PAPER

pH-Responsive biomineralization onto chitosan grafted biodegradable substrates

Catarina I. Dias,^{ab} João F. Mano^{*ab} and Natália M. Alves^{ab}

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Bioactive composites that enable the formation of an apatite layer onto the surface are important in the development of osteoconductive biomaterials in orthopaedic applications. In this work, the surface of biodegradable and bioactive substrates, composed of poly(L-lactic acid) (PLLA) reinforced with Bioglass[®], was modified by coupling chitosan to the surface, using plasma activation. The wettability of the modified films was analysed by contact angle (CA) measurements as a function of pH. It was found that this surface property changed significantly with pH. Moreover, the apatite formation on the surface upon immersion of the modified films in a simulated body fluid (SBF) solution was analysed at pH 5.4 and pH 7.4 by scanning electron microscopy/energy dispersive spectroscopy (SEM/EDS). It was found that such modification, together with the effect of pH, could block the formation of apatite onto the biodegradable substrate upon immersion in a simulated body fluid solution when the pH changed to 5.4. On the other hand, a dense apatite layer was formed at pH 7.4. For the unmodified substrates an apatite layer was formed at both pHs. These results suggest that the formation of apatite or possibly other kinds of minerals could be controlled by such a smart surface, in this case pH-responsive.

Introduction

Biomineralization is the process of forming minerals in organisms, where minerals nucleate and grow from a supersaturated aqueous solution under the mediation of an organic matrix secreted by the cells.¹ There has been an effort to mimic the mechanisms used by distinct living organisms to produce highperformance minerals²⁻⁴ especially because they are produced at mild pressures and temperatures and are stable in an aqueous medium, and may find several applications in the biomedical and technological fields.⁵⁻¹⁰

Also, among the stimuli that can be used to control the response of a system, temperature and pH have been extensively studied in the biomedical field because these two factors can be easily controlled and are applicable in both *in vitro* and *in vivo* conditions.^{11–13} In the particular case of pH-responsive polymers the large variations in physiological pH at various body sites in normal and pathological conditions can be used to modulate their behaviour.^{14,15} pH-Responsive surfaces have been developed by immobilizing this type of polymer on different substrates^{16–18} and have been applied to different fields such as drug delivery,¹⁹ tissue engineering²⁰ and separation techniques.^{16,17}

In this work chitosan, a natural and pH-responsive polymer, was grafted onto a biodegradable bioactive composite and the effect of pH on the biomineralization process was investigated. It is shown for the first time that surface biomimetic mineralization may be triggered by a pH change onto a biodegradable substrate. As far as we know, the only work that presented this concept of smart biomineralization was recently published, in this case using temperature as the trigger.²¹

In the development of medical devices for bone-related applications (filling bone defects, fixation of scaffolds for bone tissue engineering) it is important to promote the production of a bone-like mineral layer. This is a calcium phosphate (CaP) mineral, more specifically dahlite (carbonated apatite). Kokubo and Takadama showed that *in vivo* bioactivity of materials could be directly related to the rate at which the material forms apatite *in vitro* when immersed in a simulated body fluid (SBF) solution with ion concentrations nearly equal to those of human plasma.²² This biomimetic apatite coating technique has been successfully used to coat implants and scaffolds.^{23,24}

The pH-responsive surfaces developed in our work were used to simultaneously control and analyse the production of biomimetic apatite with pH. From a more practical point of view, this study could be relevant because when the body suffers any kind of lesion or inflammatory process, the pH of the medium in the affected area decreases from 7.4 (the normal pH of body fluid) to approximately 5.225 and until now, no systematic experimental study was conducted to analyse the effect of this pH change on the formation of biomimetic apatite. Some works that show evidence of the pH effect on CaP formation can be found in the literature.^{26–30} However, the majority of these works are not related to biomimetic apatite. In fact, regarding the formation of apatite in SBF, only a theoretical analysis of CaP formation²⁷ and some works where the pH of supersaturated (5 \times SBF) solutions was decreased with the purpose of accelerating the formation of the mineral layer^{29,30} can be found.

