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Biomimetic coating on bioactive glass-derived scaffolds mimicking bone tissue

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Abstract: Bioceramic "shell" scaffolds, with a morphology resembling the cancellous bone microstructure, have been recently obtained by means of a new protocol, developed with the aim to overcome the limits of the conventional foam replication technique. Because of their original microstructure, the new samples combine high porosity, permeability, and manageability. In this study, for the first time, the novel bioactive glass shell scaffolds are provided with a gelatin-based biomimetic coating to realize hybrid implants which mimic the complex morphology and structure of bone tissue. Moreover, the presence of the coating completely preserves the *in vitro* bioactivity of the bioactive glass samples, whose surfaces are converted into hydroxyapatite after a few days of immersion in a simulated body fluid solution (SBF). © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 100A: 3259–3266, 2012.

Key Words: bone tissue engineering, scaffold, genipin, gelatin, bioactive glass

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INTRODUCTION

A critical step in the framework of bone tissue engineering is the design of porous structures, that is "scaffolds," able to guide and promote the tissue regeneration process.[1,2] The main objective is to reproduce the architecture of natural bone, which is characterized by an outer compact shell (cortical bone) providing mechanical support to the internal spongy bone, also referred to as cancellous or trabecular bone.[3] Acting as an artificial extracellular matrix, scaffolds should support cells and release signalling molecules to activate specific pathways, which control the pattern and the extent of bone formation. Moreover, scaffolds provide cells with an adequate mechanical stability prior to synthesis of new extracellular matrix by cells themselves and, at least ideally, scaffolds are resorbed at rates appropriate to tissue regeneration.[4–6] Unfortunately, most materials are not bioresorbable and mechanically strong at the same time, in particular when they are employed to realize highly porous networks. For example, scaffolds for bone tissue regeneration and repair should have an open and interconnected porosity of at least 80%, with a pore size ranging between 100 and 500 μm, to ensure an adequate cell infiltration, vascularization, and transport of nutrients toward the cells.[5] Such a high porosity is likely to limit the mechanical stability of scaffolds.

On the other hand, scaffolds for bone regeneration should mimic not only the bone morphology, but also its structure and function. In fact, natural bone is composed of large amounts of biological hydroxyapatite, collagen (17–20 wt %) and other components (water, proteins, etc).[7] Synthetic hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$), which has been used for clinical applications since the 1980s, is considered the structural template for the mineral phase of bone, although—in contrast to stoichiometric HA (Ca/P molar ratio ~1.67)—biological hydroxyapatites are usually calcium-deficient (Ca/P molar ratio < 1.67) and substituted (i.e., Mg^{++}, Cl^-, Na^+, etc…, can substitute Ca^{2+} ions, while PO_4^{3-} groups are primarily substituted by CO_3^{2-}).[8]

Among ceramics for bone tissue engineering, bioactive glasses[9,10] offer remarkable advantages as they are able to develop a stable chemical bond with the surrounding bone tissue once they are implanted into the body, through the formation of a HA surface layer. In particular, the so-called 45S5 Bioglass® (45 wt % SiO_2; 24.5 wt % CaO; 24.5 wt % Na_2O; 6 wt % P_2O_5), which is able to bond to hard and soft connective tissues, has had a long history of applications in many biomedical implants since it was first introduced by Hench and coworkers.[11–13]

Highly porous bioceramic scaffolds, with a morphology resembling the microstructure of cancellous bone, have been recently obtained by means of a new protocol, aiming to overcome the limits of the conventional foam replication technique,[14,15] which is widely used notwithstanding the

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final samples are difficult to be handled due to their brittleness. Briefly, in the classical replication method,[16] a polymer foam resembling the cancellous bone microstructure is first infiltrated with a ceramic slurry and then it is thermally removed during the sintering of the ceramic phase. In this way, the final structure will correspond to a replica of the initial polymeric sponge. Although these systems look rather promising as a possible alternative to allografts and autografts, which are presently the best treatments for bone substitution,[17] bioceramic scaffolds obtained by the replication method are very breakable and scarcely manageable, especially due to their brittle surface,[18] which makes it difficult to employ them both *in vitro* and *in vivo*. On the contrary, the samples obtained by means of the new protocol, named "shell scaffolds," combine a high internal porosity (average total porosity of at least 80 vol %) and an external resistant surface, which is able to ensure both an excellent permeability to fluids and an adequate mechanical strength.[14] In fact, shell scaffolds possess suitable mechanical properties for bone regeneration, as discussed in a recent work specifically focused on the mechanical behavior of the new scaffolds.[15] In particular, the positive effect of the external shell on the scaffolds mechanical strength results in a higher compressive strength (i.e., 0.3–0.8 MPa) for the new samples[15] compared to standard scaffolds possessing the same porosity and obtained by means of the traditional replication technique.[19] It should be noted that the mechanical strength of shell scaffolds falls in the range usually reported for cancellous bone.[20] As a further step, gelatin-modified composite systems could be considered to tune the properties of the new shell scaffolds. Generally speaking, composites based on bioceramics and natural polymers[21–23] have received increasing attention in bone tissue engineering applications, due to their ability to preserve the structural and biological functions of the damaged hard tissues in a biomimetic way. Gelatin[24–26] is a nonexpensive and commercially available biomaterial that has gained interest in biomedical engineering, mainly because of its biodegradability. Gelatin is obtained by thermal denaturation or physical and chemical degradation of collagen, the most widespread protein in the body occurring in most connective tissues as skin, tendon and bone. With respect to collagen, gelatin does not express antigenicity in physiological conditions, it is completely resorbable *in vivo* and its physicochemical properties can be suitably modulated.[23] Because biodegradable polymers may be rapidly reabsorbed *in vivo*, crosslinking strategies have been applied to enhance gelatin resistance *in vivo* and to improve its mechanical properties. Much interest has been recently addressed towards enzymatically or naturally derived crosslinking agents, with a low toxicity. Genipin,[27,28] the aglycone of geniposide (an iridoid glucoside isolated from the fruits of Genipa Americana and Gardenia jasminoides Ellis) has been shown to possess crosslinking activity toward amino-containing materials. Genipin has been recently used as a crosslinking agent of gelatin microcapsules for drug delivery, of gelatin conduits for peripheral nerve regeneration[29–31] and of composite films for Guided Bone Regeneration.[32] This natural crosslinking reagent has been reported to be less toxic than glutaraldehyde and ideal for clinical usage.[33,34] In this work, for the first time, the novel bioactive glass-derived shell scaffolds are coated by gelatin to obtain a biomimetic porous scaffold to reproduce carefully the complex morphology of bone, which is composed by natural apatite and collagen. Gelatin release tests have been performed to investigate at best the coating dissolution. Moreover, the effect of the biomimetic coating on the *in vitro* bioactivity of the samples has been addressed.

