Electronic Supplementary Information

Bioactive glass hybrids: a simple route to Gelatin/SiO₂-CaO System

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1. Solid-state NMR

All solid-state NMR experiments were performed on a Bruker Avance I spectrometer operating at a magnetic field of 9.4 T (¹H and ²⁹Si Larmor frequencies of 400.2 and 79.5 MHz) using 4 mm and 7 mm double resonance MAS probehead. The ²⁹Si quantitative MAS spectrum of the pure BG sample was recorded at a spinning frequency of 14 kHz (4 mm rotor) with a pulse duration of 1µs (corresponding to a flip angle of 30°) and a recycle delay of 10s. The ²⁹Si quantitative MAS spectra of the class I and class II hybrid samples were acquired at a spinning frequency of 6 kHz (7 mm rotor) with a pulse duration of 2µs (30° flip angle) and a recycle delay of 10s. The ²⁹Si-¹H cross-polarization (CP) MAS^{1,2} spectra of all samples were recorded at a spinning frequency of 14 kHz. The ¹H and ²⁹Si nutation frequencies were set to 62 and 48 kHz during the CP time. The ¹H 90° pulse length was 4 µs and the recycle delay was set to 1s. In all case ¹H SPINAL-64 decoupling³ (¹H nutation frequency of 46 kHz) was applied during acquisition. ²⁹Si chemical shifts were referenced relative to TMS. All spectra were fitted with the DMfit program.⁴

The ²⁹Si CP MAS spectra of the pure BG recorded at various CP times are shown in Figure S1. As mentioned in the text, all spectra were fitted simultaneously and to account for the observed lineshape modifications, five individual Gaussian contributions were required. According to their ²⁹Si chemical shifts, these five resonances, located at -109.2, -99.6, -93.0, -89.0 and -83.0 ppm, are assigned to Q⁴, Q³_H, Q³_{Ca}, Q²_H and Q²_{H/Ca} units respectively.^{5,6} The intensity variations of these resonances as a function of the CP times are shown in Figure S2. The initial raises of these curves directly depend on the CP constant time (cross-relaxation time, T_{SiH}) which is inversely proportional to the ²⁹Si-¹H heteronuclear dipolar couplings. Because the magnitude of the dipolar coupling is inversely proportional to the cube of the inter-atomic distance, T_{SiH} values can in turn provide information about the proximities between ¹H and ²⁹Si.^{1,7} The results were analyzed using the classical model^{1,8} assuming that the ²⁹Si longitudinal relaxation time in the rotating frame (T_{1ρ}^{Si}) is much longer than the cross-relaxation time T_{SiH}. Accordingly, the experimental curves were fitted to the expression:

 $I(t) = I_0 (1 - T_{SiH} / T_{1\rho}^{H})^{-1} [exp(-t/T_{1\rho}^{H}) - exp(-t/T_{SiH})],$

where $T_{1\rho}^{H}$ is the ¹H longitudinal relaxation time in the rotating frame.^{7,8}

From the fits of the experimental values to the classical model, very similar T_{SiH} values of 1.5 and 1.3 ms are found for the Q⁴ and Q³_{Ca} units, respectively. In the case of the hydroxylated Q³_H and Q²_H units which exhibit stronger Si-H dipolar interactions, shorter T_{SiH} of 0.8 and 0.5 ms are obtained, as it could be expected. The analysis of the CP curves thus confirms the resonance assignment based on ²⁹Si isotropic chemical shift values.

Evidence for the presence of Q³_{Ca} units then indicates that Ca ions are incorporated into the silicate network of the BG obtained at low temperature. ²⁹Si-¹H CP MAS experiments with different CP times were also performed for class I and class II hybrid materials. The obtained spectra are shown in Figure S3. As in the case of the pure BG, the lineshape variations of these spectra give evidence for the presence of Q³_{Ca} units, indicating that calcium ions were successfully incorporated into the inorganic part of the network of both class I and class II hybrids. For the pure BG, class I and class II hybrids, the relative proportions of the various Qⁿ and Tⁿ units determined from simulations of the ²⁹Si quantitative MAS spectra are reported in table S1 (positions and linewidths of the Qⁿ resonances were fixed to the values previously determined from CP MAS experiments with different CP times).



