exhibits suitable mechanical properties at the initial stage of implantation due to the absence of macroporosity. In a later *in vivo* stage, a porous structure develops by specific enzymes and reactive species present in the human body and associated to the inflammatory response (Fig. 1).

An alternative approach, involving the concept of bioactivity and osteoconduction, is to let degradation proceed along a coordinate of the healing process by making the material sensitive to the feedback provided by the cells involved in the healing response.[109] A biomimetic scaffold for bone tissue engineering can be any scaffolding material that mimics one or more characteristics of the natural ECM.[110] Functional materials such as calcium phosphate (CaP)[111] and hydroxyapatite[112] are often mixed with bulk materials to mimic bone ECM composition. CaP has a composition similar to bone mineral and can induce a biological response identical to that generated in bone remodelling, which is the process of resorption of old bone mineral and formation of new bone.[113] The biomimetic technique for coating biomaterials with a bone-like apatite layer is well

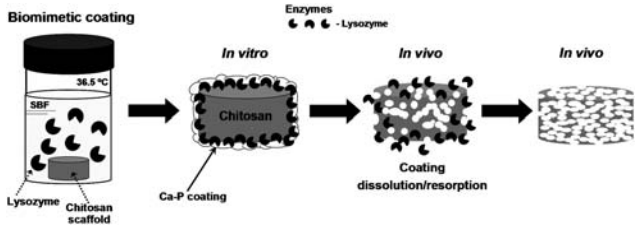

Fig. 2 Schematic representation of a self-regulated degrading material with gradual *in situ* pore formation ability.

described[18,114–116] and has been applied for the first time to biodegradable substrates and then to scaffolds by Reis *et al.*[18,51,58,115,117,118] Biomimetic CaP coating involves immersion of polymers in simulated body fluid (SBF), a solution with an ion concentration identical to human plasma. CaP coatings have shown high efficiency in expediting bone osteoconduction.[119] However, for the regeneration of bone, osteoconduction is also important.[100] The incorporation of proteins and enzymes[18,120–127] into CaP coatings is well documented. Further studies proposed an innovative self-regulated degrading material with gradual *in situ* pore formation ability for bone tissue engineering applications. In this study[127] one of the main aims was to improve the osteoconductive properties of chitosan scaffolds. For that, lysozyme was incorporated into CaP coatings, prepared on the surface of chitosan scaffolds using a biomimetic coating technique, with the aim of controlling their degradation rate and subsequent formation of pores. Furthermore, since lysozyme has antibacterial properties, these coatings may act as carriers for its sustained release, preventing infection upon implantation. Moreover, CaP coatings will enhance the osteoconductive properties of the chitosan scaffolds. Mineral deposition has been shown to slow scaffold degradation, probably by creating a barrier between the scaffold surface and surrounding environment.[128] In order to avoid this, one possible solution was the incorporation of the enzyme lysozyme to enhance degradation of chitosan scaffolds and subsequent formation of pores *in situ* (Fig. 2).

Responsive scaffolds for bone tissue engineering do not need to show the stimulus-dependent change in a reversible fashion. For example, after the cells or bioactive molecules are delivered, the scaffolds do not need to reverse the process. As was discussed above, biodegradability and lack of cytotoxicity are required characteristics of bone tissue engineering scaffolds.

"Smart"/responsive polymers may offer promise for revolutionary improvements in tissue engineering scaffolds. Beyond the physical properties of polymers, a major goal is to improve the mechanical properties at the initial stage of implantation and if possible to avoid one of the most critical steps of the tissue engineering approach, that is the pre-seeding of the scaffolds with cells. Creating scaffolds with specific properties that *per se* could recruit cells to the site of implantation is in our opinion one of the main current challenges in the field.

7. Conclusions and final remarks

Therapies for the treatment of lost tissue include tissue transplantation, surgical reconstruction, drug therapy, synthetic prostheses and medical devices or associations of those. The efforts to address their limitations have elicited the development of different biomaterials and therapies. In this review we aimed at presenting as alternative approaches to the engineering of responsive and *in situ*-forming scaffolds for bone regeneration.

Chitosan is one of the most promising natural polymers for bone tissue engineering due, in part, to its particular ability to form various shapes and structures. The degradation properties and the ability of forming porous structures and scaffolds *in situ* makes chitosan an interesting alternative biomaterial for orthopaedic applications.