MATERIALS AND METHODS

Scaffold fabrication

The protocol to prepare glass-ceramic shell scaffolds has been recently described in detail[14,15] and it is just summarized here. Briefly, a polyurethane sponge was employed as an organic template to provide the final sample with the desired shape and basic porosity. 45S5 Bioglass® powder was sieved under 45 μm ($d_{0.5} = 12.6$ μm as confirmed by a laser granulometer analysis), and used to produce a slurry. A polyvinylic binder was added to improve the slurry adhesion to the polyurethane sponge and a polyethylene powder was introduced as an additional porogen. Several tests were performed to achieve a proper balance between the viscosity of the slurry and the amount of porogen added and to ensure an adequate adhesion between the sponge structures and the glass and porogen particles. The following recipe was selected: 51 wt % distilled water, 34 wt % 45S5 Bioglass®, 5 wt % polyethylene powder (particle size between 90 and 150 μm) and 10 wt % polyvinylic binder were mixed in a beaker under magnetic stirring for 30 min. The sponge blocks were dipped into the glass slurry, impregnated, and retrieved without squeezing, unlike the standard replication method (SRM).[16] Then the samples, fully loaded with the slurry, were dried using a multidirectional air flux at 150°C for 5 min. It should be noted that the impregnation procedure is completely different in the SRM, where the green bodies, usually squeezed before drying to remove the exceeding slurry, are dried very slowly (i.e., for 24 h). Moreover, the addition of polyethylene particles to the slurry is completely new; it aims to increase further the basic porosity of the samples which derives from the polyurethane sponge architecture, in particular at their surfaces. Finally, the samples were heat-treated at 1050°C for 3 h to consolidate the glass structure and to eliminate the organic phase. The heating rate was 5°C min^{-1} up to 500°C and subsequently 10°C min^{-1} up to the maximum temperature. At the end of the thermal treatment, the samples were extracted from the furnace and let to cool down naturally.

Surface coating of gelatin on bioactive glass-based scaffolds

The sintered porous scaffolds were deaerated and infiltrated with 5% w/v gelatin solution (Type A gelatin from porcine skin, Sigma–Aldrich, Italy). After drying in an air-circulating oven at 37°C, each coated scaffold was immersed into 10 mL of 5% w/w genipin (GP) solution (Challenge Bioproducts, Taiwan) for 24 h to obtain a stable crosslinked gelatin

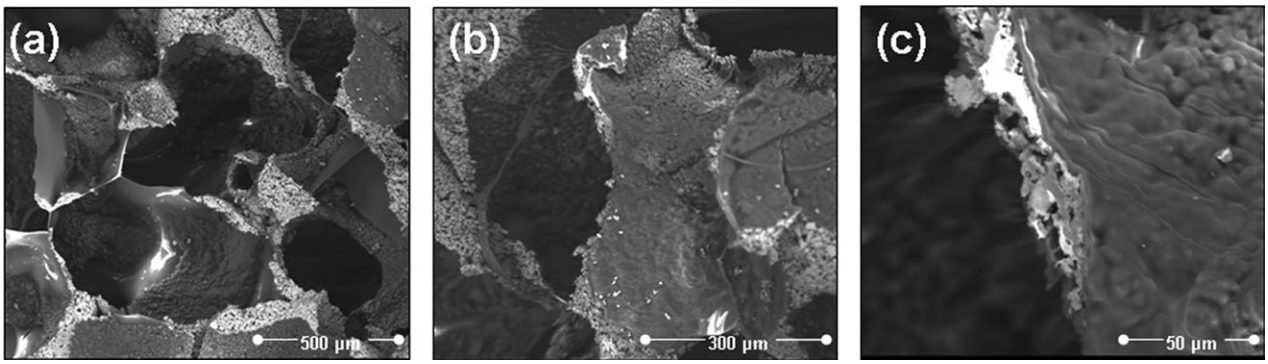

FIGURE 1. Micrographs of the shell scaffold surface before soaking in SBF at different magnification degrees.

coating. According to previous studies,[27,28] genipin was chosen to confirm the gelatin crosslinking, thanks to the blue coloring obtained by spontaneous reaction of genipin with amino acids and proteins, containing amino groups.