[&]quot;3B's Research Group—Biomaterials, Biodegradables and Biomimetics, Department of Polymer Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. E-mail: jmano@dep.uminho.pt; Fax: (+351)253 510339

^bIBB—Institute for Biotechnology and Bioengineering, Braga, Portugal

Materials and methods

Materials

Poly(L-lactic acid) (PLLA) ($M_n = 69\ 000,\ M_w/M_n = 1.734$) was supplied by Cargil Dow and used as received. A bioactive glass (BG) (45S5 Bioglass[®]) with the following composition: 45 SiO₂, 24.5 CaO, 24.5 Na₂O and 6.0 P₂O₅ in wt%, was supplied by US Biomaterials Corp. (Florida, USA). Chitosan (medium molecular weight), Chloroform (CHCl₃) and acetic acid were purchased from Sigma-Aldrich.

Preparation of PLLA/BG (or PLLA) films

The PLLA/BG films were prepared by solvent-casting in a glass Petri dish. PLLA pellets (200 mg) were dissolved in 20 ml of CHCl₃. BG was dispersed in the above solution with different glass contents (10%, 20%, 30% and 40%). Afterward, the dispersions were poured into a Petri dish (for one PLLA film, the PLLA solution was poured into a Petri dish). The Petri dish was aligned into a horizontal position to facilitate the formation of a cast film with uniform thickness. The assembly was kept in a chemical hood for 24 h, and CHCl₃ was allowed to evaporate at a very slow rate. Then, the films were vacuum dried for 48 h at 40 °C. The average thickness of the film (measured with a micrometer) formed by this procedure was 25 μ m. When the composite films were prepared by this method we found that most of the BG deposited at the bottom surface of the film and this happened for all the BG contents. In most of the cases the gradient in BG concentration was so high that the top side of composite films never showed calcification ability, even when 30% of BG was added. So, in all the experiments and measurements described in this work it was always the same surface of the film (the bottom surface) that was analysed, in order to not compromise our findings by introducing one more variable.

Plasma treatment of PLLA/BG (or PLLA) film surface and plasma coupling of chitosan to the film

The films were activated by plasma for 60 s in argon atmosphere, with a power of 32 W at a frequency of 40 kHz with an argon flux of 40%. A Plasma Prep5 reactor from Gala Instruments was used in this process. After surface activation, the PLLA/BG films were exposed to air for 30 min and a chitosan solution of 1 wt% was added to the pre-treated films. After the reaction, the films were vacuum dried at room temperature for 24 h. The plasma polymerization, *i.e.*, the second plasma treatment, was carried out with the same plasma apparatus and the same conditions used in the first plasma treatment were adopted. Finally the samples were extensively washed with distilled water and dried under vacuum.

Immersion of modified films in a simulated body fluid (SBF) solution

The SBF solution was prepared by dissolving reagent grade NaCl, NaHCO₃, KCl, K₂HPO₄· $3H_2O$, MgCl₂· $6H_2O$, CaCl₂· $2H_2O$ and Na₂SO₄ in distilled water, and buffered with (CH₂OH)₃CNH₂ and HCl to achieve pH 7.4. SBF with

a pH = 5.4 was also prepared by adjusting the pH with HCl (1 M). A corrected SBF composition was used.³¹ The concentration of different ionic species in SBF closely resembles that of human blood plasma. Each film was immersed in 40 ml of SBF for different times (7, 14 and 21 days) at 37 °C and at two distinct pHs: 5.4 and 7.4. Upon removal from SBF, the films were gently rinsed with distilled water and dried overnight at room temperature, and then vacuum dried at 40 °C for 48 h.

Surface analysis

The virgin and modified samples were characterized by Fourier transform infrared spectroscopy (FTIR) to examine the changes in their functional groups. The spectra of the samples were recorded with a Perkin-Elmer 1600 FT-IR spectrometer in the range of 4000-400 cm⁻¹. FTIR was also used to characterize the mineral deposited onto the surface of the films after immersion in SBF. The atomic composition of the virgin and modified samples was measured by X-ray photoelectron spectroscopy (XPS). These measurements were carried out using a VG Escalab 250iXL ESCA instrument (VG Scientific), equipped with monochromatic Al Ka radiation at 1486.92 eV X-ray source. The take-off angle value at XPS measurements was 90° relative to the sample surface. Water contact angle measurements were also performed for the virgin and modified surfaces by the sessile drop method, at the two pHs and at room temperature (23 °C), by using the contact angle system OCA 20 (DataPhysics). Data are expressed as the average of three measurements for each sample. The morphology and composition of the film surface after being immersed in SBF for distinct times and pHs were examined by scanning electron microscopy coupled with energy dispersive spectroscopy (SEM/EDS, Leica Cambridge S360). SEM experiments were performed at an accelerated voltage of 15 kV on previously gold coated films.

