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In vitro apatite forming ability of type I collagen hydrogels containing bioactive glass and silica sol-gel particles

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Type I collagen hydrogel containing bioactive glass (CaO-P_2O_5-SiO_2) and silica sol-gel micrometric particles were prepared and their *in vitro* apatite-forming ability in simulated body fluid assessed. X-ray diffraction and scanning electron microscopy analysis showed that bioactive glass particles entrapment in collagen matrix did not inhibit calcium phosphate formation and induced morphology variations on the crystalline phase precipitated on the hydrogel surface. The silica—collagen hydrogel composite precipitated calcium phosphate whereas silica particles and collagen hydrogel alone did not, indicating a possible synergetic effect between collagen and silica on the apatite-forming ability. Mechanisms of calcium phosphate precipitation and its relevance to biomaterial development are discussed.

1. Introduction

Extracted collagen has a long history as material for biomedical applications due to its natural abundance, biodegradability, biocompatibility and role in tissue formation. In fact, collagen-based biomedical devices under varied physical forms, including nanoparticles, fibres, film, hydrogel and pellet have been developed and used as drug carriers, wound dressings, skin replacements and bone substitutes [1]. This last application is probably the most challenging as these materials should not only present the usual biocompatibility and non-antigenic properties, but also exhibit bioactive and mechanical properties matching those of implanted tissues during all steps of bone regeneration. In the most recent advances, a collagen matrix is usually combined to a reinforcing phase such as a water-soluble organic polymer, or a calcium phosphate powder [2, 3]. For the latter, biomimicking the association of collagen and calcium phosphate should improve both the mechanical properties and the bioactivity.

Collagen itself, used as bone substitutes, has shown *in vitro* and *in vivo* osteoinductive activity when associated or not, to bone morphogenetic proteins (BMP) [4, 5]. A step further is the mixing of a bioactive glass or ceramic particles with collagen [6, 7]. Since the seminal work of L. L. Hench, it is known that glass or ceramic containing CaO-P_2O_5-SiO_2 show *in vivo* osteonconductive and osteoinductive properties [8]. Basically, rapid physico-chemical interactions between the biomaterial surface and the close surrounding biological environment; release of silica species, formation of a silica layer, adsorption of Ca and PO_4 and CO_3 and crystallisation of hydroxyapatite (HA) materials, are the first stages leading to implant success. Materials with such property are called osteoconductive or class B materials following Hench's nomenclature [9]. The rate of apatite formation (induction time) depends on the bioactive glass formulation but also its texture (porosity). A 45S5 Bioglass® has a typical *in vitro* induction time of half a day and a porous silica gel around 35 days [10]. In a second step, interaction with cells (attachment, differentiation), generation and mineralization of collagen matrix may occur. Those materials (Class A), which exhibit the capacity to induce bone regeneration, are called osteoproductive or osteoinductive.

Pohunkova and Adam reported the *in vivo* biocompatibility study of Bioglass® particles-collagen hydrogel