Fourier transform infrared-attenuated total reflectance spectroscopy (FTIR-ATR)

Chemical analysis of uncoated and crosslinked-gelatin-coated scaffolds was performed by ATR-FTIR spectroscopy over a range of 4000–400 cm^{-1} using a Perkin Elmer equipment (resolution 2 cm^{-1}; 16 scans).

Gelatin release tests

Weighed amounts of gelatin-coated scaffold were stored in closed plastic flasks containing 20 mL of phosphate buffer saline (PBS); specimens were and left in incubation at 37°C in a controlled environment chamber at 90% humidity (Control AG System, Fratelli Galli, Italy). After fixed time intervals (3, 5, and 7 days), 100 μL of the release liquid was withdrawn and diluted with distilled water at a final volume of 10 mL. Then, 1 mL of the solution was utilized for bicinchoninic acid assay test (BCA). Protein concentration of the release solutions was determined with BCA kit (Sigma–Aldrich, Italy), using bovine serum albumin (provided with the BCA kit) for calibration. Briefly, 1 mL of the bicinchoninic acid reagents was added to 1 mL of the diluted release liquid and, after 1-h incubation at 60°C, UV absorbance of the solution was measured under an UV-2100 recording spectrophotometer (Shimadzu; resolution 1 nm) at 560 nm wavelength. All measurements were carried out using standard 1-cm-thick quartz cells, at room temperature. Three measurements were carried out at each time and average results and standard deviations were reported.

Microstructural characterization

The surface morphology of the obtained scaffolds was observed with a scanning electron microscope, SEM (ESEM Quanta 2000, FEI, Eindhoven, The Netherlands), after gold coating. The microscope was operated in low-vacuum mode, with a pressure of 0.5 Torr. Chemical analyses were performed by means of X-ray Energy Dispersion Spectroscopy, EDS (Inca, Oxford Instruments, UK).

Assessment of bioactivity in simulated body fluid

The *in vitro* reactivity of the scaffolds was tested in a Simulated Body Fluid solution according to the procedure developed by Kokubo et al. and accurately described in Ref. [35]; SBF contains similar ion concentrations to those in human blood plasma.[35] The soaking tests were conducted in closed plastic flasks with reaction time periods of 3, 7, and 14 days, since bone-bonding materials usually form apatite *in vitro* on their surfaces within 4 weeks.[35] The flasks were held at a constant temperature of 37°C and 90% humidity in a controlled environmental chamber (MPM Instruments S.R.L., Bernareggio, Mi, Italy). The SBF was refreshed every 48 h. The soaked samples were withdrawn from the test solution, rinsed with distilled water and left to dry at room temperature. Subsequently, their surface was investigated by means of SEM and Raman spectroscopy. The precipitation of HA, in fact, can be further confirmed by means of this technique because of the high intensity of the Raman peaks of the P–O vibration modes.[36] A Jobin-Yvon Raman Microscope (Horiba Jobin-Yvon, Langjumeau, France) with a 632.8 nm diode laser emitting, an output power of 20 mW at the sample and a 5 μm resolution was employed. Light scattered by the samples was dispersed by a 1800 lines/mm grating monochromator and collected on a CCD camera. The 200–1200 cm^{-1} wave numbers range was investigated; the collection optic was set at 100× ULWD objective, and a spectrum collection setup of 15 acquisitions, each of them taking 60 s, was used.

RESULTS AND DISCUSSION

Shell scaffolds characterization

On the basis of the excellent preliminary results obtained by the morphological and mechanical characterization of shell scaffolds,[14,15] the next step, which is presented and discussed here for the first time, was to obtain new gelatin-coated hybrid samples, with the aim to mimic the complex bone morphology and its structure. Figure 1 reports some micrographs of the shell scaffold internal structure after coating with gelatin. A uniform gelatin layer coats the internal surface of pores and their struts. In particular, the gelatin adheres to the walls of pores without clogging them, so that the porosity remains open and fully interconnected. The treatment with genipin, which produced a colored