Results and discussion

Bioactivity, *i.e.*, the formation of a CaP layer at a biomaterial's surface upon implantation, is a fundamental property for bone–implant bonding.^{32,33} Bioglass[®], BG, in the Na₂O–CaO–SiO₂– P_2O_5 system, is a bioactive glass that was found to spontaneously bond to living bone without forming a fibrous tissue at the interface³⁴ and has been used clinically since 1985. BG may be combined with biocompatible and biodegradable polymers in order to produce bioactive composites.³¹

In this work BG was compounded with poly(L-lactic acid), PLLA, a well known biodegradable and biocompatible polyester, in order to produce composite films with different BG contents. Chitosan was grafted onto the film surface by plasma coupling. Chitosan is a pH-responsive polymer that contains both hydrophobic (-CH₃) and hydrogen bonding favouring groups (-OH, -NH₂ and -C=O). However, in an acidic medium this polymer becomes positively charged due to the protonation of the free amine groups (the p K_a is ~6)³⁵ and polymer–polymer interactions *via* hydrophobic effect and/or hydrogen bonding junctions can be hindered due to electrostatic repulsion.³⁶ So, this polymer is normally insoluble in neutral or basic pH conditions and soluble in acidic pH.³⁷ Polymeric surfaces were already modified with chitosan through plasma

methods³⁸ and in the particular case of PLLA it was as a way to combine its excellent mechanical properties with the known biological properties of chitosan.³⁸ The biocompatibility of chitosan is mainly attributed to its structural similarity to glycosaminoglycans, which are the main components of the extracellular matrix, and therefore this polymer has been used to conduct the extracellular matrix formation in tissue regeneration.³⁹ Moreover this polymer may be chemically modified, for example through grafting, in order to extend the range of its properties.40,41 The changes at the PLLA surface with two consecutive argon plasma treatments, as was used in our work, were analysed in detail by Ding and co-workers.38 They analysed the PLLA films by contact angle and XPS analysis and found that after the first plasma treatment and subsequent exposure to air the film turned more hydrophilic and chemically active by introducing polar groups such as peroxides and other oxygenated species onto the C-C bonds.38 The peroxides and other species formed would subsequently initiate the surface free radical coupling reaction with active chitosan species that are activated by the second plasma treatment.38 More information about the effect of plasma treatments on PLLA films can be found in reference 38.

The modified film surface was characterized by FTIR. Fig. 1 shows a representative FTIR spectrum of the chitosan modified PLLA/BG films. The spectra of chitosan and unmodified PLLA/BG films are also shown as control materials. After modification, the more visible differences are between 3000–3700 cm⁻¹, due to the strong and large adsorption band assigned to the stretching vibrations of O–H and to the peak assigned to the stretching vibrations of N–H (3363 cm⁻¹), both characteristic bands of chitosan.^{42,43} Similar changes were found in the FTIR spectra of PLLA/BG films with different BG contents after modification with chitosan.

XPS analysis was also conducted with unmodified and chitosan-modified composite films. The C1s multiplex scans and survey spectra of both samples are shown in Fig. 2. The spectra in Fig. 2A and B were decomposed into Gaussian peaks by using a conventional curve-fitting procedure: 3 and 4 peaks were obtained for the unmodified and for the modified film,



Fig. 1 FTIR spectra of PLLA composite (20% BG), chitosan and grafted PLLA composite films.

respectively. Fig. 2C shows a clear contribution related to N1s after modification. The new peak at 288 eV for the modified sample is attributed to the amide peak (NH–CO) of chitosan.⁴⁴ Furthermore, the new peak at 400.28 eV observed in the survey spectrum, which is characteristic of nitrogen,⁴⁴ indicated that chitosan coupled to PLLA. The calculated amount of N in the modified film was 4.3%. So, XPS and FTIR results confirm that chitosan was effectively grafted onto the film surface.