In the first part of this review, biodegradation and mechanical properties were highlighted requirements for the implantation of acellular scaffolds. In the second part, we presented developments and examples of naturally derived responsive scaffolds for bone tissue engineering. Finally, we described a new strategy for bone tissue engineering: the *in situ* pore-forming concept. The success of this approach is mainly dictated by the presence of lysozyme incorporated into the coatings that will grant chitosan scaffolds with a gradual *in vivo* pore forming ability and antibacterial activity. Meanwhile, the presence of the CaP coating will simultaneously enable osteoconductive properties to the scaffolds. These "smart" and responsive scaffolds, with *in situ* pore forming capability and interesting mechanical properties, seem to be advantageous when compared with other presently available conventional materials. Despite the recent advances, future research focusing on the development of novel scaffolds that *per se* could recruit desirable cells, regenerate the implantation site and degrade/resorb as a function of healing time still comprises one of the most demanding and challenging strategies in the field of bone tissue engineering.

References

1 J. P. Schmitz and J. O. Hollinger, *Clin. Orthop. Relat. Res.*, 1986, 299–308.
2 M. C. Kruyt, W. J. Dhert, H. Yuan, C. E. Wilson, C. A. van Blitterswijk, A. J. Verbout and J. D. de Bruijn, *J. Orthop. Res.*, 2004, **22**, 544–551.
3 Z. S. Patel, S. Young, Y. Tabata, J. A. Jansen, M. E. Wong and A. G. Mikos, *Bone*, 2008, **43**, 931–940.
4 R. Langer and J. P. Vacanti, *Science*, 1993, **260**, 920–926.
5 A. Atala, *J. Tissue Eng. Regener. Med.*, 2007, **1**, 83–96.
6 R. Cancedda, B. Dozin, P. Giannoni and R. Quarto, *Matrix Biol.*, 2003, **22**, 81–91.
7 D. W. Hutmacher, J. T. Schantz, C. X. Lam, K. C. Tan and T. C. Lim, *J. Tissue Eng. Regener. Med.*, 2007, **1**, 245–260.
8 D. W. Hutmacher, *Biomaterials*, 2000, **21**, 2529–2543.
9 D. W. Hutmacher, *J. Biomater. Sci., Polym. Ed.*, 2001, **12**, 107–124.
10 P. B. Malafaya, M. E. Gomes, A. J. Salgado and R. L. Reis, *Adv. Exp. Med. Biol.*, 2003, **534**, 201–233.
11 G. A. Silva, F. J. Costa, O. P. Coutinho, S. Radin, P. Ducheyne and R. L. Reis, *J. Biomed. Mater. Res.*, 2004, **70a**, 442–449.
12 R. M. da Silva, J. F. Mano and R. L. Reis, *Trends Biotechnol.*, 2007, **25**, 577–583.
13 A. M. Martins, M. I. Santos, H. S. Azevedo, P. B. Malafaya and R. L. Reis, *Acta Biomater.*, 2008, **4**, 1637–1645.
14 A. M. Martins, Q. P. Pham, P. B. Malafaya, R. M. Raphael, F. K. Kasper, R. L. Reis and A. G. Mikos, *Tissue Eng. A*, 2009.
15 L. J. Fan Xia, *Adv. Mater.*, 2008, **20**, 2842–2858.
16 D. L. Kaplan, R. T. Moon and G. Vunjak-Novakovic, *Nat. Biotechnol.*, 2005, **23**, 1237–1239.
17 H. L. Holtorf, J. A. Jansen and A. G. Mikos, *Adv. Exp. Med. Biol.*, 2006, **585**, 225–241.
18 A. M. Martins, Q. P. Pham, P. B. Malafaya, R. M. Raphael, F. K. Kasper, R. L. Reis and A. G. Mikos, *Tissue Eng. A*, 2008, **14**, 795–795.

