Stability of bioactive bone graft substitutes exposed to different aging and sterilization conditions

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Abstract
Bioactive glasses have been used for many years as bone graft substitutes in orthopedic and dental applications as well as an additive in toothpastes, cosmetics, and cosmeceutical products. The interest of using bioactive glass comes from its ability to dissolve and release dissolution products that stimulate bone regeneration. Porous bioactive glass scaffolds that can provide structural support while bone is growing into the structure have generated interest. However, little data is available in the literature on the effect of environmental conditions or sterilization treatments on the structure and properties of these materials. This study presents the evolution of the structure and microstructure of bioactive foams exposed to different accelerated and real-time aging conditions and sterilization treatments. The results indicate that the material is relatively stable. For example, different sterilization methods (steam, ethylene oxide, hydrogen peroxide, gamma-rays) have limited effect on the structure and properties of the foams. However, carbonate species may form on the surface of the material when exposed to CO2 and humidity. Some carbonates dissolve rapidly in water and may impact the pH of the solution. Adequate packaging should limit the reaction of the bioactive glass with CO2 and humidity and the formation of carbonate.

KEYWORDS
aging, bioactive glass, porous structure, stability, sterilization

1 | INTRODUCTION

In the early 1970s, Hench et al1 reported that soda-lime-phosphate-silicate glass (SiO2–P2O5–CaO–Na2O) implanted in rat femurs enhanced ossification of unmineralized tissues at bone-implant interface. Since then, these materials also identified as bioactive glasses or bioactive glass-ceramics have generated significant interest due to their resorbability and capacity to stimulate bone regeneration. The 45S5 composition (24.5Na2O–24.5CaO–6P2O5–45SiO2 in wt. %) is by far the most widely investigated bioactive glass. Numerous studies reported on the dissolution of bioactive glass and glass-ceramic composites2–4 as well as their bone-bonding ability.5–10 The US Food and Drug Administration approved the first bioactive glass medical device in 1985 for ossicular reconstruction prosthesis. Since then, the organization has approved numerous products such as endosseous ridge maintenance implant (1988), particles used to restore bone loss from periodontal disease (1993), bone grafts in tooth extraction sites and alveolar ridge augmentation (1996), general orthopedic bone grafting in nonload bearing sites (2000), particulates for use in dentinal hypersensitivity treatment (2004), bioactive glass scaffold (2014),...
and a series of products based on bioactive glass fibers from 2014 and 2017.\textsuperscript{11} These materials are presently commercialized in the form of granules (Biogran®, BonAlive®, PerioGlas®, GlassBone\textsuperscript{TM}, FIBERGRAFT\textsuperscript{®} Activioss\textsuperscript{TM}) and charges in pastes and putties (Activioss\textsuperscript{TM}, BonAlive®, NanoFUSE®, BioSphere®, Vitoss\textsuperscript{TM}, NovaBone\textsuperscript{®}) and have been used in more than 1.5 million of various surgeries.\textsuperscript{12} Recently, different groups around the world have developed 3D porous structures that can support some load.\textsuperscript{13–17}

Despite the fact that the properties of bioactive glass is well documented (more than 1000 publications so far), there is little information on the stability of the material during storage or sterilization treatments. Cerruti et al\textsuperscript{18} observed calcium carbonate formation on shelf-aged 58S bioactive glass powders (60% SiO\textsubscript{2}, 36% CaO, and 4% P\textsubscript{2}O\textsubscript{5} in mol\%) using Fourier-transform infrared spectroscopy (FTIR). Different mechanisms of carbonate formation at the surface of 58S glass were investigated. Carbonate formation appeared to occur only in the presence of both CO\textsubscript{2} and excess water. Cerruti et al\textsuperscript{19} observed carbonates on 45S5 glasses, which was confirmed by the presence of a sharp peak at ~1500 cm\textsuperscript{−1} through FTIR. The carbonate formation was associated to the reaction of glass with atmospheric CO\textsubscript{2}. Magallanes-Perdomo et al\textsuperscript{20} observed the presence of carbonate on the surface of bioglass particles with different levels of crystallinity and showed that the carbonate may form both on glass and glass-ceramic particles. The carbonate formation was correlated to adsorbed atmospheric CO\textsubscript{2} and/or CO\textsubscript{2} dissolved in the melt-derived glass process. The carbonate peak observed at ~1449 cm\textsuperscript{−1} through FTIR disappeared after 2-hour immersion in simulated body fluid (SBF).

