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ARTICLE TYPE

Bioactive glass hybrids: a simple route to Gelatin/SiO_2-CaO System

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Bioactive glass hybrids are among the most promising materials for bone regeneration, but the incorporation of calcium, a key element for mineralization properties of the implant, into the inorganic part of the hybrid network is challenging. We present here a new synthesis route to both class I and II gelatin-bioactive glass hybrids allowing the efficient incorporation of calcium ions at low temperature.

Bioactive glasses (BG) are known to be the most bioactive biomaterials and to stimulate bone regeneration more efficiently than other synthetic or natural implants.[1] However they are brittle and cannot be implanted in load-bearing sites. To overcome this limitation, hybrid organic-inorganic biomaterials based on the combination of bioactive glasses and polymers appear as an ideal alternative. Such materials would benefit from both the toughness of the polymer and of the bioactivity of the glass. A congruent degradation of inorganic and organic parts of the material is also required to maintain its mechanical properties during the bone growth process. This cannot be easily achieved with conventional "raw" composite materials but can be obtained through the formation of covalent linkages between the organic and inorganic interpenetrated networks.

Calcium is one of the most important elements for osteogenic properties of the implant, as it favours osteoblast proliferation, differentiation, mineralization of the extracellular matrix, and stimulates the expression of growth factors, due to the presence of dedicated Ca-sensing receptors in bone cells.[2-4] Calcium is also required for efficient apatite-forming ability of the material, as the release of Ca from the implant favorably changes the local suspersaturation of the medium towards apatite precipitation.[5, 6] The synthesis of BG hybrids involves the use of soft chemistry routes (sol-gel process) and requires careful restrictions on thermal treatments to preserve their organic part. Under such conditions, the incorporation of calcium ions into the inorganic part of the hybrid network remains challenging to achieve as shown in the current literature[7-11].

Previously described BG hybrids [7-11] have been obtained using common sol-gel syntheses involving calcium salts as calcium source. Under such conditions, high thermal treatments (> 400 °C) are required for the formation of an inorganic calcium silicate glass network.[12] The inability to perform such thermal treatments for hybrids prevents the calcium incorporation and leads to the formation of calcium salt deposits outside the hybrid network. This feature can induce an undesirable alkaline shock damageable for cells, as a result of the fast release of these calcium deposits. It is worth noting that, to avoid alkaline shock, cytotoxicity and biocompatibility properties of BG hybrids based on calcium salts were systematically investigated after a preincubation or washing:step, [7, 9-11] which remove in this case nearly all the calcium salt deposits. After this treatment, the biomaterial consists finally in a nearly pure-SiO_2 hybrid.

As an alternate way, BG hybrids can be obtained through alkoxide routes. With the use of calcium alkoxides the homogeneous incorporation of calcium at room temperature becomes possible, but the synthesis of BG hybrids remains challenging due to the extreme sensitivity of calcium alkoxides towards hydrolysis-condensation reactions in the presence of water. As a result, premature gelation of the hybrid sol can occur leading to heterogeneity, especially regarding calcium distribution. This is particularly annoying when the polymer used in the hybrid synthesis requires dissolution in aqueous media, e.g. like gelatin used in the present study or like most biopolymers of natural origin that have the advantage of being degraded through enzymatic action. This explains that the rare studies reporting the synthesis of BG hybrids from calcium alkoxides involve the use of a synthetic polymer and remain far from ideal because the homogeneity of the obtained hybrid is questionable due to premature gelation.[13] Here, to limit the reactivity of the calcium alkoxide, the synthesis was done in alcoholic media under dilute conditions. The BG precursors, namely tetraethylorthosilicate (TEOS) and calcium ethoxide, are first mixed together in a 12.7 wt.% ethanol solution slightly acidified with 2M HCl (0.05 mL/g of sol); therefore water, which is still necessary for hydrolysis of the alkoxide precursors, is only introduced through limited addition of HCl. Under these conditions, a clear red-brown suspension is obtained, which remains stable for several hours before gelation. Drying the obtained gel at low temperature (60°C for 24 h) leads to the formation of a SiO_2–CaO BG (75 – 25 wt.% respectively in the present study without the need of further thermal treatments.

The formation of BG hybrids involves the polymerization of the silicate network around the polymer chains. Here the polymer chosen was gelatin. Gelatin is a hydrolysed form of type I collagen, which accounts for 90 % of the organic part of bones.[14] With respect to collagen, gelatin exhibits two main advantages: it does not express antigenicity in physiological conditions, and it can be handled easily, being readily dissolved in aqueous media and then reconstituted at temperatures that are not damaging for proteins. Gelatin is widely used in the medical industries, including tissue engineering applications.[15, 16] It has

binding sites for cell attachment and can be broken down by cellular action through enzymatic degradation, in a process that does not produce toxic by-products.[17] This enzymatic degradation process is much preferable to the autocatalytic chain scission mechanism of synthetic polymers like e.g. the commonly used polylactic and polyglycolic acids, because it results in a more linear degradation rate in vivo.[18] Finally, gelatin is an inexpensive polymer and its dissolution rate and mechanical properties can be easily adjusted through cross-linking with e.g. glutaraldehyde, carbodiimide, genipin or organoalcoxysilanes. [19] In the following, we describe the synthesis of both class I and class II hybrids. According to Novak[20] and Sanchez et al.[21], class I hybrids correspond to inorganic chains entangled with organic chains with only weak (hydrogen and/or Van der Waals bonding) interactions between them, while in class II hybrids the inorganic and organic networks are covalently bonded.