19 A. M. Martins, Q. P. Pham, P. B. Malafaya, R. A. Sousa, M. E. Gomes, R. M. Raphael, F. K. Kasper, R. L. Reis and A. G. Mikos, *Tissue Eng. A*, 2009, **15**, 295–305.
20 M. E. Gomes, C. M. Bossano, C. M. Johnston, R. L. Reis and A. G. Mikos, *Tissue Eng.*, 2006, **12**, 177–188.
21 M. E. Gomes, H. L. Holtorf, R. L. Reis and A. G. Mikos, *Tissue Eng.*, 2006, **12**, 801–809.
22 M. E. Gomes, V. I. Sikavitsas, E. Behravesh, R. L. Reis and A. G. Mikos, *J. Biomed. Mater. Res.*, 2003, **67a**, 87–95.
23 N. Datta, Q. P. Pham, U. Sharma, V. I. Sikavitsas, J. A. Jansen and A. G. Mikos, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 2488–2493.
24 Q. P. Pham, F. K. Kasper, L. Scott Baggett, R. M. Raphael, J. A. Jansen and A. G. Mikos, *Biomaterials*, 2008, **29**, 2729–2739.
25 C. Aulin, F. Foroughi, R. Brown and J. Hilborn, *J. Tissue Eng. Regener. Med.*, 2009, **3**, 188–195.
26 M. E. Furth, A. Atala and M. E. Van Dyke, *Biomaterials*, 2007, **28**, 5068–5073.
27 A. J. Salgado, O. P. Coutinho and R. L. Reis, *Macromol. Biosci.*, 2004, **4**, 743–765.
28 S. Yang, K. F. Leong, Z. Du and C. K. Chua, *Tissue Eng.*, 2001, **7**, 679–689.
29 J. Lee, M. J. Cuddihy and N. A. Kotov, *Tissue Eng., Part B: Rev.*, 2008, **14**, 61–86.
30 K. F. Leong, C. M. Cheah and C. K. Chua, *Biomaterials*, 2003, **24**, 2363–2378.
31 S. J. Hollister, *Nat. Mater.*, 2005, **4**, 518–524.
32 J. S. Temenoff and A. G. Mikos, *Biomaterials*, 2000, **21**, 431–440.
33 J. Wilson, A. E. Clark, M. Hall and L. L. Hench, *J Oral Implantol*, 1993, **19**, 295–302.
34 A. Gopferich, *Biomaterials*, 1996, **17**, 103–114.
35 J. A. Tamada and R. Langer, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 552–556.
36 J. A. Hubbell, *Bio/Technology*, 1995, **13**, 565–576.
37 F. Rosso, G. Marino, A. Giordano, M. Barbarisi, D. Parmeggiani and A. Barbarisi, *J. Cell. Physiol.*, 2005, **203**, 465–470.
38 I. Y. Galaev and B. Mattiasson, *Trends Biotechnol.*, 1999, **17**, 335–340.
39 B. D. Ratner and S. J. Bryant, *Annu. Rev. Biomed. Eng.*, 2004, **6**, 41–75.
40 A. S. Hoffman, P. S. Stayton, V. Bulmus, G. Chen, J. Chen, C. Cheung, A. Chilkoti, Z. Ding, L. Dong, R. Fong, C. A. Lackey, C. J. Long, M. Miura, J. E. Morris, N. Murthy, Y. Nabeshima, T. G. Park, O. W. Press, T. Shimoboji, S. Shoemaker, H. J. Yang, N. Monji, R. C. Nowinski, C. A. Cole, J. H. Priest, J. M. Harris, K. Nakamae, T. Nishino and T. Miyata, *J. Biomed. Mater. Res.*, 2000, **52**, 577–586.
41 R. Langer, *J. Controlled Release*, 1999, **62**, 7–11.
42 E. Gentleman, A. N. Lay, D. A. Dickerson, E. A. Nauman, G. A. Livesay and K. C. Dee, *Biomaterials*, 2003, **24**, 3805–3813.