Observations of bioactive glass foams (45S5 compositions) stored under uncontrolled conditions (room temperature in air) showed the presence of crystal-like structures (ie, whiskers) on the surface of the material.\textsuperscript{21} However, little attention was paid so far on the composition and structure of the whiskers, on the mechanism of formation and their impact on properties and biocompatibility of the material. The aim of this study was to evaluate the stability of bioactive glass scaffolds exposed to different environments or sterilization treatments, investigate crystal formation on the surface of the foams when aged under different conditions and estimate the potential impact of these crystals.

2 | MATERIALS AND METHODS

2.1 | Preparation of bioactive glass foam samples

Bioactive glass foams were produced using a powder metallurgy process, where bioactive glass powder (24.5Na\textsubscript{2}O–24.5CaO–6P\textsubscript{2}O\textsubscript{5}–45SiO\textsubscript{2} in wt. %, SCHOTT Vitryxx\textsuperscript{®}) is dry-mixed with a solid polymeric binder (phenolic resin, Varcum\textsuperscript{®}) and a foaming agent (p-toluenesulfonylhydrazide, Celogen\textsuperscript{®} TSH-C). The resulting mixture is poured into tubular molds (Ø32.5 mm) and subjected to a three-step thermal treatment (Figure 1). During the foaming step, the binder melts creating a suspension with the glass particles. The foaming agent then decomposes and releases a gas expanding the structure and creating porosity. Deposition is then carried out to eliminate the binder through thermal decomposition. The sintering step is performed to create bonds between glass particles and provide mechanical strength to the material. The specimens were then machined into cylinders (Ø = 10 mm, H = 7.5 mm) for further analysis.

2.2 | Morphological characterization

The microstructure was observed using scanning electron microscopy (SEM, Hitachi S-4700) and microcomputed tomography (µCT, XTek HMXST 225; Nikon) on one sample per tested condition. The µCT scanner was equipped with a Perkin-Elmer 1621 AN amorphous silicon flat panel (409.6 × 409.6 mm) coupled to a CsI scintillator. The X-ray source was operated at 104 kV and 36 μA, with an integration time of 1000 ms, 3142 projections over 360° and one frame per projection. A minimal focal spot size of 7 μm was obtained under these operating conditions. CT Pro 3D and ORS VISUAL software were used to rebuild 3D images and to analyze reconstructed images, respectively. Pore size distribution was evaluated using CLEMEX Vision PE\textsuperscript{TM} image analyzing software on 12 2D slices extracted from 3D reconstructed images.

2.3 | Chemical and physical characterization

The porosity of each cylinder was calculated using the following equation

![FIGURE 1](image-url) Thermal treatment of bioactive glass foams process
where \( P \) is the calculated porosity (%), \( m \) is the measured mass (g), \( d \) is the measured diameter (cm), \( h \) is the measured height (cm), and \( \rho \) is the theoretical density of bioactive glass (2.7 g cm\(^{-3}\)). For each tested condition, measurements and calculations were averaged on five samples.

Chemical characterization was accomplished using energy-dispersive X-ray spectroscopy (EDS) for elemental analysis, X-ray diffraction (XRD), Fourier-transform infrared (FTIR) spectroscopy, mid-infrared (MIR) reflectance spectroscopy, and pH measurements after water immersion on one sample for each tested condition.

The crystalline phase analysis was completed using XRD (AXS D8 Discover Diffractometer; Bruker) with Cu \( \kappa \alpha \) radiation. The patterns were recorded on the 20-90° 2θ range, using a 0.02° step size.

Fourier-transform infrared spectroscopy was performed using a Nicolet™ iS50R spectrometer with a diamond attenuated total reflectance module and DTGS KBr detector, as the sum of 64 accumulations with 4 cm\(^{-1}\) resolution within the 4000-525 cm\(^{-1}\) range. Data were analyzed using OMNIC™ Specta software.