Figure S1: Experimental (black) ²⁹Si CP MAS spectra of the pure BG sample recorded with CP time of (a) 0.25, (b) 0.5, (c) 1.0, (d) 2.5, (e) 5.0, (f) 7.5, (g) 10.0 and (h) 12.5 ms and their best fits (red). The individual Qⁿ resonances are shown below the experimental spectra.



Figure S2: Intensity variations of the individual Q^n resonance as a function of the CP time. Experimental values (symbols) were fitted to the classical model (lines) to obtain T_{SiH} values.



Figure S3: Experimental (black) ²⁹Si CP MAS spectra of the class I (a-c) and class II (d-f) hybrid materials recorded with CP time of (a, d) 0.5, (b, e) 2.5 and (c, f) 10.0 ms and their best fits (red). The individual Qⁿ resonances are shown below the experimental spectra.



Figure S4: ²⁹Si quantitative MAS spectra of the pure BG, class I and class II hybrids and their best fits using the parameters obtained from CP MAS experiments with different CP times.

Table S1: Chemical shifts (\pm 0.5), full width at half maximum (\pm 0.5) and relative proportions (\pm 3) of Tⁿ and Qⁿ resonances in pure BG, class I and class II hybrids determined from ²⁹Si quantitative MAS spectra.

Units	δ	FWHM	Relative intensity (%)		
	(ppm)	(ppm)	BG	Class I H	Class II H
Q^4	-109.2	9.4	26	33	24
$Q^3{}_{\rm H}$	-99.6	8.6	40	38	32
$Q^{3}Ca$	-93.0	8.7	18	14	12
$Q^2{\rm H}$	-89.0	8.3	12	11	10
$Q^2_{H/Ca} \\$	-83.0	7.0	4	4	4
T ³	-56.5	7.2	-	-	12
T^2	-65.6	7.7	-	-	6

2. In vitro bioactivity assay in SBF

The ISO-23317 standard procedure was followed. Briefly, c- SBF2, a protein-free solution of inorganic composition close to human blood plasma, was prepared following recommendations of Bohner et al.³⁸ Hybrid powders were immersed in SBF at a 1 mg/mL ratio for up to 7 days, keeping a constant 37°C temperature. After interaction, aliquots of the solution are taken for determination of the fluids composition and gelatin dissolution measurements, while the hybrid particles are carefully rinsed with pure ethanol and dried to avoid further mineralization reactions.

3. Gelatin dissolution measurements

Gelatin concentration in extracts of SBF were evaluated using the Thermo Scientific μ -BCA protein assay kit, which involves bicinchoninic acid (BCA) to detect Cu¹⁺ ions which are formed when Cu²⁺ is reduced by gelatin in an alkaline environment. A purple-colored reaction product is formed and absorbance of the solution at 562 nm is measured using a UV-visible spectrophotometer. The gelatin concentration is obtained from absorbance values which were previously calibrated using standard solutions.

4. Samples preparation for TEM and PIXE analyses

The dry hybrid powders after interaction with SBF were embedded in resin (AGAR, Essex, England). For PIXE analyses, 40 μ m cross-sections of materials were cut using a LEICA RM 2145 microtome. For TEM, 100 nm ultrathin cross-sections were cut using a LEICA EM UC6 with diamond knives.

5. PIXE nuclear microprobe analysis

PIXE (Particle-Induced X-ray Emission) quantitative microanalysis of the cross-sections was carried out using the nuclear microprobes at the AIFIRA platform, CENBG (Centre d'Etude Nucléaire De Bordeaux-Gradignan), France. The key advantage of the PIXE method is its extreme sensitivity (up to the 10^{-6} g/g level) compared to conventional electron probe microanalysis (SEM-EDXS). We used the AIFIRA nanobeam line with a 3 MeV incident proton beam and 1 µm beam diameter. X-ray detection was performed using a 80 mm² Si (Li) detector orientated at 135° with respect to the incident beam axis and equipped with a beryllium window 12 µm thick and an aluminium funny filter with a tiny hole of 2 mm in its center mounted on the detector.