43 X. B. Yang, R. S. Bhatnagar, S. Li and R. O. Oreffo, *Tissue Eng.*, 2004, **10**, 1148–1159.
44 C. Perka, O. Schultz, R. S. Spitzer, K. Lindenhayn, G. R. Burmester and M. Sittinger, *Biomaterials*, 2000, **21**, 1145–1153.
45 T. Osathanon, M. L. Linnes, R. M. Rajachar, B. D. Ratner, M. J. Somerman and C. M. Giachelli, *Biomaterials*, 2008, **29**, 4091–4099.
46 A. S. Hoffman, *Artif. Organs*, 1988, **12**, 504–505.
47 J. F. Mano, G. A. Silva, H. S. Azevedo, P. B. Malafaya, R. A. Sousa, S. S. Silva, L. F. Boesel, J. M. Oliveira, T. C. Santos, A. P. Marques, N. M. Neves and R. L. Reis, *J. R. Soc. Interface*, 2007, **4**, 999–1030.
48 J. F. Mano, *Adv. Eng. Mater.*, 2008, **10**, 515–527.
49 A. R. Costa-Pinto, A. J. Salgado, V. M. Correlo, P. Sol, M. Bhattacharya, P. Charbord, R. L. Reis and N. M. Neves, *Tissue Eng. A*, 2008, **14**, 1049–1057.
50 P. B. Malafaya, T. C. Santos, M. van Griensven and R. L. Reis, *Biomaterials*, 2008, **29**, 3914–3926.
51 A. M. Martins, R. C. Pereira, I. B. Leonor, H. S. Azevedo and R. L. Reis, *Acta Biomater.*, 2009.
52 J. M. Oliveira, M. T. Rodrigues, S. S. Silva, P. B. Malafaya, M. E. Gomes, C. A. Viegas, I. R. Dias, J. T. Azevedo, J. F. Mano and R. L. Reis, *Biomaterials*, 2006, **27**, 6123–6137.
53 J. T. Oliveira, V. M. Correlo, P. C. Sol, A. R. Costa-Pinto, P. B. Malafaya, A. J. Salgado, M. Bhattacharya, P. Charbord, N. M. Neves and R. L. Reis, *Tissue Eng. A*, 2008, **14**, 1651–1661.
54 A. Lahiji, A. Sohrabi, D. S. Hungerford and C. G. Frondoza, *J. Biomed. Mater. Res.*, 2000, **51**, 586–595.
55 S. V. Madihally and H. W. Matthew, *Biomaterials*, 1999, **20**, 1133–1142.
56 J. K. Suh and H. W. Matthew, *Biomaterials*, 2000, **21**, 2589–2598.
57 C. Chatelet, O. Damour and A. Domard, *Biomaterials*, 2001, **22**, 261–268.
58 R. A. A. Muzzarelli, *Carbohydr. Polym.*, 2009, **76**, 167–182.
59 K. Tomihata and Y. Ikada, *Biomaterials*, 1997, **18**, 567–575.
60 S. Hirano, H. Tsuchida and N. Nagao, *Biomaterials*, 1989, **10**, 574–576.
61 R. A. Muzzarelli, *Cell. Mol. Life Sci.*, 1997, **53**, 131–140.
62 A. Abarrategi, A. Civantos, V. Ramos, J. V. Sanz Casado and J. L. Lopez-Lacomba, *Biomacromolecules*, 2008, **9**, 711–718.
63 J. Ma, H. Wang, B. He and J. Chen, *Biomaterials*, 2001, **22**, 331–336.
64 J. Brouwer, T. van Leeuwen-Herberts and M. Otting-van de Ruit, *Clin. Chim. Acta*, 1984, **142**, 21–30.
65 J. Hankiewicz and E. Swierczek, *Clin. Chim. Acta*, 1974, **57**, 205–209.
66 I. Torsteinsdottir, L. Hakansson, R. Hallgren, B. Gudbjornsson, N. G. Arvidson and P. Venge, *Rheumatology*, 1999, **38**, 1249–1254.
67 C. Teijon, R. Olmo, M. Dolores Blanco, A. Romero and J. Maria Teijon, *Toxicology*, 2003, **191**, 245–258.