For the synthesis of class I gelatin-BG hybrids, a 12.7 wt.% gelatin solution is first prepared in water, while the BG sol is prepared separately as described above and left for condensation for 1 hour. After mixing the two solutions, a homogeneous hybrid sol is obtained. Here, the two solutions were mixed in equal proportions to obtain 50/50 wt.% gelatin/SiO_2–CaO BG hybrids. Thanks to the limited introduction of water and adequate dilution of the precursors, the stability of the sol is greatly improved and gelation typically occurs after a few ten minutes allowing the complete mixing of components and further processing of the sol, e.g. for obtaining particular shapes for the implant. As for the BG, the last step of the synthesis consists in drying the gel at 60°C for twenty-four hours to remove solvents. A gelatin-BG hybrid is then finally obtained in a simple way, especially when compared to previous works employing inert, and/or harmful solvents atmosphere.[13, 22]

For the synthesis of class II gelatin-BG hybrids, 3-glycidoxypropyltrimethoxysilane (GPTMS), is used as coupling agent to bridge the gelatin and silicate networks together, following the procedure first proposed by Ren et al.[9] for hybrid silica-gelatin system. The mechanism of GPTMS reaction is well documented in the literature.[23, 24] GPTMS is first added in the gelatin solution and the obtained functionalized gelatin with terminal silanol arms is then introduced in the inorganic solution allowing the bridging of the organic and inorganic networks. This procedure has been carried out to obtain class II BG hybrids with varying organic–inorganic proportions, up to 70 wt.% gelatin, the hybrid sol still remaining stable over a few ten minutes. The inorganic composition of the obtained BG hybrids was checked through SEM-EDS measurements on pressed pellets to avoid errors due to uncontrolled surface geometry. The measured inorganic compositions are very close to the expected values (73% SiO_2 and 27% CaO) for both class I and II hybrids.

As mentioned above, the incorporation of calcium ions into the silicate network at low temperature is a key point in the synthesis of hybrid BG. This was investigated by ^{29}Si solid-state NMR which allows evidencing the various kinds of Si units forming the inorganic network of the hybrid BG materials. The ^{29}Si MAS NMR spectra of the pure BG, class I and class II gelatin–BG hybrid samples (obtained in the same conditions regarding pH of the sol, temperature, ageing and drying conditions) are shown in Fig. 1a.

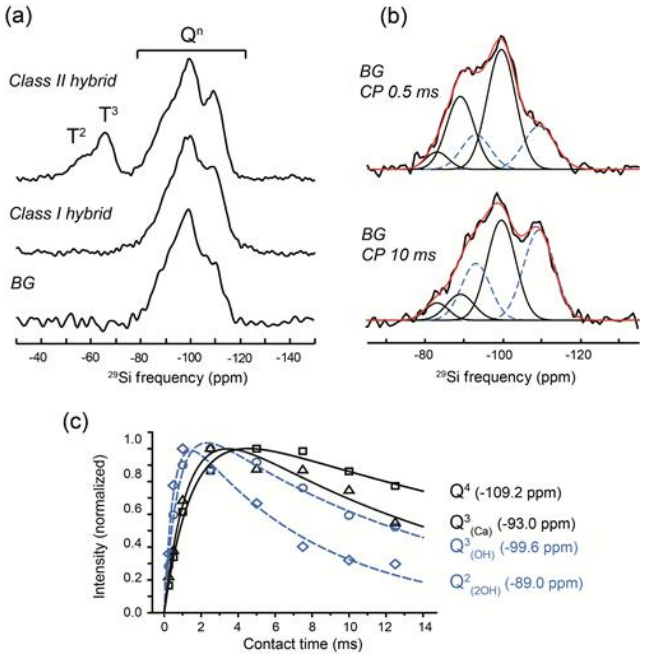

Fig. 1 (a) ^{29}Si MAS NMR spectra of pure bioactive glass (bot-tom), class I hybrid (middle) and class II hybrid (top) bioactive glasses. (b) ^{29}Si CP MAS NMR spectra of the pure bioactive glass (black) recorded with CP times of 0.5 (top) and 10 ms (bottom) and their best simultaneous fits (red). (c) Variations of the intensities of the individual Q^n resonance as a function of the CP time