Mid-infrared reflectance spectroscopy was measured using a bi-directional laser-based reflectance setup with a tunable external cavity quantum cascade laser (EC-QCL) source (Block Engineering, LaserTune). The tunable laser source (wavelength of 5.2-13.4 μm) was operated at a pulse repetition rate of 10 kHz and a pulse duration of 100 ns. The MIR laser beam was focused on the sample with a spot diameter of 400 μm × 500 μm using a gold parabolic mirror at normal incidence angle. The working distance was 10 cm. The reflected light was collected and focused on a 1 x 1 mm\(^2\) MCT detector (Infrared Associate, FTIR-16), The detector was operated at 77 K using liquid nitrogen. The spectra were formed by continuously tuning the EC-QCL emission wavelength and by recording each pulse amplitude value at different wavelengths. The spectrum measurement was repeated at 100 different positions on the sample. The raw spectra were smoothed using a moving average filter and normalized using the reflectance spectrum of a KBr pellet.

pH measurements, samples were soaked in Milli-Q water at 37°C with a ratio of 1.5 g of bioactive glass foam per liter of water. Measurements were taken every 10 seconds up to a total of 180 seconds (Mettler-Toledo T50 Titratror).

2.4 | Mechanical characterization

The mechanical characterization was completed using a universal tester (Instron 5582) equipped with a 100 kN load cell and a crosshead speed of 2.5 mm min\(^{-1}\). Uniaxial compression strength was defined as the maximum on stress-strain curves and completed on five samples for each tested condition.

2.5 | Accelerated aging

Aging of the foams was done under different conditions. Accelerated aging was completed following ASTM F1980 standard where samples were stored at 55°C and 10% relative humidity (RH) for 45 days, corresponding to 1 year of real-time aging at ambient conditions. Real-time aging was completed at room temperature (23°C) with 50% RH. Specimens were also aged in an incubator at 37°C with 90% RH and 5% CO\(_2\) to monitor the combined effect of carbon dioxide and humidity. Specimens were characterized before and after aging to evaluate the impact on material properties.

2.6 | Sterilization

Sterilization treatments were done according to ISO standards, except for the hydrogen peroxide treatment, which is not covered by standard procedures. Gamma-ray sterilization was done according to ISO 11137-2 standard at 25 kG\(\gamma\). Hydrogen peroxide sterilization was done at 50°C for 33 minutes using STERIS V-PRO system. Steam sterilization was done according to ISO 11134 standard for 5 minutes at 134°C and 33 psi. Ethylene oxide treatment was done according to ISO 11135 for 1 hour at 55°C.

3 | RESULTS AND DISCUSSION

3.1 | Sample preparation and properties of bioactive glass foams

The foam specimens showed good structural integrity and could be handled and machined easily without damage. The initial compressive strength of the foams was 10.2 ± 1.6 MPa. The calculated average porosity was 67%. The open network was relatively uniform across the diameter of the cylinders, as shown on the 2D cross section extracted from 3D microtomography reconstruction (Figure 2).

The pore size distribution ranged between 50 and 700 μm, with a median pore size (D50) of 275 μm (Figure 3) as required for appropriate bone ingrowth.\(^{22}\) XRD analysis of foam specimens showed the presence of crystalline peaks (Figure 4), identified as combeite (\( \text{Na}_4\text{.5Ca}_3\text{.5Si}_6\text{O}_{18}\)) with a small amount of rhenanite (\( \text{CaNaPO}_4\)). This observation is coherent with previous
studies on the crystallization of bioactive glass, where nanocrystals of combeite and rhenanite precipitate in a matrix of residual glass during sintering.  

3.2 Accelerated aging and sterilization

Bioactive glasses have been used for many years in various orthopedic and dental applications. Little information is nonetheless available in the literature on the stability of this material with time, its sensitivity to the environment or sterilization treatments.

Accelerated aging at 55°C/10% RH for 45 days was chosen to reproduce 52 weeks of real-time aging. The results obtained in the present study showed no significant impact on microstructure of aged samples as shown by SEM analysis (Figure 5). The mechanical properties were also similar between aged and as produced samples (11.5 ± 0.7 MPa vs 10.2 ± 1.6 MPa, respectively).

Sterilization techniques tested in this study also had limited impact on microstructure (Figure 5). The mechanical properties of samples sterilized through hydrogen peroxide (11.7 ± 1.4 MPa), gamma-ray (11.7 ± 1.3 MPa), and ethylene oxide (10.2 ± 1.6 MPa) were similar to as produced samples (10.2 ± 1.6 MPa). Steam sterilization had a slightly higher compression strength (13.1 ± 2.0 MPa), although the difference was not statistically significant.