68 J. Y. Lee, S. H. Nam, S. Y. Im, Y. J. Park, Y. M. Lee, Y. J. Seol, C. P. Chung and S. J. Lee, *J. Controlled Release*, 2002, **78**, 187–197.
69 E. Ruel-Gariepy and J. C. Leroux, *Eur. J. Pharm. Biopharm.*, 2004, **58**, 409–426.
70 A. Di Martino, M. Sittinger and M. V. Risbud, *Biomaterials*, 2005, **26**, 5983–5990.
71 R. A. Muzzarelli, M. Mattioli-Belmonte, C. Tietz, R. Biagini, G. Ferioli, M. A. Brunelli, M. Fini, R. Giardino, P. Ilari and G. Biagini, *Biomaterials*, 1994, **15**, 1075–1081.
72 C. I. Dias, J. F. Mano and N. M. Alves, *J. Mater. Chem.*, 2008, **18**, 2493–2499.
73 M. Prabaharan, *J. Biomater. Appl.*, 2008, **23**, 5–36.
74 M. Prabaharan, J. J. Grailer, D. A. Steeber and S. Gong, *Macromol. Biosci.*, 2008, **8**, 843–851.
75 I. Y. Kim, S. J. Seo, H. S. Moon, M. K. Yoo, I. Y. Park, B. C. Kim and C. S. Cho, *Biotechnol. Adv.*, 2008, **26**, 1–21.
76 C. Aimin, H. Chunlin, B. Juliang, Z. Tinyin and D. Zhichao, *Clin. Orthop. Relat. Res.*, 1999, **366**, 239–247.
77 D. Cohn, A. Sosnik and S. Garty, *Biomacromolecules*, 2005, **6**, 1168–1175.
78 R. Marchand and S. Woerly, *Neuroscience*, 1990, **36**, 45–60.
79 B. Balakrishnan and A. Jayakrishnan, *Biomaterials*, 2005, **26**, 3941–3951.
80 A. C. Wan, E. Khor and G. W. Hastings, *J. Biomed. Mater. Res.*, 1998, **41**, 541–548.
81 Y. D. Park, N. Tirelli and J. A. Hubbell, *Biomaterials*, 2003, **24**, 893–900.
82 H. Park, J. S. Temenoff, Y. Tabata, A. I. Caplan and A. G. Mikos, *Biomaterials*, 2007, **28**, 3217–3227.
83 D. W. Lim, D. L. Nettles, L. A. Setton and A. Chilkoti, *Biomacromolecules*, 2008, **9**, 222–230.
84 D. L. Nettles, K. Kitaoka, N. A. Hanson, C. M. Flahiff, B. A. Mata, E. W. Hsu, A. Chilkoti and L. A. Setton, *Tissue Eng. A*, 2008, **14**, 1133–1140.
85 G. Tae, Y. J. Kim, W. I. Choi, M. Kim, P. S. Stayton and A. S. Hoffman, *Biomacromolecules*, 2007, **8**, 1979–1986.
86 K. A. B, C. W. P.J, C. E. S and Jennie Baier Leach, *Biotechnol. Bioeng.*, 2003, **82**, 578–589.
87 J. A. Burdick, R. F. Padera, J. V. Huang and K. S. Anseth, *J. Biomed. Mater. Res.*, 2002, **63**, 484–491.
88 A. Hatefi and B. Amsden, *J. Controlled Release*, 2002, **80**, 9–28.
89 G. Chandrashekar and N. Udupa, *J. Pharm. Pharmacol.*, 1996, **48**, 669–674.
90 S. Kumar, B. O. Haglund and K. J. Himmelstein, *J. Ocul. Pharmacol.*, 1994, **10**, 47–56.
91 L. Klouda, M. C. Hacker, J. D. Kretlow and A. G. Mikos, *Biomaterials*, 2009.
92 J. Kirkham, A. Firth, D. Vernals, N. Boden, C. Robinson, R. C. Shore, S. J. Brookes and A. Aggeli, *J. Dent. Res.*, 2007, **86**, 426–430.
93 Y. Zhang, H. H. Xu, S. Takagi and L. C. Chow, *J. Mater. Sci.: Mater. Med.*, 2006, **17**, 437–445.