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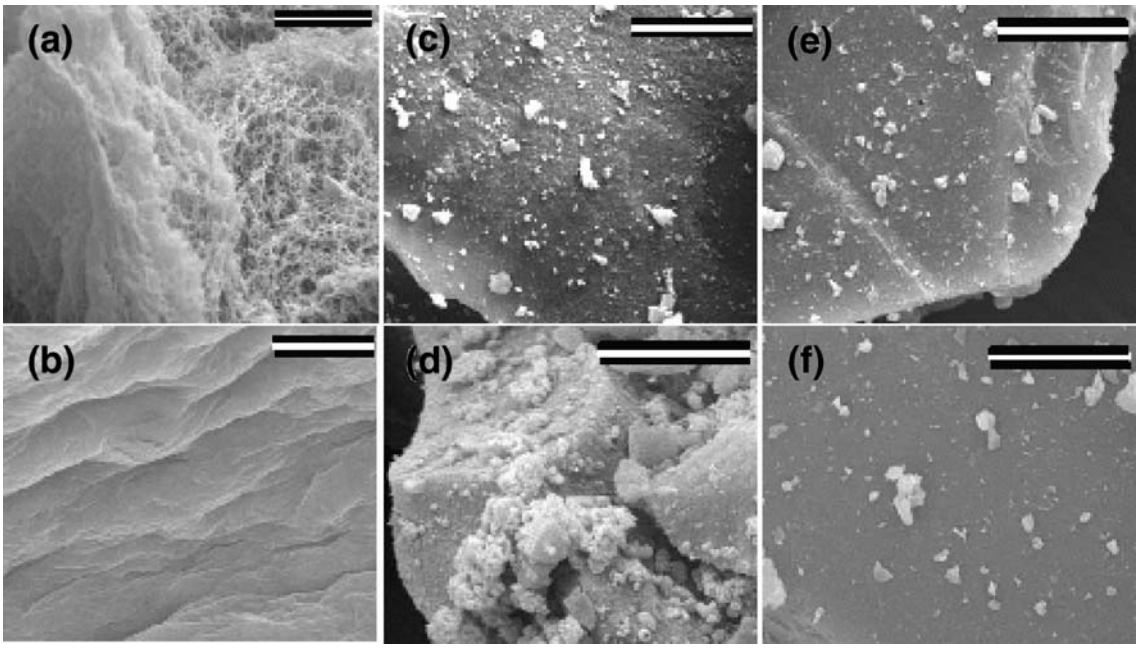

Figure 1 SEM photographs of collagen (a, b), BG (c, d) and silica (e, f) surface, soaked in SBF solution for 0 and 14 days, top to bottom (scale bar = 5 μm).

composite [7]. However, *in vivo* and *in vitro* osteoconductivity of bioactive glass-collagen hydrogel and silica-collagen hydrogel have not yet been reported to our knowledge.

Therefore, the scope of this work was to prepare bioactive glass- and silica-collagen hydrogel composites and to study their *in vitro* apatite forming ability. Their properties were compared to pure collagen, bioactive glass, silica and alumina-collagen composites. Relevance of these studies to biomaterials preparation and *in vivo* bioactivity of silica containing materials was drawn.

2. Materials and methods

2.1. Materials

A 10 mg $\cdot$ mL^{-1} solution of type I collagen from rat tail tendon in 0.5 M acetic acid was obtained following a method of extraction and purification reported elsewhere [11]. Silica particles were prepared using sol-gel method, *ie* tetraethoxysilane hydrolysis-condensation reactions in ethanol/water acidic solution [12]. The aged and dried monolith obtained was crushed in a mortar to a powder material. Colloidal alumina was precipitated out by increasing the pH of $Al(NO_3)_3$ solution, washed with deionised water and dried at 130°C. The bioactive glass (BG) CaO-P_2O_5-SiO_2 ternary oxide particles, prepared following a sol-gel method described by Jaakkola *et al*. [13], were a gift from Dr. Sami Areva (Turku University-Finland). Particles size range evaluated by scanning electron microscopy, Figs. 1, 3 and 6, were 2–200 μm, 70–90 μm and 2–20 μm for silica, BG and alumina, respectively.

2.2. Hydrogel composites synthesis

In a 10 ml plastic vial punctured 4 times with a 0.5 mm diameter needle at middle height, 2 ml of collagen solution was added to 0.2 g of aluminium hydroxide, BG or silica particles (inorganic:collagen weight ratio = 10:1). The viscous mixture was thoroughly mixed until a homogenous solution was obtained. Then, the closed plastic vial was introduced in a 50 ml glass vial containing 1 ml of 30% ammonia solution. The glass vial was tightly closed and ammonia allowed to diffuse into the collagen solution through the plastic vial pinhole at room temperature. After 6 h, the obtained hydrogel disks were collected and soaked in deionised water until the pH of renewed washing water remained constant. The white dense hydrogels were flexible and showed enough mechanical strength to be manipulated without any precaution.