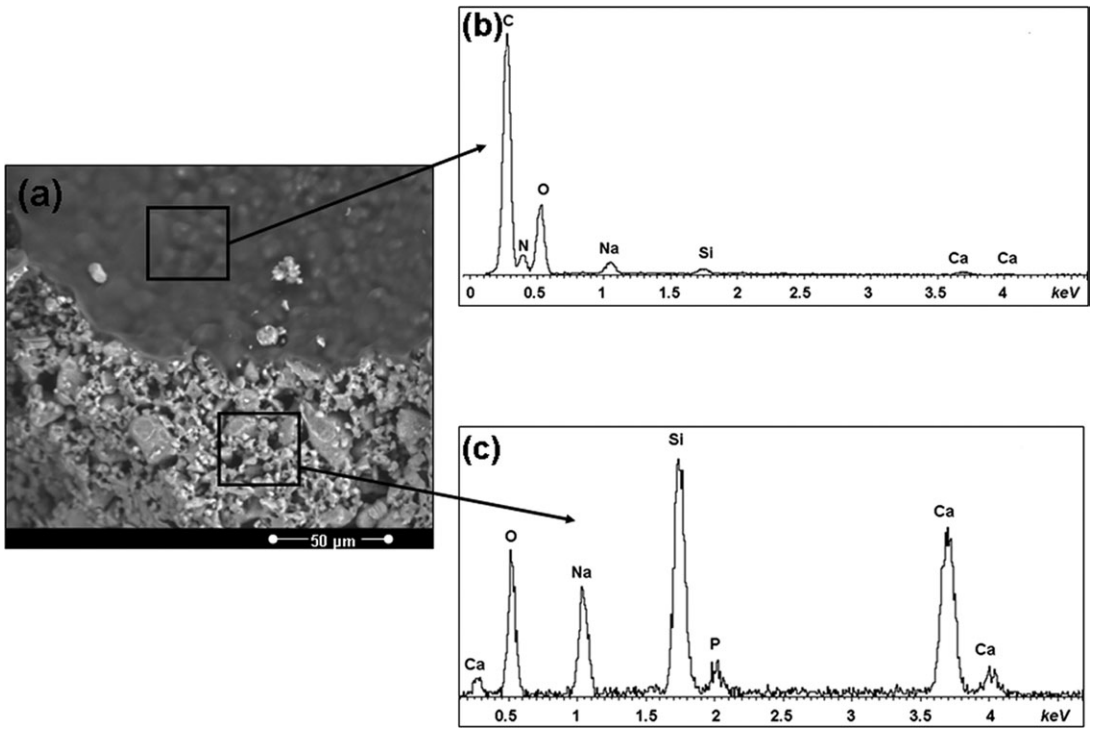

FIGURE 2. (a) Micrograph of the section a pore wall before soaking in SBF and (b,c) results of the EDS analysis performed on the areas reported in (a).

crosslinkage of gelatin, clearly confirmed the presence of an organic phase in the whole sample. At the same time, the effectiveness of the crosslinking treatment was demonstrated. A micrograph of the section of a pore wall, before soaking in SBF, is shown in Figure 2, together with EDS results which reports the gelatin [Fig. 2(b)] and the 45S5-derived glass-ceramic composition [Fig. 2(c)]. The presence of the typical gelatin elements [Carbon (C), Nitrogen (N), and Oxygen(O)] on the scaffold walls is revealed. Also in this case, it should be noted that gelatin completely covers the internal surface of the pore, and the peaks which correspond to the underlying glass-ceramic phase are hard to be distinguished. As described in literature,[27,28] the genipin crosslinking mechanism consists of two reactions, involving different sites on the genipin molecule thus improving the stability of the gelatin coating in aqueous solution and allowing sample characterization by release tests. Table I reports the UV absorbance of the release solutions from the gelatin coated scaffolds after fixed times and the corresponding released protein concentrations calculated by means of the calibration curve (data not shown). Initial gelatin weight was evaluated by the difference between the weight of the gelatin-coated scaffold and the corresponding uncoated scaffold before immersion in the gelatin solution. The degradation of the coated scaffolds, evaluated by measuring the weight loss of the gelatin coating over time, was undetectable up to the first week of immersion in PBS. The weight loss was ascribed only to the gelatin dissolution since bioactive glass did not contribute. Gelatin dissolution started to dissolve after just a few days: the amount released was 18 and 50 % after 3 and 5 days, respectively, and the degradation was almost completed after 1 week. This behavior was due to genipin crosslinking, able to reduce the dissolution rate of the polymeric coating. The evaluation of gelatin release from uncrosslinked gelatin-coated scaffold was not performed due to their quick loss of consistency in water media. FTIR-ATR analysis was performed to confirm the obtainment of a gelatin coating on the scaffold walls. Figure 3 compares the chemical structure of the coated (in blue) and uncoated (in black) scaffolds. The FT-IR spectrum of the bioactive glass-based scaffold revealed the typical band of silica-based bioactive glasses[37]: wide vibrational bands corresponding to Si—O—Si stretch and Si—O—Si bending at 1060 and 480 cm^{-1}, while the vibrational band at 900–950 cm^{-1} has been credited to the presence of silanol groups (Si—OH), respectively, and vibrational bands at ~571 and 602 cm^{-1}, which correspond to

TABLE I. UV Absorbances of the Release Solutions from Coated Scaffolds After Different Immersion Time and the Corresponding Released Protein Concentrations

Time of Immersion (Days)	Abs at 560 nm (–)	Protein Concentration of Release Solution (mg mL^{-1})	Weight Loss (%)
3	0.234 ± 0.057	10.5 ± 2.5	18.2 ± 2.6
5	0.674 ± 0.101	29.5 ± 4.4	50.4 ± 3.9
7	1.234 ± 0.285	56.7 ± 12.3	94.8 ± 4.9

Data are the average values ($n = 3$).