94 M. H. Cho, K. S. Kim, H. H. Ahn, M. S. Kim, S. H. Kim, G. Khang, B. Lee and H. B. Lee, *Tissue Eng. A*, 2008.
95 V. Crescenzi, L. Cornelio, C. Di Meo, S. Nardecchia and R. Lamanna, *Biomacromolecules*, 2007, **8**, 1844–1850.
96 M. S. Kim, S. K. Kim, S. H. Kim, H. Hyun, G. Khang and H. B. Lee, *Tissue Eng.*, 2006, **12**, 2863–2873.
97 E. Ruel-Gariepy, A. Chenite, C. Chaput, S. Guirguis and J. Leroux, *Int. J. Pharm.*, 2000, **203**, 89–98.
98 I. S. Kim, J. W. Park, I. C. Kwon, B. S. Baik and B. C. Cho, *Plast. Reconstr. Surg.*, 2002, **109**, 1966–1977.
99 J. K. Leach and D. J. Mooney, *Expert Opin. Biol. Ther.*, 2004, **4**, 1015–1027.
100 P. Habibovic and K. de Groot, *J. Tissue Eng. Regener. Med.*, 2007, **1**, 25–32.
101 T. Albrektsson and C. Johansson, *Eur. Spine J.*, 2001, **10**(Suppl. 2), S96–101.
102 J. D. Kretlow and A. G. Mikos, *Tissue Eng.*, 2007, **13**, 927–938.
103 V. P. Shastri, P. Hildgen and R. Langer, *Biomaterials*, 2003, **24**, 3133–3137.
104 G. Chan and D. J. Mooney, *Trends Biotechnol.*, 2008, **26**, 382–392.
105 A. P. Marques, R. L. Reis and J. A. Hunt, *J. Mater. Sci.: Mater. Med.*, 2003, **14**, 167–173.
106 A. P. Marques, R. L. Reis and J. A. Hunt, *J. Mater. Sci.: Mater. Med.*, 2005, **16**, 1029–1043.
107 A. P. Marques, R. L. Reis and J. A. Hunt, *Macromol. Biosci.*, 2005, **5**, 775–785.
108 D. F. Williams and S. P. Zhong, *Int. Biodeterior. Biodegrad.*, 1994, **34**, 95–130.
109 J. A. Hubbell, *Curr. Opin. Biotechnol.*, 1999, **10**, 123–129.
110 P. X. Ma, *Adv. Drug Delivery Rev.*, 2008, **60**, 184–198.
111 M. J. Mondrinos, R. Dembzynski, L. Lu, V. K. Byrapogu, D. M. Wootton, P. I. Lelkes and J. Zhou, *Biomaterials*, 2006, **27**, 4399–4408.
112 D. A. Wahl, E. Sachlos, C. Liu and J. T. Czernuszka, *J. Mater. Sci.: Mater. Med.*, 2007, **18**, 201–209.
113 F. Barrere, C. A. van Blitterswijk and K. de Groot, *Int. J. Nanomed.*, 2006, **1**, 317–332.
114 Y. Abe, T. Kokubo and T. Yamamuro, *J. Mater. Sci.: Mater.Med.*, 1990, **1**, 233–238.
115 R. L. Reis, A. M. Cunha, M. H. Fernandes and R. N. Correia, *J. Mater. Sci.: Mater. Med.*, 1997, **8**, 897–905.
116 K. Tuzlakoglu and R. L. Reis, *J. Mater. Sci.: Mater. Med.*, 2007, **18**, 1279–1286.
117 I. B. Leonor and R. L. Reis, *J. Mater. Sci.: Mater. Med.*, 2003, **14**, 435–441.
118 A. L. Oliveira, P. B. Malafaya and R. L. Reis, *Biomaterials*, 2003, **24**, 2575–2584.
119 C. N. Cornell, *Orthop. Clin. North Am.*, 1999, **30**, 591–598.
120 H. S. Azevedo, I. B. Leonor, C. M. Alves and R. L. Reis, *Mater. Sci. Eng. C*, 2005, **25**, 169–179.
121 I. B. Leonor, C. M. Alves, H. S. Azevedo and R. L. Reis, *Mater. Sci. Eng., C*, 2009, **29**, 913–918.
122 Y. Liu, K. de Groot and E. B. Hunziker, *Bone*, 2005, **36**, 745–757.
123 Y. Liu, E. B. Hunziker, P. Layrolle, J. D. De Bruijn and K. De Groot, *Tissue Eng.*, 2004, **10**, 101–108.
124 Y. Liu, E. B. Hunziker, N. X. Randall, K. de Groot and P. Layrolle, *Biomaterials*, 2003, **24**, 65–70.
125 Y. Liu, P. Layrolle, J. de Bruijn, C. van Blitterswijk and K. de Groot, *J. Biomed. Mater. Res.*, 2001, **57**, 327–335.
126 Y. Liu, J. P. Li, E. B. Hunziker and K. de Groot, *Philos. Trans. R. Soc. London, Ser. A*, 2006, **364**, 233–248.
127 A. M. Martins, R. L. Pereira, I. B. Leonor, H. S. Azevedo and R. L. Reis, *Acta Biomater.*, 2009, **5**, 3328–3336.
128 S. Maeno, Y. Niki, H. Matsumoto, H. Morioka, T. Yatabe, A. Funayama, Y. Toyama, T. Taguchi and J. Tanaka, *Biomaterials*, 2005, **26**, 4847–4855.