The pH-responsive behaviour of the modified films was analysed by contact angle measurements, Fig. 3. The unmodified PLLA and PLLA/BG films revealed a quite hydrophobic character, presenting a water contact angle (WCA) of $\sim 82^{\circ}$, independently of the pH. After modification, the WCA of the films changed from $88.9^{\circ} \pm 4.05^{\circ}$ at pH 7.4 to $67.6^{\circ} \pm 2.3^{\circ}$ at pH 5.4 (Fig. 3C). It should be noted that these contact angle measurements could be significantly affected by several parameters, namely surface roughness and temperature. By looking at the SEM images of Fig. 4 and 5 it is evident that the roughnesses of both original and grafted materials are similar and very low, at least at the micron/submicron length scale. Regarding temperature, in this study all the measurements were conducted at the same controlled temperature (23 °C). Therefore it can be clearly concluded that wettability depends on pH just for the modified samples. This significant change in the WCA is consistent with the increase of the hydrophilic nature of the surface at low pH. In fact at this pH, due to the ionization of the amine groups of chitosan, polymer-water interactions are favourable and the chains exhibit a hydrophilic behaviour and, therefore an extended conformational state. At pH 7.4, the amine groups of chitosan are uncharged and the chains change to a collapsed state. Furthermore, we investigated if the conformational changes below and above the critical pH of chitosan, previously described, could affect the biomineralization of the PLLA/BG composites. The apatite formed on bioactive materials, after being in contact in SBF, exhibits important characteristics of the bone mineral phase. The precipitate consists of nano-crystals of carbonate ion containing apatite that has a defective structure and low crystallinity.³³ Fig. 4 shows representative SEM pictures probing the apatite formation for the unmodified 30% and 40% composites after soaking in SBF during different time periods at pH 5.4 and pH 7.4. For the composite with 30% of BG it can be seen that, independently of pH, after 7 days no calcification occurred (Fig. 4A1 and A2), whereas a partially mineralized surface was always detected after 3 weeks (Fig. 4B1 and B2). Regarding PLLA with 40% of BG, this quantity is high enough to enable calcification to occur at pH 7.4 after only one week (Fig. 4C2) and even at pH 5.4 (Fig. 4D1) after 14 days.

Fig. 5 shows that for the chitosan modified composite films with 30% BG no apatite formation could be observed at pH 5.4 either after 1 or 3 weeks (Fig. 5A1 and B1). At pH 7.4 and even after only one week the treated film could form dense precipitates with the typical cauliflower morphology, containing needle-like nanometric structures, characteristic of biomimetic-formed apatite (Fig. 5A2 and B2). The images corresponding to the modified PLLA with 40% BG also suggest that even with such a high quantity of BG the chitosan chains are effective in blocking the apatite formation at pH 5.4 for all the immersion times studied (Fig. 5C1 and D1).



Fig. 2 XPS C1s multiplex scans of the A) unmodified and B) chitosan-modified films. C) XPS survey spectra for the unmodified and modified film. The XPS results refer to PLLA with 15% BG. The modified films were obtained after a grafting time of 60 s.



Fig. 3 Micrographs of: virgin PLLA, unmodified composites and chitosan modified composites at different pHs.

For the films with 20% of BG the obtained results were similar to the ones found for the sample with 30% (not shown). For both unmodified and modified PLLA reinforced with 10% of BG, no apatite formation could be detected at both pH (not shown), *i.e.*, for composites with BG contents less than 20% no calcification occurred at both pH, even after 21 days of immersion in SBF.

By looking at Fig. 4 and 5 it can also be said that a denser apatite layer was typically formed with increasing immersion time for the unmodified composites, as expected. The same result was obtained for the modified composites at pH 7.4. On the other hand, an apatite layer was never formed at pH 5.4, even after 21 days, for the modified composites.

The presence of calcium and phosphate at the surface of these coated samples was confirmed by EDS (Fig. 6). The TF-XRD profile of the coating formed at 37 °C and pH 7.4 is also shown in Fig. 6. Besides the diffraction peaks at $2\theta = 16.7^{\circ}$ and $2\theta = 19.2^{\circ}$, which are characteristic of the PLLA substrate, the characteristic peaks of hydroxyapatite, $2\theta = 32^{\circ}$ (overlapping of (211), (112) and (300) diffraction peaks) and $2\theta = 22.9^{\circ}$ ((111) diffraction peak) are detected. The small intensity of the TF-XRD peaks indicates a partially crystalline structure, and their broadness is indicative of small crystalline structures, similar to the ones found in bone. The TF-XRD was poorly resolved, mainly due to the low crystallinity of the coating, and more data would be necessary to fully elucidate the nature