2.3. *In vitro* apatite forming ability tests

Static *in vitro* apatite forming ability tests and simulated body fluid solution (SBF) preparation were performed following the protocol described by Kokubo *et al.* [14]. The composite hydrogel disks or 0.2 g of BG, silica and aluminium hydroxide powder materials were soaked in closed plastic containers containing 30 ml of the SBF solution at 37°C $\pm$ 1°C. The samples were removed after 3, 7 and 14 days, soaked in a large volume of deionised water for at least 12 h, to remove all salts from hydrogels. Then, in order to preserve hydrogel structure and shape, they were freeze-dried at -30°C prior to analysis.

2.4. Scanning electron microscopy (SEM)

Scanning electron microscopy and energy dispersive X-ray micro-analysis were carried out on gold coated samples, using a Cambridge Stereoscan 120 instrument operating at an accelerating voltage of 20.0 kV for microscopy observation and an EDAX microanalysis instrument operating at an accelerating voltage of 15.0 kV at a working distance equal to 5–6 mm for microanalysis.

2.5. X-ray diffraction analysis (XRD)

The surface of the freeze-dried samples was analysed with a Phillips X-ray diffractometer. The X-Ray diffraction spectra were recorded from 8° to 55° 2θ at a rate of 0.5°/min, using a Cu $K\alpha$ radiation at 20 kV. The phase composition was determined by comparing acquired spectra with peaks identified in the joint committee on Powder Diffraction Standards (JCPDS) database of standards [15]. Of particular relevance to this study are the XRD spectra of $Ca_5(PO_4)_3(OH)$ Hydroxyapatite (HA) [09-432], $Ca_8H_2(PO_4)_6 \cdot 5H_2O$ orthocalcium phosphate (OCP) [26-1056], α-$Ca_3(PO_4)_2$ alpha-tricalcium phosphate (α-TCP) [09-348], $Ca_4(Si_3O_9)(OH)_2$ calcium silicate [74-0360] and aluminium hydroxide $Al(OH)_3$ [74–1119]. Following the collection of XRD data, the background noise was subtracted and the integrated intensities of peaks measured for comparison with standard values.

2.6. Silicic acid colorimetric assays

Silicic acid, $Si(OH)_4$, released in the SBF solution was measured following the blue silicomolybdate method described in the literature [16]. This method is based on the ability of silicic acid to form a coloured complex with molybdate ions in an acidic solution. The optimum accuracy of this method is obtained in the 10^{-4}–10^{-5} mol $\cdot$ L^{-1} $Si(OH)_4$ concentration range. The measurements were performed in duplicate.

