Calcium carbonate: Adored and ignored in bioactivity assessment

Calcium Carbonate: Adored and Ignored in Bioactivity Assessment

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Abstract

The title of this article could sound a bit curious to some readers since a layer of apatite – and not calcium carbonate – is well-known to form on the surface of bioactive glasses upon immersion in simulated body fluids. However, calcium carbonate (commonly reported as calcite crystals) can form on the surface of bioactive glasses as well, instead of or in competition with hydroxyapatite, during *in vitro* tests. Major factors that govern calcium carbonate formation are a high concentration of Ca^{2+} ions in the testing solution – and, in this regard, glass composition/texture and type of medium play key roles – along with the volume of solution used during *in vitro* tests. To date, this phenomenon has received relatively little attention and is still partly unexplored. This article provides a critical overview of the available literature on this topic in order to stimulate constructive discussion among biomaterials scientists and further research for better understanding the mechanisms involved in glass bioactivity.

Keywords: Calcium carbonate; Hydroxyapatite; Bone bonding; Bioactivity; Simulated body fluid; Bioactive glass; Calcium phosphate

1. A brief history of in vitro bioactivity assessment

Three decades ago, Kokubo et al. proposed a series of simulated body fluids (SBFs) to achieve *in vitro* simulations of animal studies [1]. This strategy has been further developed by many researchers as a standard test method for bioactivity assessment. In 2006, Kokubo and Takadama reviewed the topic in a paper entitled ‘‘How useful is SBF in predicting in vivo bone bioactivity?’’ [2] and reiterated that SBF could be still useful for bioactivity evaluation. Afterward, the biomaterials community declared that the formation of an apatite layer on the surface of a material soaked in SBF confirms its bioactivity and guarantees its bone bonding ability, *in vivo*. However, in 2009, Bohner and Lemaitre [3] criticized the topic in an article entitled “Can bioactivity be tested *in vitro* with SBF solution”, suggesting that the basic principles of the method suffer from serious elaboration to predict bone bonding capability.

It has been suggested that some aspects of the proposed protocol by Kokubo and Takadama for the preparation of SBF solutions might be doubtful. This is because of the complexity of the procedure which is time-consuming, tricky, and some contents (e.g., carbonate ions) are not controlled. Additionally, bioactivity measurement using SBF solutions may result in false positive and false negative results. Although there are many criticisms toward the use of SBF as a standard testing method for bone bonding ability of biomaterials *in vitro*, there is an increasing interest in this area as shown by the growing number of publications testing biomaterials in SBF solutions.

It is known that the developed SBFs are supersaturated of calcium and phosphorous solutions with respect to apatite formation. However, there are several studies in the literature on the formation of calcium carbonate as the calcite crystals before or along with the apatite

formation. It is worth mentioning that calcium carbonate has three different crystallographic forms including calcite, aragonite, and vaterite [4]. In the case of bioactive glasses contacted with physiological media, calcium carbonate usually precipitates as the calcite crystals [5-7]. More specifically, calcite formation has been frequently reported during the bioactivity assessment of bioactive glasses. This is another proof of concept toward better understanding the essential factors governing different steps of crystalline phases' nucleation and growth during bioactivity assessment. There are several factors responsible for the formation of calcite during the *in vitro* bioactivity assessment. Although some researchers have previously confirmed this phenomenon, recent debates failed to comprehensively discuss and acknowledge calcium carbonate formation and its impact on the accuracy of bioactivity tests.

2. Bioactive glasses and bioactivity assessment

Bioactive glasses, first developed by Larry Hench in Florida fifty years ago [8], revolutionized biomedical science and technology by concreting the idea that an artificial material could bond to living tissues without undergoing long-term rejection. Research on bioactive glasses still continues today opening new scenarios that expand the potential of these bioactive materials from "traditional" bone and dental regeneration [9-12] to a wide range of applications in contact with soft tissues [13-18]. *In vivo* studies showed that the Hench's original 45S5 Bioglass®, as well as many other formulations developed over the years, can bond to bone and stimulate more bone growth than synthetic apatitic phases [19]. The bone-bonding ability of bioactive glasses has been ascribed to a series of surface chemical reactions occurring in contact

with physiological fluids, which result in the formation of a surface layer of crystalline apatite [20], similar to the mineral phase of bone [21, 22].