 δ0 μm
 A1

 50 μm
 B1

 50 μm
 C1

 50 μm
 C1

 50 μm
 D1

 50 μm
 D1

рН 5.4

pH 7.4

Fig. 4 SEM images of unmodified composites immersed in SBF for different periods of time at two distinct pHs. Sample with 30% BG: 7 days, A1 and A2; 21 days, B1 and B2; sample with 40% of BG: 7 days, C1 and C2; 14 days, D1 and D2.

of the CaP formed. Thus, the coating was also characterized by FTIR; see Fig. 7. The spectrum exhibited the characteristic peaks of carbonated apatite: the phosphate absorption bands at 1190–1020 cm⁻¹ (ν_3 band), at 960 cm⁻¹ (ν_2 band) and at 606 and 565 cm⁻¹ (ν_4); the absorption peaks corresponding to the carbonate groups were also detected, namely at 878 cm⁻¹ (ν_2 band) and a broad absorption at 1650–1300 cm⁻¹ (ν_3); the broad peak at 3700–3300 cm⁻¹ is mainly due to the stretching

Fig. 5 SEM images of modified composites immersed in SBF for different periods of time at two distinct pHs. Sample with 30% BG: 7 days, A1 and A2; 21 days, B1 and B2; sample with 40% of BG: 7 days, C1 and C2; 14 days, D1 and D2.

of absorbed water. These results are in agreement with the EDS and TF-XRD data, confirming the formation of carbonated apatite mineral similar to the major mineral component of vertebrate bone tissue.

It is known that pH may influence the precipitation of apatite onto the surface of bioactive substrates. Lu and Leng analysed the CaP formation in SBF based on classical crystallization theories of thermodynamics and kinetics and found that



Fig. 6 EDS (left) and TF-XRD (right) characterization of the CaP layer of the composite film modified with chitosan and soaked in SBF for two weeks.



Fig. 7 Representative FTIR spectrum of the CaP coating formed on the modified composite at pH 7.4.

hydroxyapatite precipitation is thermodynamically favourable only when $pH \ge 5.4$ and that the nucleation rate is significantly affected by the pH value.²⁷ They theoretically showed that a higher pH (in our case pH 7.4) is more favourable for HA nucleation and that the nucleation rate is higher at pH 7.4 than at pH 5.4.²⁷ The experimental results obtained in this work also confirm this feature. For instance, for PLLA with 30% BG and at pH 5.4 an apatite layer was only observed after 3 weeks, whereas a dense apatite layer was formed after just 1 week when the pH changed to 7.4.

However, just the pH effect alone cannot explain the results found in the chitosan modified composite films. In fact, taking again PLLA with 30% BG as a representative example, an apatite layer is formed in the unmodified composites at both pHs after 3 weeks, whereas after one week no apatite formation is observed for these materials. Therefore, the different conformational states of the chitosan chains at the PLLA surface could also have an important influence in blocking the apatite formation at pH 5.4, even for times as long as 3 weeks. For pH-responsive polymers, the transition from a collapsed to an expanded state is explained by the osmotic pressure exerted by mobile counterions neutralizing the network charges.45 This kind of conformational change related to phase transitions of responsive polymers and the formation of globular structures with nanometric sizes in the collapsed state was already shown by AFM for a pH- and temperature-responsive copolymer of poly(N-isopropylacrylamide) and acrylic acid.46

Such conformational changes with pH lead to a significant change in the topography of the surface. Several works revealed that the increase of surface roughness can enhance the formation and stability of a CaP coating on bioactive substrates,^{47–50} probably due to an increase of nucleation sites on rougher surfaces.⁴⁷ So, the different capability for apatite formation of the modified films at the two studied pHs could be explained by the different surface topography. The results found in this work are in agreement with the behaviour found by Shi *et al.*²¹ In that study the ability to form apatite was also more pronounced in hydrophobic surfaces resulting from the collapse of grafted polymeric chains.