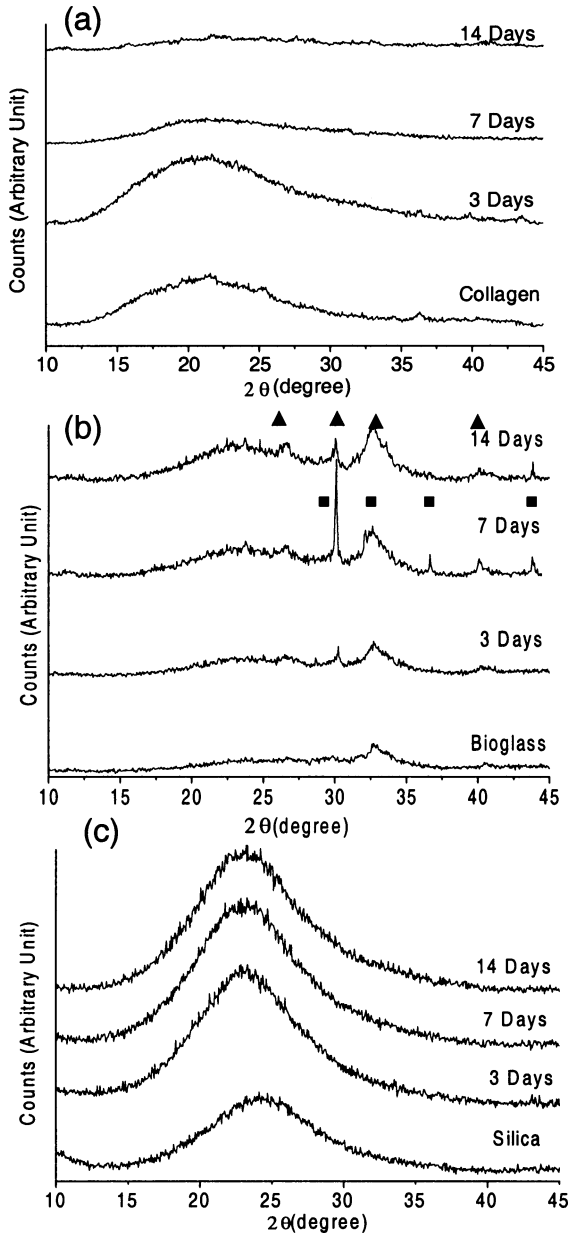

Figure 2 XRD patterns of collagen hydrogel (a), BG (b) and silica (c) soaked in SBF solution for 0, 3, 7 and 14 days (▲HA, ■ α-TCP and/or OCP).

3. Results

3.1. Silica, BG and collagen materials

Figs. 1 and 2 present scanning electron micrographs and XRD diffractograms of silica, BG and collagen hydrogel surfaces, soaked in SBF for 0, 3, 7 and 14 days. As shown on Fig. 1, calcium phosphate deposition on silica and collagen hydrogel is not observed even after 14 days of soaking in SBF. The absence of new peaks in the corresponding X-ray diffractograms collected at different soaking time, Fig. 2, confirms that no apatite-like material is precipitated on materials surface in the experiment time-scale. Meanwhile, BG particles precipitate crystalline phases on its surface, as shown by SEM and XRD analysis (Figs 1 and 2). New diffraction peaks appear in BG X-ray diffraction patterns after 3 days of soaking in SBF (Fig. 2b). The intensity of the peaks, notably at $2\theta = 32.0°$ ($d = 2.79$ Å), and $2\theta = 36.0°$ ($d = 2.49$ Å) increases up to 7 days and then decreases. Signal at 36° even disappears at 14 days of soaking. These reflections may be attributed to the presence of a transient OCP or an α-TCP type phase.

By comparison with JCPDS standard files, the diffraction peaks on BG after 14 days in SBF, can be