Accelerated aging at 55°C/10% RH for 45 days had limited effect and may not be a good indicator of the stability of the material in a humid environment. Hydrogen peroxide, gamma-ray, and ethylene oxide sterilization techniques also had limited impact on mechanical properties and microstructure of the foams. Only steam sterilization could be an indicator of the stability of the material in a humid environment, since this technique involves the use of saturated steam under pressure. A small increase of mechanical properties was observed after steam sterilization, although the duration of the treatment (5 minutes) was not sufficient to significantly alter the properties of the foams. These observations suggest that the chosen sterilization technique has limited impact on the properties of the foams.

3.3 Real-time aging

Specimens were subjected to real-time aging at room temperature. While the general aspect of the foams seemed
similar after 4 weeks at 23°C/50% RH, some crystal-like structures (ie, whiskers) were observed using SEM analysis (Figure 6).

Energy-dispersive X-ray spectroscopy analysis on these outgrowths showed large amount of Na and O, and also the presence of Si, P, and Ca. However, due to the size of the crystal-like structures (approximate length of 10 µm and thickness <1 µm) and the spatial resolution of the technique, it was difficult to confirm if Si, P, and Ca signals came from the whiskers or the surrounding matrix.

**FIGURE 4** X-ray diffraction patterns of as-produced samples (black) and aged 4 wk at 23°C/50% relative humidity (red)

**FIGURE 5** Scanning electron microscopy micrographs of specimens (A) as produced, (B) aged 45 d at 55°C/10% relative humidity and after (C) gamma-ray, (D) hydrogen peroxide, (E) steam, and (F) ethylene oxide sterilization
XRD analysis of the foams aged 4 weeks at 23°C/50% RH showed similar diffraction pattern to the as-produced samples (Figure 4). The volume of the crystal-like structures as observed by SEM was most likely too small to be detected by XRD (detection limit around 1%) with the equipment, method, and specimens used.

Fourier-transform infrared spectroscopy analysis was then conducted on samples aged 4 weeks at 23°C/50% RH to determine if the technique would be more sensitive than XRD (Figure 7). A peak at 1460 cm⁻¹ was detected on the aged sample, corresponding to a carbonate specie. This observation is consistent with Cerruti et al who associated peaks at 1460 cm⁻¹ on FTIR spectrum with calcium carbonate developed during shelf-aging of 58S bioactive glasses powder (60% SiO₂, 36% CaO, 4% P₂O₅ mol %).18

Specimens aged at room temperature (23°C/50% RH) in air were covered by crystal-like structures, which was not the case for specimens treated at higher temperature and lower humidity content (55°C/10% RH). These observations suggest that temperature is not critical in activating the reaction and the effect of humidity is much more important. The amount of crystal-like structures formed on specimens aged 4 weeks at 23°C/50% RH was too small to allow precise characterization by EDS, FTIR, and XRD.

The present observations are consistent with those from Magallanes-Perdomo et al who observed carbonate on bioactive glass particles with different levels of crystallinity.20 Carbonate formation can be attributed to the interaction between bioactive glass and atmospheric CO₂ in the presence of excess water.

Carbonate formation on bioactive glass powders has been reported in the literature,18-20 but not on foams. While some studies suggested that the material might react with CO₂ in the presence of humidity, information of the effect of aging conditions on the formation of carbonate was not available. The impact on carbonate formation on the microstructure (ie, formation of crystal-like structures) or dissolution was not disclosed either.

3.4 | Accelerated aging with high humidity

Additional aging at 37°C/90% RH with 5% CO₂ was performed to accelerate the kinetics of whisker formation. A total humid mass increase of 30.2%, 46.6%, and 49.0% was observed after 1, 2, and 4 weeks of aging, respectively, confirming the reactivity of the bioactive glass in the presence of humidity and CO₂.

Scanning electron microscopy observations of samples aged 1 week at 37°C/90% RH with 5% CO₂ confirmed the presence of crystal-like structures (Figure 8). However, the appearance of the crystals was different from those observed on the foams aged at 23°C/50% RH. This suggests that aging conditions not only impact the kinetics of formation of the crystals but also their morphology. Aging at 37°C/90% RH with 5% CO₂ significantly increased the amount of crystal-like structures found on the surface, which decreased the overall porosity of bioactive glass foams and thus increased the average compression strength by 31.5% after 4 weeks when compared to unaged samples (11.2 ± 1.2 vs 8.5 ± 1.1 MPa).