In 1991, Kokubo [23] proposed that the formation of bone-like apatite crystals on biomaterials' surfaces is a key requirement to allow interfacial bonding to the bone, upon implantation in the living body. He also suggested that the apatite formation could be simulated in a solution with ion concentrations nearly equal to those of human blood plasma. The validity of this approach has been the matter of debate over the past decade and, today, most scientists agree that *in vitro* conditions cannot exactly match those *in vivo* [3]. In spite of these limitations, assessment of *in vitro* bioactivity of biomaterials indeed remains important and an ISO standard [24] has been proposed for the testing of the apatite-forming ability of bioactive medical implants using Kokubo's SBF [2]. However, there is still scope for further research and refinement of the methods as well as a better understanding of the mechanisms of bioactivity. In this context, the present article aims to shed light on a relatively unexplored phenomenon that may occur during *in vitro* testing of bioactive glasses, i.e., the formation of calcium carbonate instead of, or in competition with apatite upon immersion in the testing solution. The authors believe that glass dissolution, ion-release, and the consequent calcium carbonate and/or apatite formation may be influenced by many factors, as schematically indicated in Fig. 1. These factors including glass composition, glass structure, powder or bulk state of the material and the immersion media will be discussed in details in the next parts. To the best of the authors' knowledge, this is the first review article in which such issue is comprehensively covered and critically discussed in the light of the existing experimental studies.

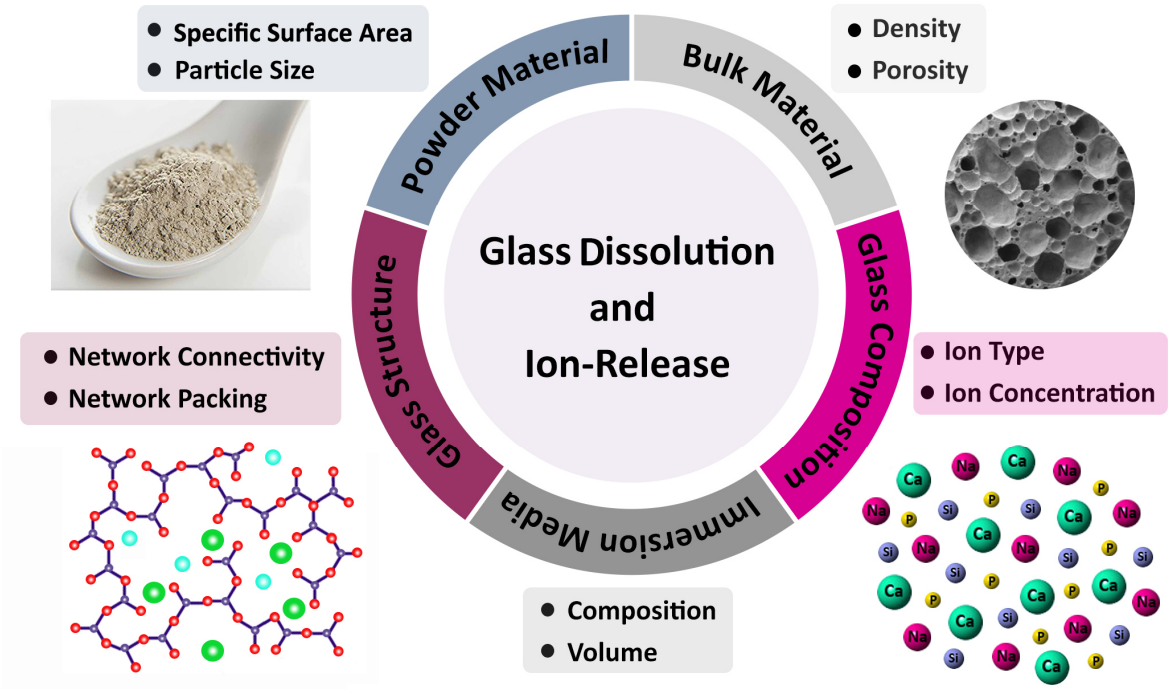

Fig. 1. A schematic overview of different factors affecting the glass dissolution, ion-release behavior, and biomineralization. The type and concentration of ions released from a bioactive glass are governed by the chemical composition of glass as well as its network connectivity and network packing. Bioactive glasses with less network connectivity and network packing release ions more easily due to the facilitated movement of ions through the glass structure. Moreover, the physical features of a bioactive glass as a bulk or powder material have a leading role in its dissolution behavior. The composition and volume of immersion media can concurrently control the glass dissolution behavior and the rate of ion release which consequently affect the biomineralization process. (Source; Authors)

2. Formation of calcium carbonate on the surface of bioactive glasses: an overview

2.1 Basics of the mechanism and role of calcium ions

Formation of calcium carbonate on the surface of bioactive glass-derived materials during *in vitro* tests has been reported in a relatively few numbers of studies. From a general viewpoint, there are many factors responsible for the precipitation of calcium carbonate, among which the high concentration of calcium ions (Ca^{2+}) in the surrounding medium plays a key role.