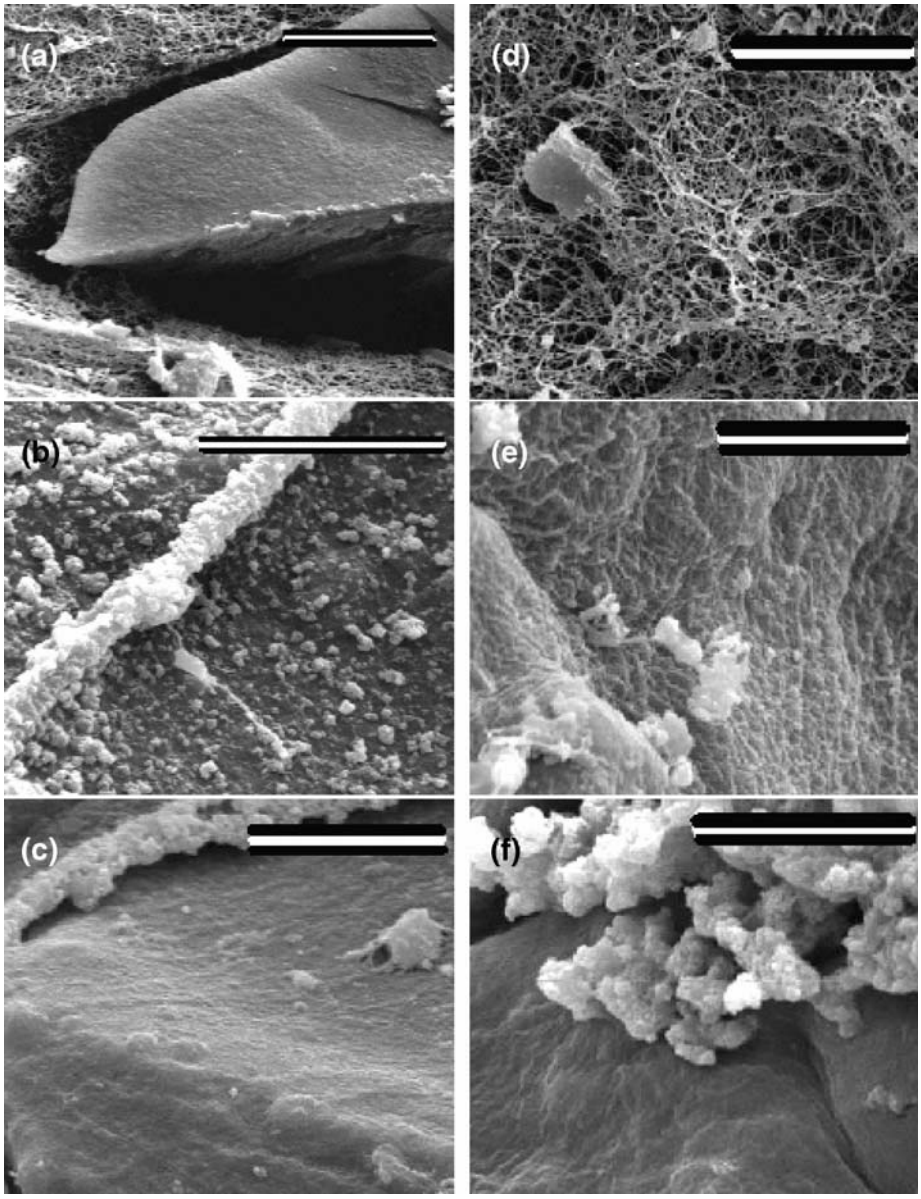

Figure 3 SEM photographs of the surface of BG-collagen (a–c) and silica-collagen (d–f) hydrogel composites soaked in SBF solution for 0, 7 and 14 days (scale bar = 5 μm).

attributed to crystalline HA material. SEM photography (Fig. 1d) shows 1 to 5 μm diameter spheres, typical of the morphology obtained by precipitation of calcium phosphate material on osteoconductive surface in SBF solution [17].

3.2. Collagen-based composites

Prepared composite materials can best be described as inorganic particles embedded in fibrous collagen hydrogels. Figs 3 and 4 show SEM and XRD analysis results for the BG-collagen and the silica-collagen hydrogels soaked in SBF. BG-collagen and, more surprisingly, silica-collagen hydrogels precipitate crystalline materials on their surface as shown by SEM (Fig. 3). Compared to BG particles alone, the precipitation of materials on BG-collagen surface appears more homogeneous and significant (compare Figs 1d and 3c) and consists of compact aggregates of 0.5 to 1 μm diameter particles (Fig. 3b and c). For silica-collagen composites, a very different morphology is observed after 14 days with spherical particles, 2 to 5 μm in diameter forming grape-like aggregates on the surface.

X-ray diffraction patterns after soaking, Fig. 4, indicate the formation of a HA-like phase on BG-collagen hydrogel surface similar to the phase precipitate on pure BG particles (Fig. 2b). The induction time of both materials is below 3 days and similar variations in diffraction peaks intensities with soaking time are observed. Again,