For all samples, the ^{29}Si MAS spectra exhibit several overlapping contributions associated to Q^n units (SiO_4 tetrahedra with n bridging oxygen atoms): two resonances at -109.2 and -99.6 ppm which can be assigned to Q^4 and Q^3_H units respectively,[24] and a broader component at about -92 ppm covering the chemical shift ranges of Q^2_H and Q^3_{Ca} units.[21,22] Evidence for the presence of both Q^2_H and Q^3_{Ca} units was obtained from ^{29}Si-^{1}H cross-polarization (CP) MAS experiments. The ^{29}Si CP MAS spectra of the pure BG obtained for short and long CP times are shown in Fig. 1b. To account for the observed lineshape modifications and obtain good fits of the spectra recorded with different CP times (Fig. S1), two peaks at -93.0 and -89.0 ppm and a peak of much weaker intensity at -83.0 ppm are required in addition to the Q^4 and Q^3_H resonances. According to their chemical shifts, these resonances are assigned to Q^3_{Ca}, Q^2_H and $Q^2_{H/Ca}$ units, respectively.[24, 25] This assignment is also consistent with the CP time constants (T_{SiH}) determined from the intensity variations of each individual resonance as a function of the CP time (Fig. 1c): similar T_{SiH} of 1.5 and 1.3 ms are found for Q^4 and Q^3_{Ca} units, while Q^3_H and Q^2_H units (with stronger Si-H dipolar interactions) exhibit shorter T_{SiH} of 0.8 and 0.5 ms. These ^{29}Si NMR spectra thus indicate that Ca ions take part in the silicate network of the BG obtained at low temperature. Similar results were obtained for both class I and class II hybrids (Fig. S3), indicating that calcium ions were successfully incorporated into the inorganic part of the hybrid network, as in the case of the pure BG. In addition to these Q^n resonances, the ^{29}Si MAS NMR spectra of the class II hybrid show two peaks at -56.5 and -65.6 ppm characteristic of T^2 and T^3 units resulting from the hydrolysis/condensation of the terminal methoxysilane groups (T^0) of GPTMS. Despite polycondensation between GPTMS

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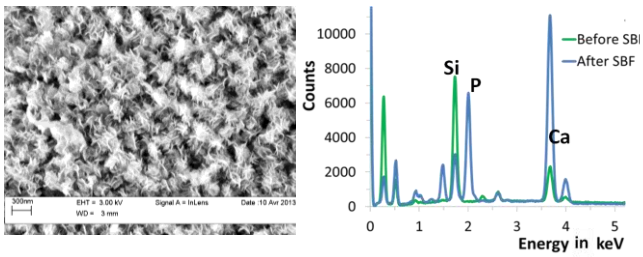

Fig. 2 SEM image of 50 wt.% SiO_2-CaO (75-25 wt.%) – 50 wt.% gelatin class II hybrid surface after7 days soaking in SBF (bar is 300 nm), and corresponding SEM-EDS spectrum. EDS spectrum before soaking in SBF is also shown for comparison.

chains cannot be excluded, the high occurrence of T^3 units suggests that, to some extent, GPTMS is successfully grafted to the BG network, thereby coupling the organic and inorganic parts of the hybrid.

The in vitro apatite-forming ability of a material is the commonly retained criteria for assessing its bioactivity and hypothesizing that it will be able to bond to living bone. Here it was investigated by immersing BG hybrid powders in Kokubo's simulated body fluid (SBF)[26] for up to 7 days. SEM images (Fig. 2 and Fig. S7) show that the surfaces of class I and class II BG hybrids are uniformly covered by flakes and needle-like materials after a few days in SBF. Deposition of needle-like and flakes precipitates typically occur during apatite-like formation.[27, 28] SEM-EDS measurements investigated the resulting changes regarding inorganic elements: after 7 days soaking in SBF, high amounts of both calcium and phosphorus are detected at the surface of the initially phosphorus-free BG hybrids, the oxide composition being measured as 27% SiO_2- 49% CaO - 24% P_2O_5 and 11% SiO_2- 56% CaO - 32% P_2O_5 for class I and class II BG hybrids respectively (see Fig. S5 and S6). The calcium versus phosphorus ratio ranges between 1.6 - 1.9 and matches the characteristic values of bone mineral and "biological" apatites.[29] These results confirm the in-vitro bioactivities of synthesized BG hybrids.

To conclude, the alkoxide route has been successfully used to elaborate BG hybrid materials under a simple and versatile synthesis. For the first time gelatin can be mixed at the molecular level with an inorganic BG while a great control of calcium incorporation within the BG network is allowed. Importantly, thanks to dilute conditions and limited introduction of water, a very stable inorganic sol is obtained. It allows further manipulation and handling of the sol to produce hybrid scaffolds by one of the many available manufacturing techniques. This process is also compatible with a variety of other biopolymers that can be easily dissolved in water. Both class I and class II BG hybrids with promising bioactive behaviors can be obtained, allowing a further control of the mechanical properties and degradation rates by coupling the organic and inorganic phases.

Notes and references

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† Electronic Supplementary Information (ESI) available: [NMR experimental conditions, additional ^{29}Si-^{1}H CP MAS spectra, details of the simulations of the CP curves, chemical shifts and relative proportions of T^n and Q^n species for the different samples, SEM-EDX analyses of BG hybrid before and after soaking in SBF]. See DOI: 10.1039/b000000x/

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