XRD analysis of the foam aged 2 weeks at 37°C/90% RH with 5% CO₂ showed the presence of new crystalline species (Figure 9). The peaks were referenced to sodium and calcium carbonates (essentially NaHCO₃ with small amount of CaCO₃) and confirmed the reaction of the bioactive glass with atmospheric CO₂ during aging since no other source of carbon was present in the foams. The absence of carbon in samples after sintering and before aging was confirmed through elemental analysis using a LECO analyzer, with 0.03% of carbon content compared to 0.26% for the bulk bioactive glass powder.

Mid-infrared reflectance spectroscopy measurements conducted on bioactive glass foams aged 4 weeks at 37°C/90% RH with 5% CO₂ also showed peaks associated to sodium bicarbonate (NaHCO₃) and calcium carbonate (CaCO₃) (Figure 10). These peaks related to carbonates were not observed on unaged specimens, as for XRD measurements.

Dissolution tests were carried out to evaluate the potential impact of carbonates in aqueous solutions. SEM observations of bioactive glass foam aged 6 months at 23°C/50% RH showed a substantial amount of crystal-like structures covering the surface (Figure 11). The sample was immersed in water at room temperature for 2 minutes and SEM observations showed that the whiskers completely dissolved. These results are coherent with observations done by Cerruti et al through FTIR analysis showing the
disappearance of carbonates on 45S5 powders after soaking 30 seconds in 0.05 mol L$^{-1}$ TRIS-buffered solution (pH 7.4).\textsuperscript{19}

Rapid dissolution of carbonates leads to a release of ions and increase in pH. This was confirmed by tests showing the variation of pH with time of aged and unaged specimens immersed in water (Figure 12). The results obtained showed that the carbonates (essentially sodium bicarbonate) dissolved very quickly in water, leading to faster pH increase. This is consistent with the water solubility of sodium bicarbonate,
which varies between 6.5 and 19.1 g cc\(^{-1}\) for temperatures between 0 and 100°C, respectively.\(^{25}\)

This observation may be of importance when conducting in vitro or in vivo assays. In fact, inadequate storage of the specimens prior to testing can lead to carbonate formation, affect pH after implantation and thus impact the surrounding environment and the results. Care must be taken accordingly to store the devices in humidity-free and CO\(_2\)-free packaging.
However, it is believed that aging of bioactive glass foams should not be clinically significant since bone graft substitutes are usually packaged under inert environment after fabrication. Caution must also be taken to control aging of the raw material (ie, powder). The observations reported in this study with foam specimens should likely happen when the material is in powder form, as reported in the literature. Consequently, the presence of carbonates may affect the manufacturing process of the material (eg, sintering, pH of the material when preparing putty or paste, etc). Once again, this could be easily controlled using appropriate storage and packaging.

4 | CONCLUSIONS

The present study shows that 45S5 bioactive glass foams are not stable at room temperature in atmosphere containing humidity and CO2. The material reacts with atmospheric CO2 in the presence of humidity to form crystal-like structures composed of sodium bicarbonate and calcium carbonate. The shape and density of the crystal-like structures vary with aging conditions (temperature, humidity, CO2 concentration) and appear like platelets when aged at 37°C/90% RH with 5% CO2. Dissolution of sodium bicarbonate in water is very rapid and affects the pH of the solution. The pH increase is faster in the presence of carbonates on the surface of the material. Standard sterilization treatments (ie, gamma-rays, ethylene oxide, steam, hydrogen peroxide) seem to have limited impact on microstructure and mechanical properties of the scaffolds. These observations are based, however, on preliminary trials and additional studies should be conducted to confirm the results.

The presence of carbonates on foam specimens could impact the pH, the surrounding environment, and influence the results. Using an appropriate packaging could limit carbonate formation on specimens. Caution should be taken if the material is used clinically as a bone graft substitute.

Besides the aging of the foam scaffolds, aging of the powder should also be investigated further. Carbonate formation might also impact manufacturing processes like sintering. It could also influence the interaction of the particles with their environment, such as pH of glass particle suspensions used in toothpastes, cosmetics, or graft substitute putties. The presence of carbonates within in vivo conditions should also be addressed to understand their impact on biocompatibility.

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