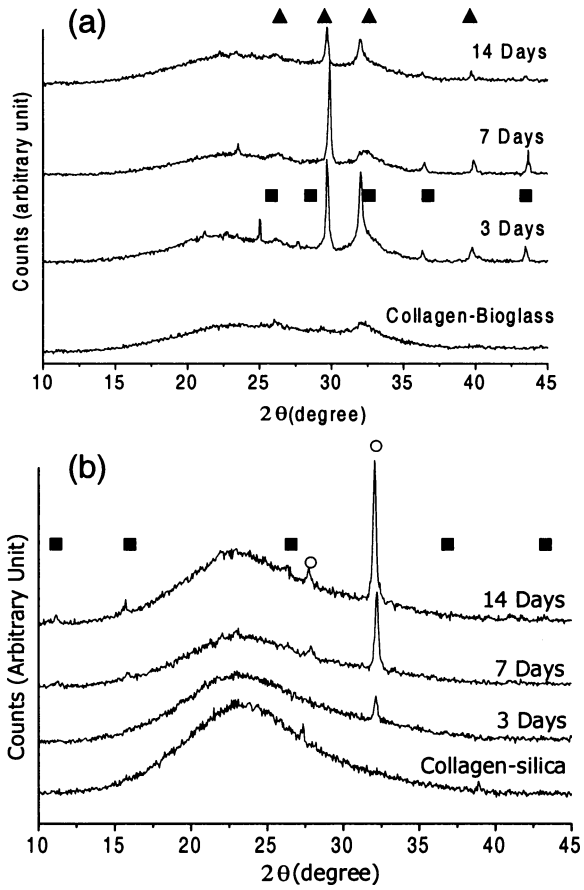

Figure 4 XRD Patterns of BG-collagen (a) and silica-collagen (b) hydrogel composites surfaces soaked in SBF solution for 0, 3, 7 and 14 days (▲ HA, ■ α-TCP or OCP and ○ $Ca_4(Si_3O_9)(OH)_2$).

these reflections may be attributed to an OCP or an α-TCP type phase. We note that some of the characteristic diffraction peaks of these crystalline structures are not observed, but this may be attributed to the high signal-to-noise ratio of the collected diffractograms. Such an evolution of calcium phosphate phases was already reported by others authors [18].

In contrast, d-spacing and relative intensities of diffraction peaks observed for material precipitated on silica-collagen composite cannot be easily attributed. After 3 days, only a single diffraction peak at $2\theta = 32.0^\circ$ ($d = 2.79$ Å) is observed. Comparison with JCPDS files strongly suggests formation of calcium silicate hydroxide or some highly silica-substituted HA materials. This last possibility appears very unlikely as the EDX analysis, performed on materials surfaces, gives a calcium-to-phosphorus molar ratio equal to 1.09, much lower than the Ca/P = 1.68 ratio for HA. After 7 days, and more clearly after 14 days, new signals appear at $2\theta = 11.2^\circ$ ($d = 7.89$ Å), 15.7° ($d = 5.63$ Å), and 27.0° ($d = 3.29$ Å), that could be assigned to calcium phosphate materials, α-TCP and/or OCP.

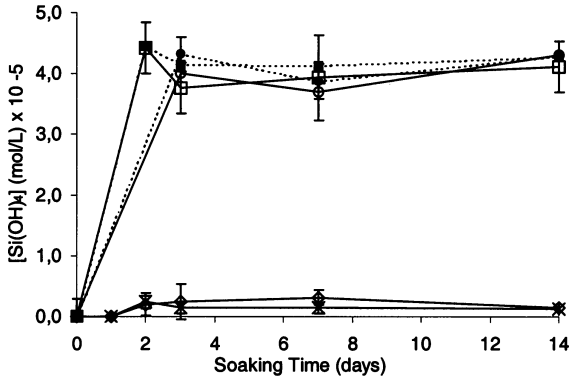

Figure 5 Plot of silicic acid concentration as function of soaking time in SBF (◇); collagen (X), silica (○), BG (□), silica-collagen (●) and BG-collagen (■).

3.3. Complementary studies

In order to investigate the possible effect of collagen on silica and BG dissolution, the concentration of silicic acid released as a function of the soaking time in SBF solution was measured using the blue silicomolybdate method (Fig. 5). It shows significant release of silicic acid for all the materials containing silica:silica and BG particles, silica-collagen and BG-collagen composites. The plot indicates similar release of silicic acid up to around 4×10^{-5} mol $\cdot$ L^{-1}, a plateau being reached in less than three days.

Additionally, in order to understand the specificity of silica toward apatite formation, aluminium hydroxide particles were synthesized and their reactivity in SBF, either in a pure phase or within collagen hydrogel, was studied. SEM micrographs of the corresponding surfaces before and after soaking for 14 days are shown on Fig. 6. Alumina particles did not undergo any significant change during their soaking in SBF, revealing the absence of precipitation in agreement with previous report [17]. Comparison of micrographs, Fig. 6c and d susggests that calcium phosphate precipitate was not formed on alumina-collagen hydrogel. This was confirmed by XRD studies (not shown) revealing diffraction peaks of the pristine aluminium hydroxide phase only. Aluminium Al^{3+} release in SBF was also studied by spectroscopic measurements but the experimental values obtained were below the detection limit (10^{-5} mol $\cdot$ L^{-1}) of the chemical assay.