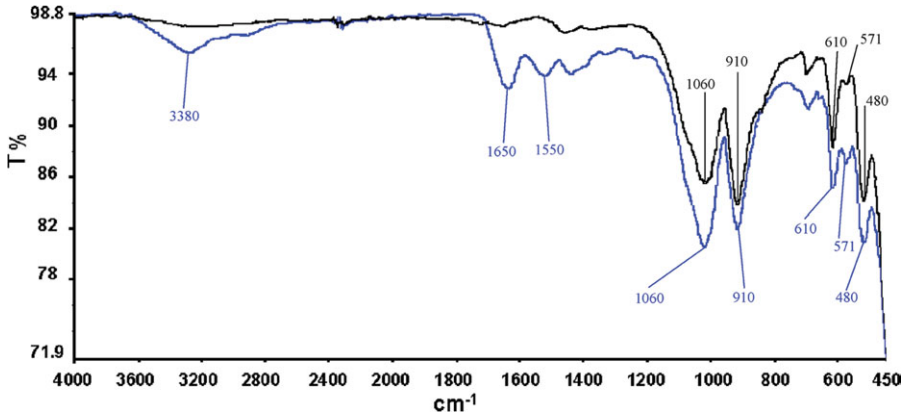

FIGURE 3. FTIR spectra of uncoated scaffold (evidenced in black) and gelatin-coated scaffold (evidenced in blue). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the P—O bending vibrations of PO_4^{-3}. On the other hand, in the blue spectrum the characteristic gelatin band amide I and II bands, at 1650 and 1550 cm^{-1}, respectively, were evidenced. The observation of these characteristic amide bands of gelatin in the ATR-FT-IR spectrum of the gelatin-coated scaffolds validates the presence of gelatin in the coating.

Assessment of *in vitro* bioactivity

Preliminary studies have recently shown that uncoated 45S5 Bioglass®-derived shell scaffolds possess an excellent *in vitro* bioactivity already after a few days in SBF.[15] It is essential to verify that gelatin, being progressively dissolved while the scaffold surface reacts with the physiological environment, has no negative effects on the *in vitro* bioactivity of bioactive glass derived scaffolds. The *in vitro* bioactivity, in fact, is considered as a fundamental prerequisite for an *in vivo* biointegration of the implant. The conversion into hydroxyapatite of 45S5 Bioglass®-derived glass-ceramics during exposure to SBF has been extensively studied by Boccacini et al.,[38] who based their considerations on the model proposed by Hench at the beginning of the 70s, which was originally developed for bioactive glasses.[39,40] Briefly, as a first step, the 45S5 Bioglass®-derived glass-ceramic exchanges alkaline or alkaline-earth cations (in particular Na^+) with H^+ or H_3O^+ from the simulated body fluid. The ion leaching results in slow amorphization of the crystalline network by point defects which form during the ion exchange. At the same time, a silica gel layer forms on the sample surface from silanol groups (Si—OH) produced at the interface between solution and sample. In particular, surface Si—OH functional groups have been reported to be effective for apatite nucleation.[41,42] Micrographs of the gelatin-coated scaffold surface after 3 days in SBF are reported in Figure 4. Rare traces of residual gelatin can be seen [Fig. 4(a)—bottom]. The sample surface is covered by sparsely distributed apatite precipitates. After 7 (data non-reported) and 14 days in SBF (Fig. 5) the scaffolds are completely covered by a large amount of apatite agglomerates. The EDS analysis reported in Figure 5(c) reveals a

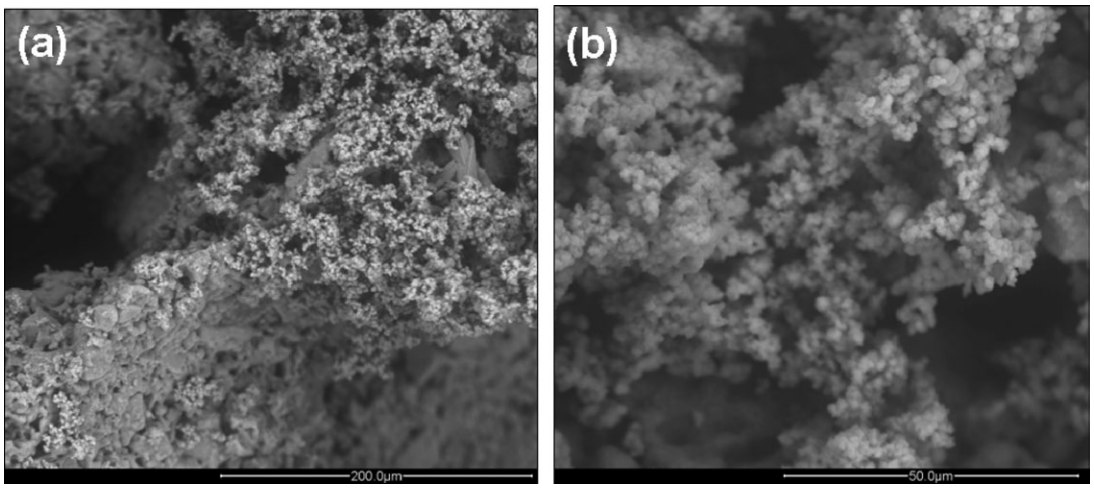

FIGURE 4. Micrographs of the hydroxyapatite formed on the shell scaffold surface after 3 days in SBF.