It should be pointed that plasma modification could alter the surface permeability and the corresponding dissolution rate of the ions from the BG particles in the composite. However, in a previous work it was investigated whether the plasma treatment alone has some effect on the ability for apatite formation with the same composites, and it was confirmed that this process was not able to promote calcification after the used immersion times in SBF.²¹ Also, the bioactivity of chitosan grafted PLLA films was investigated at both pHs but in this case no apatite was formed (not shown). This indicates that the conformational changes of chitosan chains do not promote by themselves the mineralization, *i.e.*, for apatite formation the release of phosphate and calcium ions from the BG particles into the solution is also necessary. It was shown before that chitosan can be modified, for example through combination with siloxane, in order to exhibit bioactive characteristics.⁵¹

The results presented in this work show that the smart nature of this biodegradable surface can switch the ability for apatite formation depending on the pH change. This concept of pH-responsive surface could be, in principle, used in the future to control the deposition of other minerals besides apatite.

Conclusions

In this work smart biodegradable surfaces that respond to pH and that could be used to control the biomineralization process were successfully developed. These surfaces were prepared by grafting chitosan, a pH-sensitive biodegradable and natural polymer, to the surface of PLLA/BG films. It was found that the formation of biomimetic apatite was dependent on the conformational changes of chitosan across its critical pH and, hence, can be controlled by pH switching.

This work suggests that the used methodology could be applied to develop smart coatings of different minerals. Moreover, these coatings could be produced in substrates with more complex geometries, by using other methodologies (*e.g.* chemical activation of the substrate surface) instead of plasma for coupling chitosan to the surface.

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References

- 1 S. Weiner and C. R. Crit, Rev. Biochem., 1986, 20, 365.
- 2 L. Addadi and S. Weiner, Angew. Chem., Int. Ed. Engl., 1992, 31, 153.
- 3 S. Weiner and L. Addado, J. Mater. Sci., 1997, 7, 689.
- 4 S. Mann, Nature, 1993, 365, 499.
- 5 G. F. Xu, N. Yao, I. A. Aksay and J. T. Groves, J. Am. Chem. Soc., 1998, **120**, 11977.
- 6 Z. Y. Tang, N. A. Kotov, S. Mogonov and B. Ozturk, Nat. Mater., 2003, 2, 413.
- 7 A. L. Oliveira, J. F. Mano and R. L. Reis, Curr. Opin. Solid State Mater. Sci., 2003, 7, 309.
- 8 Y. J. Han and J. Aizenberg, Adv. Mater., 2004, 16, 1295.
- Y. Gao and K. Koumoto, *Cryst. Growth Des.*, 2005, 5, 1983.
 F. Nudelman, B. A. Gotly, L. Addadi and S. Weiner, *J. Struct. Biol.*, 2006, 153, 176.
- 11 M. Matsusaki and M. Akashi, Biomacromolecules, 2005, 6, 3351.
- 12 C.-L. Lo, K.-M. Lin and G.-H. Aslue, J. Controlled Release, 2005, 104, 477.
- 13 H. K. Ju, S. Y. Kim and Y. M. Lee, Polymer, 2001, 42, 6851.