4. Discussion

Table I summarizes the major results of this study. A comparison of the materials *in vitro* osteoconductivity indicates that the presence of silica is a necessary condition for calcium phosphate precipitation on particles and hydrogel composites in the assay time-scale. However, it is not a sufficient condition to induce apatite precipitation on the silica gel particles since no *in vitro* osteoconductivity could be observed after 14 days of soaking in SBF.

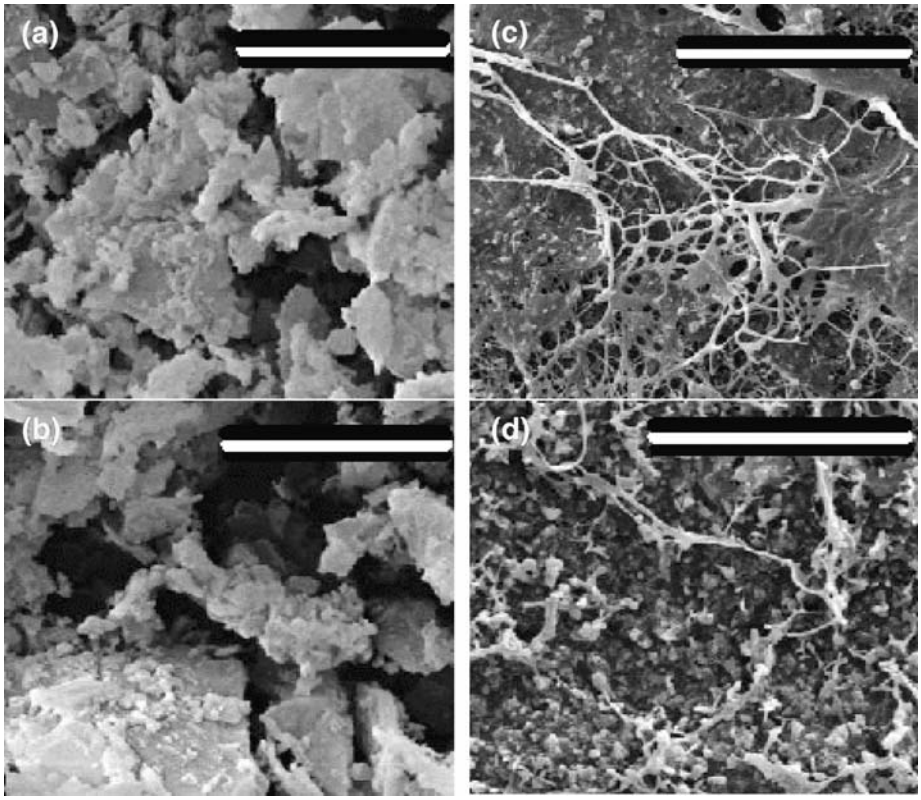

Figure 6 SEM photographs of the surface of alumina (a, b) and alumina-collagen hydrogel (c, d) soaked in SBF solution for 0 and 14 days (scale bar $= 5\ \mu$m).

TABLE I Silicic acid concentration after 3 days of soaking, induction time and calcium to phosphorous molar ratio after 14 days of soaking.