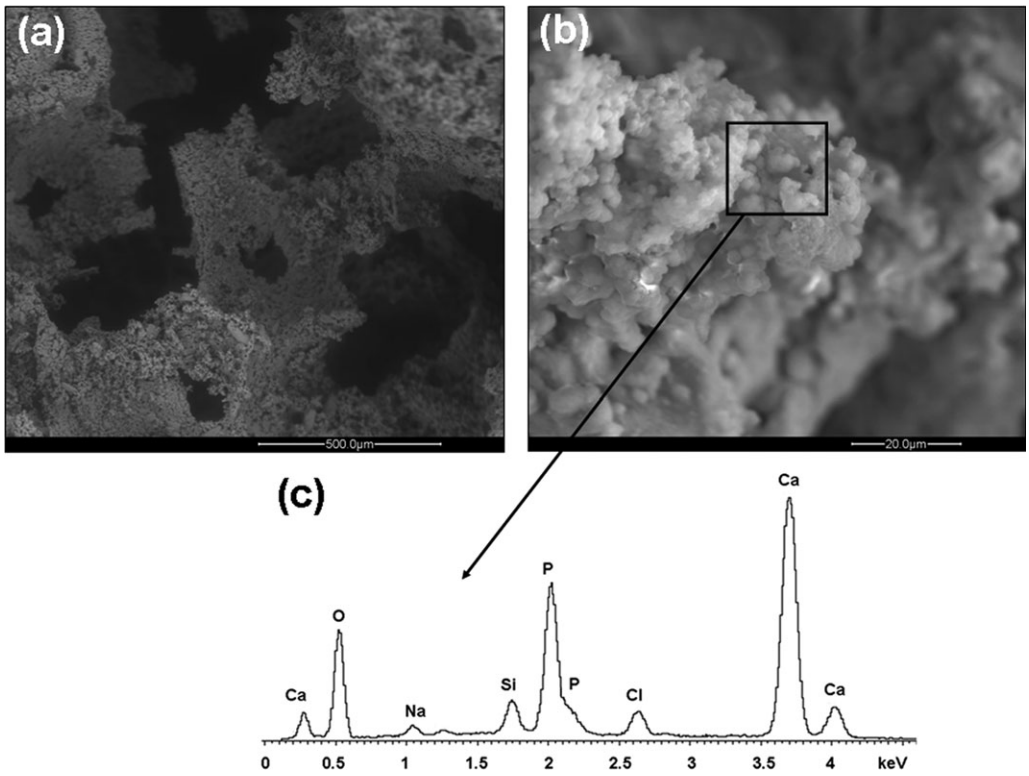

FIGURE 5. (a,b) Micrographs of the hydroxyapatite formed on the shell scaffold surface after 14 days in SBF; results of the EDS analysis performed on the area reported in (b).

Ca/P ratio (~1.8) analogous, apart from local fluctuations, to that of stoichiometric hydroxyapatite (~1.67[43]). It should be noted that the typical gelatin elements (carbon or nitrogen) are no longer present, while the signal of Silicon should be referred to the silica gel layer. To support the identification of the precipitates with hydroxyapatite, Raman spectroscopy was performed; the P—O vibration modes, in fact, are related to particularly strong Raman peaks.[44,45] In addiction, by means of this technique, which is sensitive to the presence of carbonate groups, it is possible to study in detail the chemistry of the *in vitro* deposited hydroxyapatite, which is expected to be carbonated. The Raman spectra acquired on the gelatin-coated shell scaffolds after soaking in the SBF solution for different time periods are presented in Figure 6. The spectra are characterized by a high peak at about 960 cm^{-1}, which is associated to the PO_4 group, and a peak at about 1070 cm^{-1}, which does not belong to pure apatite, but is due to the stretching of carbonate groups.[36,44,45] In particular, Awonusi et al. ascribed this peak (which is observed at 1071 cm^{-1}) to a superposition of the carbonate and phosphate mode, which can be linearly correlated to the carbonate content of apatite.[46] Therefore, the apatite precipitated *in vitro* on the gelatin-coated shell scaffolds is carbonated. The spectra reported in Figure 6 are analogous to that commonly reported in literature for hydroxyapatite.[45] In fact, no other peaks were detected (i.e., relative to glass-ceramic or gelatin) except those attributable to apatite. For these reasons, it can be concluded that the presence of gelatin does not affect the *in vitro* bioactivity of the 45S5 Bioglass®-derived shell scaffolds, which remain bioactive and look particularly promising to reproduce the complex hybrid structure of natural bone. It should be noted that the proposed protocol to realize biomimetic porous scaffolds is particularly interesting also thanks to its versatility, since it allows to directly control the samples porosity by increasing the amount of polyethylene powder added to the glass slurry the sponges are immersed in.