- 14 P. Gupta, K. Vermani and S. Garg, Drug Delivery Today, 2002, 7, 569.
- 15 X. Qu, A. Wirsen and A. C. Albertsson, Polymer, 2000, 41, 4589.
- 16 H. Iwata, I. Hirata and Y. Ikada, Macromolecules, 1998, 31, 3671.
- 17 L. Ying, P. Wang, E. T. Kang and K. G. Neoh, *Macromolecules*, 2002, **35**, 673.
- 18 D. W. Jenkins and S. M. Hudson, Chem. Rev., 2001, 101, 3245.
- 19 D. Schmaljohann, Adv. Drug Delivery Rev., 2006, 58, 1655.
- 20 C. Alexander and K. M. Shakesheff, *Adv. Mater.*, 2006, **18**, 3321. 21 J. Shi, N. M. Alves and J. F. Mano, *Adv. Funct. Mater.*, 2007, **17**,
- 3312.
- 22 T. Kokubo and H. Takadama, Biomaterials, 2006, 27, 2907.
- 23 A. L. Oliveira, I. B. Leonor, C. Elvira, M. C. Azevedo, I. Pashkuleva and R. L. Reis, *Surface treatments and pre-calcification routes to enhance cell adhesion and proliferation*, NATO/ASI series, ed. R. L. Reis and D. Cohn, Kluwer, Drodercht, 2002.
- 24 I. B. Leonor, H. M. Kim, F. Balas, M. Kawashita, R. L. Reis, T. Kokubo and T. Nakamura, J. Mater. Chem., 2007, 17, 4057.
- 25 E. Y. Kawachi, C. A. Bertran and L. T. Kubota, *Biomaterials*, 1998, 19, 2329.
- 26 X.-D. Sun, Y.-L. Zhou, J. Y. Ren, F.-Z. Cui and H.-D. Li, Curr. Appl. Phys., 2007, 7S1, e75.
- 27 X. Lu and Y. Leng, Biomaterials, 2005, 26, 1097-1108.
- 28 C. S. Liu, W. Huang, W. Shen and J. H. Cui, *Biomaterials*, 2001, 22, 301.
- 29 Y.-F. Chou, W.-A. Chiou, Y. Xu, J. C. Y. Dunn and B. M. Wu, *Biomaterials*, 2004, 25, 5323–5331.
- 30 F. Barrere, C. A. Blitterswijk, K. Groot and P. Layrolle, *Biomaterials*, 2002, 23, 1921.
- 31 K. Rezwan, Q. A. Chen, J. J. Blaker and A. R. Boccaccini, *Biomaterials*, 2006, 27, 3413.
- 32 L. L. Hench, J. Am. Ceram. Soc., 1991, 74, 1487.
- 33 T. Kokubo, H.-M. Kim and M. Kawashita, *Biomaterials*, 2003, 24, 2161.

- 34 L. L. Hench, R. J. Splinter, W. C. Allen and T. K. Greenlee, J. Biomed. Mater. Res., 1972, 2, 117.
- 35 A. Denuziere, D. Ferriera and A. Domard, *Carbohydr. Polym.*, 1996, 29, 317.
- 36 J. Cho, M.-C. Heuzey, A. Bégin and P. J. Carreau, *Carbohydr. Polym.*, 2006, 63, 507.
- 37 S. V. Madihally and H. W. T. Matthew, *Biomaterials*, 1999, **20**, 1133.
- 38 Z. Ding, J. Chen, S. Gao, J. Changa, J. Zhang and E. T. Kang, *Biomaterials*, 2004, 25, 1059.
- 39 A. Lahiji, A. Sohrabi, D. S. Hungerford and C. G. Frondoza, J. Biomed. Mater. Res., 2000, 51, 586.
- 40 R. Jayakumar, M. Prabaharan, R. L. Reis and J. F. Mano, Carbohydr. Polym., 2005, 62, 142.
- 41 M. Prabarahan and J. F. Mano, Macromol. Biosci., 2006, 6, 991.
- 42 A. Sionkowska, H. Kaczmarek, M. Wisniewski, J. Skopinska, S. Lazare and V. Tokarev, *Surf. Sci.*, 2006, 600, 3775.
- 43 Z. Ma, C. Gao, Y. Gong, J. Ji and J. Shen, J. Biomed. Mater. Res., 2002, 63, 838.
- 44 A. Zhu, M. Zhang, J. Wu and J. Shen, *Biomaterials*, 2002, 23, 4657–4665.
- 45 S. R. Tongue and B. J. Tighe, Adv. Drug Delivery Rev., 2001, 53, 109–122.
- 46 H. M. Zareie, E. V. Bulmus, A. P. Gunning, A. S. Hoffman, E. Piskin and V. J. Morris, *Polymer*, 2000, **41**, 6723.
- 47 F. Barrere, M. M. E. Snem, C. A. van Blitterswijk, K. de Groot and P. Layrolle, *Biomaterials*, 2004, 25, 2901.
- 48 J. Peltonen, M. Järn, S. Areva, M. Linden and J. B. Rosenholm, Langmuir, 2004, 20, 9428.
- 49 M. Järn, S. Areva, V. Pore, J. Peltonen and M. Linden, *Langmuir*, 2006, 22, 8209.
- 50 Q. Z. Chen, K. Rezwan, D. Armitage, S. N. Nazhat and A. R. Boccaccini, J. Mater. Sci.: Mater. Med., 2006, 17, 979.
- 51 S. S. Silva, R. A. S. Ferreira, L. Fu, L. D. Carlos, J. F. Mano, R. L. Reis and J. Rocha, J. Mater. Chem., 2005, 15, 3952.