Quantification was done using the Gupixwin software and was calibrated against NIST 620 (soda-lime glass) standard reference material.



Fig. S5 PIXE chemical imaging of cross-sections of gelatin/SiO₂-CaO (50wt.%/50wt.%) hybrid particles as synthesized.



Fig. S6 PIXE chemical imaging of cross-sections of class I gelatin/SiO₂-CaO (50wt.%/50wt.%) hybrid particles after 3 days immersion in SBF showing the distribution of Si, Ca, and P respectively. Corresponding inorganic composition is measured as follows: Si = 0.34 wt.%, P = 18.70 wt.%, Ca = 33.49 wt.%.

6. TEM

A Phillips CM 20 microscope (LaB₆ thermoelectronic gun) operating at 200 kVwas used to study the structure and morphology of hybrid particles after soaking in SBF. The images were recorded with a Keenview CCD camera with 18.67 μ m pixel size, and processed with the analySIS software. Home standards were used for calibration, and the SAED diffraction patterns were analyzed using ImageJ and CaRine softwares.

A shown in Figure S6a, the TEM image of a cross-section of gelatin/SiO₂-CaO class-II hybrid after 3 days immersion in SBF evidences agglomerates of needle-like nanocrystals. The corresponding electron diffraction pattern (Fig. S6b) is characteristics of hexagonal-HA (space group P 63/m) nanocrystals smaller than 500 nm.⁹ The 11 most intense characteristic reflections of HA are all observed in Fig. S6b. The first ring belongs to the (002) planes with $d_{002} = 0.34$ nm, the second one to (012) planes ($d_{012} = 0.32$ nm), the strong and broad third ring is formed by several reflections, namely (211), (112) and (030) with interplanar spacings ranging from 0.27 to 0.28 nm.^{9, 10} Other reflections are (022) ($d_{022} = 0.26$ nm), (130) ($d_{130} = 0.23$ nm), (222) ($d_{022} = 0.19$ nm). The seventh ring has two contributions from (132) and (213) planes ($d_{213} = 0.18$ nm, $d_{132} = 0.19$ nm). Finally the eighth ring corresponds to (004) ($d_{004} = 0.17$ nm).¹¹.



Fig. S7 (left) TEM image of a cross-section of gelatin/SiO₂-CaO class-II hybrid after 3 days immersion in SBF, (right) Corresponding electron diffraction pattern showing the presence of hydroxyapatite nanocrystals (the 11 most intense reflections characteristic of the HA hexagonal structure are indicated).

7. SEM-EDS characterizations

A field emission scanning electron microscope (FEG-SEM) Model Zeiss Supra 55VP was used to image the sample surfaces surface before and after soaking in SBF. The samples were mounted on carbon tape and sputter coated with carbon. The inorganic compositions of the pure BG, class I and class II hybrids were checked by Energy-Dispersive Spectroscopy (EDS) which allows qualitative chemical analysis of the sample surfaces. The NIST SRM 1411 (soft borosilicate glass) has been used as standard reference material for EDS quantitative analysis. For the EDS analysis, the powdered samples were pressed into a pellet mounted on a carbon tape and covered with carbon.



Figure S8. SEM-EDS spectra of class I hybrid bioactive glass powder (50 wt.% SiO₂-CaO (75-25 wt.%) - 50 wt.% gelatin) before and after 7j soaking in simulated body fluid (SBF).



Figure S9. SEM-EDS spectra of class II hybrid bioactive glass powder (50 wt.% SiO₂-CaO (75-25 wt.%) – 50 wt.% gelatin) before and after 7j soaking in simulated body fluid.



Figure S10. SEM images of class I (left) and class II (right) hybrids after 3 days soaking in SBF.

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