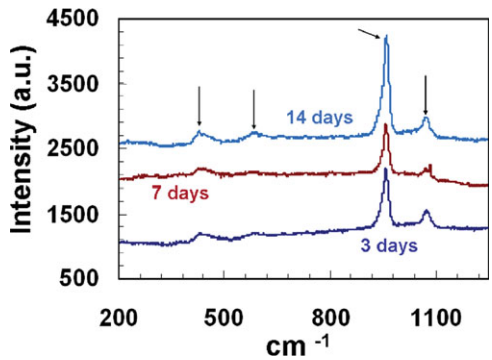

FIGURE 6. Raman spectra acquired on gelatin-coated shell scaffolds immersed in SBF for different times. Main peaks are marked with arrows. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CONCLUSIONS

Bioactive glass-derived shell scaffolds coated by bio-resorbable gelatin have been developed to reproduce at best the natural bone structure, which is composed by natural apatite (i.e., a mineral phase) and collagen. The biomimetic coating does not affect either the sample porosity, which remains open and fully interconnected, or the scaffold bioactivity in SBF. Gelatin release tests demonstrated that the coating started to dissolve after just a few days and its degradation was almost completed after 1 week. *In vitro* tests confirmed the excellent bioactivity of the gelatin-coated samples, since a significant apatite precipitation in SBF was observed. Potential applications of the gelatin coating as drug-delivery medium are presently under investigation. As a further step, it will be interesting to study the effect of the biomimetic coating on the mechanical strength of the obtained samples; moreover, it would be crucial to investigate the possible enhancement of the biological cell activity.

REFERENCES

1. Petite H, Viateau V, Bensaïd W, Meunier A, de Pollak C, Bourguignon M, Oudina K, Sedel L, G. Guillemin. Tissue-engineered bone regeneration. Nat Biotech 2000;18:959–963.
2. Salgado AJ, Coutinho OP, Reis RL. Bone tissue engineering: State of the art and future trends. Macromol Biosci 2004;4: 743–765.
3. Asahina I, Seto I, Oda M, Marukawa E, Imranul AM, Enomoto S. Bone Engineering, 1st ed. Toronto: Em squared; 1999.
4. Burg KGL, Porter S, Kellam JF. Biomaterial developments for bone tissue engineering. Biomaterials 2000;21:2347–2359.
5. Rezwan K, Chen QZ, Blaker JJ, Boccaccini AR. Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering, Biomaterials 2006;27:3413–3431.
6. Karageorgiou V, Kaplan D. Porosity of 3D biomaterial scaffolds and osteogenesis. Biomaterials 2005;26:5474–5491.
7. Yaszemski MJ, Oldham JB, Lu L, Currier BL. Clinical needs for bone tissue engineering technology. In: Davis JE, editor. Bone Engineering. Toronto: Em Squared; 2000. pp. 541–547.
8. Wopenka B, Pasteris JD. A mineralogical perspective on the apatite in bone. Mater Sci Eng C 2005;25:131–143.
9. Hench LL. Bioceramics: From concept to clinic. J Am Ceram Soc 1991;74:1487–1510.
10. Hench LL, Wilson J, editors. Introduction to Bioceramics. Singapore: World Scientific; 1993.
11. Hench LL. The story of Bioglass®. J Mater Sci: Mater Med 2006; 17:967–978.
12. Oonishi H, Kushitani S, Yasukawa E, Iwaki H, Hench LL, Wilson J, Tsuji E, Sugihara T. Particulate bioglass compared with hydroxyapatite as a bone graft substitute. Clin Orthop Relat Res 1997;334: 316–325.
13. Schepers EJG, Ducheyne P, Barbier L, Schepers S. Bioactive glass particles of narrow size range: A new material for the repair of bone defects. Implant Dent 1993;2:151–156.
14. Bellucci D, Cannillo V, Sola A. Shell scaffolds: A new approach towards high strength bioceramic scaffolds for bone regeneration. Mater Lett 2010;64:203–206.
15. Bellucci D, Chiellini F, Ciardelli G, Gazzarri M, Gentile P, Sola A, Cannillo V. Processing and characterization of innovative scaffolds for bone tissue engineering. J Mater Sci: Mater Med. 2012;23:1397–1409.
16. Chen QZ, Thompson ID, Boccaccini AR. 45S5 Bioglass®-derived glass–ceramic scaffolds for bone tissue engineering. Biomaterials. 2006;27:2414–2425.
17. Damien C, Parsons R. Bone graft and bone graft substitutes: A review of current technology and applications. J Appl Biomater 1991;2:187–208.
18. Fu Q, Saiz E, Rahaman MN, Tomsia AP. Bioactive glass scaffolds for bone tissue engineering: State of the art and future perspectives. Mater Sci Eng C Mater 2011;31:1245–1256.
19. Miao X, Lim G, Loh KH, Boccaccini AR. Preparation and characterization of calcium phosphate bone cement. Mater Proc Prop Perf (MP3) 2004;3:319–324.
20. Gibson LJ, Ashby MF. Cellular Solids: Structure and Properties, 2nd ed. Oxford: Pergamon; 1999. p 429–452.
21. Landi E, Valentini F, Tampieri A. Porous hydroxyapatite/gelatine scaffolds with ice-designed channel-like porosity for biomedical applications. Acta Biomater 2008;4:1620–1626.
22. Ciardelli G, Gentile P, Chiono V, Mattioli-Belmonte M, Vozzi G, Barbani N, Giusti P. Enzymatically crosslinked porous composite matrices for bone tissue regeneration. J Biomed Mater Res A 2010;92:137–151.
23. Liu X, Smith LA, Hu J, Ma PX. Biomimetic nanofibrous gelatin/apatite composite scaffolds for bone tissue engineering. Biomaterials 2009;30:2252–2258.
24. Mozafari M, Rabiee M, Azami M, Maleknia S. Biomimetic formation of apatite on the surface of porous gelatin/bioactive glass nanocomposite scaffolds. Appl Surf Sci 2010;257:1740–1749.
25. Harrington WF, Vonhippel PH. The structure of collagen and Gel. Adv Protein Chem 1961;16:1–138.
26. Boedtker H, Doty P. A study of gel molecules, aggregates and gels. J Phys Chem 1954;58:968–983.
27. Touyam R, Inoue K, Takeda Y, Yatsuzuka M, Ikumoto T, Moritome N, Shingu T, Yokoi T, Inouye H. Studies on the blue pigments produced from genipin and methylamine. II: On the formation mechanisms of brownish-red intermediates leading to the blue pigment formation. Chem Pharm Bull 1994;42:1571–1578.
28. Tonda-Turo C, Gentile P, Saracino S, Chiono V, Nandagiri VK, Muzio G, Canuto RA, Ciardelli G. Comparative analysis of gelatin scaffolds crosslinked by genipin and silane coupling agent. Int J Biol Macromol 2011;49:700–706.
29. Song F, Zhang LM, Yang C, Yan Li. Genipin-crosslinked casein hydrogels for controlled drug delivery. Int J Pharm 2009;373: 41–47.
30. Muzzarelli RAA. Genipin-crosslinked chitosan hydrogels as biomedical and pharmaceutical aids. Carbohydr Polym 2009;77:1–9.
31. Yang Y, Zhao W, He J, Zhao Y, Ding F, Xiaosong Gu. Nerve conduits based on immobilization of nerve growth factor onto modified chitosan by using genipin as a crosslinking agent. Eur J Pharm Biopharm 2011;79:519–525.
32. Gentile P, Chiono V, Boccafoschi F, Baino F, Vitale-Brovarone C, Vernè E, Barbani N, Ciardelli G. Composite films of gelatin and hydroxyapatite/bioactive glass for tissue-engineering applications. J Biomater Sci Polym Ed 2010;21:1207–1226.
33. Sung HW, Liang IL, Chen CN, Huang RN, Liang HF. Stability of a biological tissue fixed with a naturally occurring crosslinking agent (genipin). J Biomed Mater Res 2001;55:538–546.
34. Chang Y, Hsu CK, Wei HJ, Chen SC, Liang HC, Lai PH, Sung HW. Cell-free xenogenic vascular grafts fixed with glutaraldehyde or genipin: In vitro and in vivo studies. J Biotechnol 2005;120: 207–219.
35. Kokubo T, Takadama H. How useful is SBF in predicting *in vivo* bone bioactivity? Biomaterials 2006;27:2907–2915.
36. Antonakosa A, Liarokapisa E, Leventouri T. Micro-Raman and FTIR studies of synthetic and natural apatites. Biomaterials 2007; 28:3043–3054.
37. LaTorre G, Hench LL. Analysis of bioactive glass interfacial reactions using Fourier-transform infrared reflection spectroscopy. In: Adair JH, Casey JA, Venigalla S, editors. Handbook on Characterization Techniques for the Solid-Solution Interface. Westerville: American Ceramic SOC; 1993. p 177–195.
38. Boccaccini AR, Chen QZ, Lefebvre L, Gremillard L, Chevalier J. Sintering, crystallization and biodegradation behaviour of Bioglass®-derived glass-ceramics. Faraday Discuss 2007;136:27–44.
39. Hench LL, Splinter RJ, Allen WC, Greenlee TK. Bonding mechanisms at the interface of ceramic prosthetic materials. J Biomed Mater Res Symp 1971;2 (Part I):117–141.
40. Hench LL, Paschall HA. Direct chemical bonding between bioactive glass-ceramic materials and bone. J Biomed Mater Res Symp 1973;4:25–42.