Sample	$[Si(OH)_4]$ ($mol \cdot L^{-1}$)	Induction time (days)	Ca/P molar ratio
Alumina	–	$\gg 14$	–
Silica gel	$3.7 * 10^{-5}$ (0.4)	$\gg 14$	–
BG	$4{,}0 * 10^{-5}$ (0.3)	$3<$	1.68
Collagen Hydrogel	$0.2 * 10^{-5}$ (0.2)	$\gg 14$	–
Collagen-Alumina	–	$\gg 14$	–
Collagen-Silica	$4.14 * 10^{-5}$ (0.4)	$3<$	1.09
Collagen-BG	$4.32 * 10^{-5}$ (0.3)	$3<$	1.60

BG particles present HA formation on their surfaces in less than 3 days, as reported by many others studies [19]. BG-collagen hydrogel shows a similar behaviour, suggesting that the presence of the collagen network was not detrimental to *in vitro* osteoconductivity. However, it affects the morphology and homogeneity of HA deposit. This is not surprising since the collagen fibrils structure has already been suggested to play a role in the oriented deposition and subsequent growth of OCP [19].

More surprisingly, silica particles-collagen composites precipitate a crystalline phase on its surface when soaked in SBF for 3 days, whereas the collagen hydrogel and silica particles alone did not, even after 14 days of soaking in SBF. It is difficult to interpret the apparent synergetic effect of silica and collagen hydrogel. First, silicic acid concentration results indicate similar release profiles, independent of the respective apatite-forming ability of silica-containing materials. Then, XRD and EDX data suggest the initial formation of calcium silicate and subsequent precipitation of some calcium phosphate crystalline phases.

Even though the combined effect of silica and collagen hydrogel on HA precipitation has not been reported yet, the addition of polyacrylic acid in a modified SBF solution (saturated in Ca^{2+}) has been shown to inhibit or modify the morphology of the HA materials that precipitate on collagen fibers [20]. Combes *et al.* also reviewed the effect of a protein, albumin (BSA), on the calcium phosphate precipitation on type I collagen powder in SBF solution at 37°C, with calcium concentrations 1.5 times superior to normal SBF [21]. On the basis of experimental observations and theoretical considerations, they proposed that protein adsorption on a mineral surface increases or decreases nucleation and crystal growth rates depending on their concentration. In fact, the property of a surface to precipitate calcium phosphate from SBF depends of its ability to decrease the activation barrier of the spontaneous precipitation [13]. This may occur via homogeneous nucleation in solution, induced by modification of the solution critical saturation via calcium ion and silicic acid release

for example. Alternatively, specific surface sites, such as silanol or carboxylate groups, can be involved in heterogeneous nucleation [21–23].

In the case of silica-containing bioactive glasses, it was suggested that the release of silicic acid upon material dissolution led to the formation of a bioactive silica layer on its surface [17, 24]. Calcium and phosphate ions also released during this process increase the ionic content of the SBF, favouring HA precipitation. In the case of pure silica particles, such release is absent but, within the composites, collagen appears to counterbalance this loss in HA precursors. As a matter of fact, this protein exhibits acidic groups (aspartic acid, glutamic acid) whose carboxylate functions can efficiently bind Ca^{2+} cations. As suggested by XRD experiments, these calcium ions can associate with silicic acid to form calcium silicate phase. Such species may serve as nucleation centers for further calcium phosphate deposition. The fact that collagen can provide additional nucleation sites is supported by the better homogeneity of HA deposition on BG-collagen composites when compared to BG particles alone. Finally, the absence of mineralization of aluminium hydroxide-containing composite can be correlated to the absence of Al^{3+} release in solution, preventing the formation of Ca^{2+}-rich nucleating site.

5. Conclusion

The role of silicon species in bone formation [25, 26] and implants bioactivity [17] is still a matter of debate. Silicic acid is known to play a key role in biomaterials properties both *in vitro* and *in vivo* [23, 24]. Additionally, it was recently shown that $Si(OH)_4$ influences the collagen self-assembly process [27].

This work demonstrates that silica-collagen composites exhibit *in vitro* osteoconductivity properties, whereas their components alone do not. This synergetic effect is tentatively attributed to the ability of the protein to bind calcium ions, which can further associate with silicic acid to form a bioactive layer.

Further investigations are now needed to get a better insight of the properties of these materials, but these first results suggest that such silica-collagen composites could be useful for bone repair and tissue engineering applications [28]. The possibilities to design new collagen-silica hybrid materials where the protein and the mineral phase are associated at the nanometer scale are also in progress [30].

Acknowledgments

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