41. Kokubo T. Apatite formation on surfaces of ceramics, metals and polymers in body environment. Acta Mater 1998;46: 2519–2527.
42. Kokubo T, Kushitani H, Sakka S, Yamamuro T. Solutions able to reproduce in vivo surface-structure changes in bioactive glass-ceramic A/W. J Biomed Mater Res 1990;24:721–734.
43. Liu H, Yazici H, Ergun C, Webster TJ, Bermek H. An in vitro evaluation of the Ca/P ratio for the cytocompatibility of nano-to-micron particulate calcium phosphates for bone regeneration. Acta Biomater 2008;4:1472–1479.
44. Penel G, Leroy G, Rey C, Bres E. MicroRaman spectral study of the PO_4 and CO_3 vibrational modes in synthetic and biological apatite. Calcif Tissue Int 1998;63:475–481.
45. Bellucci D, Bolelli G, Cannillo V, Cattini A, Sola A. In situ Raman spectroscopy investigation of bioactive glass reactivity: Simulated body fluid solution vs. TRIS-buffered solution. Mater Character 2011;62:1021–1028.
46. Awonusi A, Morris MD, Tecklenburg MMJ. Carbonate assignment and calibration in the Raman spectrum of apatite. Calcif Tissue Int 2007;81:46–52.