Calcium ions originate from both bioactive glasses and immersion media. The concentration of calcium ions released from bioactive glasses is in turn influenced by several factors including glass composition, glass structure and glass particle size. A role on glass dissolution could also be played by the pH of the medium, but no specific study on this aspect is available in the literature. This factor deserves to be investigated in the future as the pH of fluids at surgical wound sites (i.e., around the implant) could be different compared to physiologic conditions.

SiO_2-CaO-P_2O_5–based bioactive glasses have been deeply explored for bioactivity assessment *in vitro*; in general, calcium carbonate was observed to form on both melt-derived and sol gel bioactive glasses Mami *et al.* [25] investigated the surface reactivity of a sol-gel derived bioactive glass with a composition of 60SiO_2-35CaO-5P_2O_5 (in mol %), *in vitro*. For this purpose, uniaxially-compacted glass pellets were soaked in 8 ml of SBF for up to 7 days at 37 °C. The results of this study showed a high reactivity of the glass with a continuous release of calcium ions into the testing solution. Furthermore, Fourier-transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) analysis revealed that, upon soaking in SBF, the glass surface was progressively covered by a carbonated apatite layer as the main phase and calcium carbonate as the secondary phase. At 4 h of soaking in SBF, the analyses confirmed the presence of a well-crystallized calcium carbonate phase, which remained present over the whole soaking period (up to 7 days). In order to check whether the presence of calcium carbonate was merely related to the low volume of SBF used (supersaturation of the solution) or not, a second experiment was performed in which the glass samples were soaked in 30 ml of SBF for 4 h and 1 day [25]. The XRD patterns obtained from these samples confirmed the presence of the calcium carbonate phase on the glass surface at both time points. These findings suggest that the

simultaneous formation of apatite and calcium carbonate may occur on the surface of bioactive glass upon soaking in biological fluids when it releases a high content of calcium ions.

As previously described, the concentration of the released calcium ions could be greatly influenced by the variation of the glass composition. Through modification of similar bioactive glasses' composition and incorporation of zinc ions, Fan Goh et al. [26] could change the trend of calcite and apatite formation on the surface of bioactive glasses upon immersion in the same medium. They examined the simultaneous formation of calcite and apatite on the surface of bioactive glasses ($52SiO_2$-$45CaO$-$3P_2O_5$, in mol %) with 5 and 10 mol % Zn of the different overall composition during *in vitro* examinations at 14 and 21 days soaking in SBF. On the basis of microstructural evaluation, spherical apatite crystals with Ca/P ratios close to 1.67 precipitated on the surface of low Zn-containing glasses at both time intervals. The increase of Zn content to 10 mol % led to the formation of flake-like crystals of calcite with much higher Ca/P ratio. Fig. 2 indicates the FESEM micrographs of both glasses at 14 and 21 days' immersion in SBF as well as the EDS spectra taken from different crystal morphologies. Presumably, an increase of Zn content in the studied glass compositions weakened the glass structural bonds and promoted the dissolution rate. As a result, the SBF solution enriched with calcium ions which further raised the possibility of calcium carbonate precipitation (as the calcite) on the glass surface. It should be noticed that the apatitic surface layer formed during the immersion of bioactive glasses in physiological solutions usually adopts a "cauliflower-like" morphology. However, the apatite crystals actually consist of several needles aligned parallel to the c crystallographic axis [27].

It was pointed out that the formation of calcite nanocrystals could occur within the early hours of immersion in SBF [28-31]. The high release of calcium ions from the glass structure and

the presence of hydrogen carbonate ions in the SBF solution allow the precipitation of calcium carbonate according to the following reaction (1):

$$Ca^{2+} + 2HCO_3^- \rightarrow CaCO_3 + CO_2 + H_2O \quad (1)$$

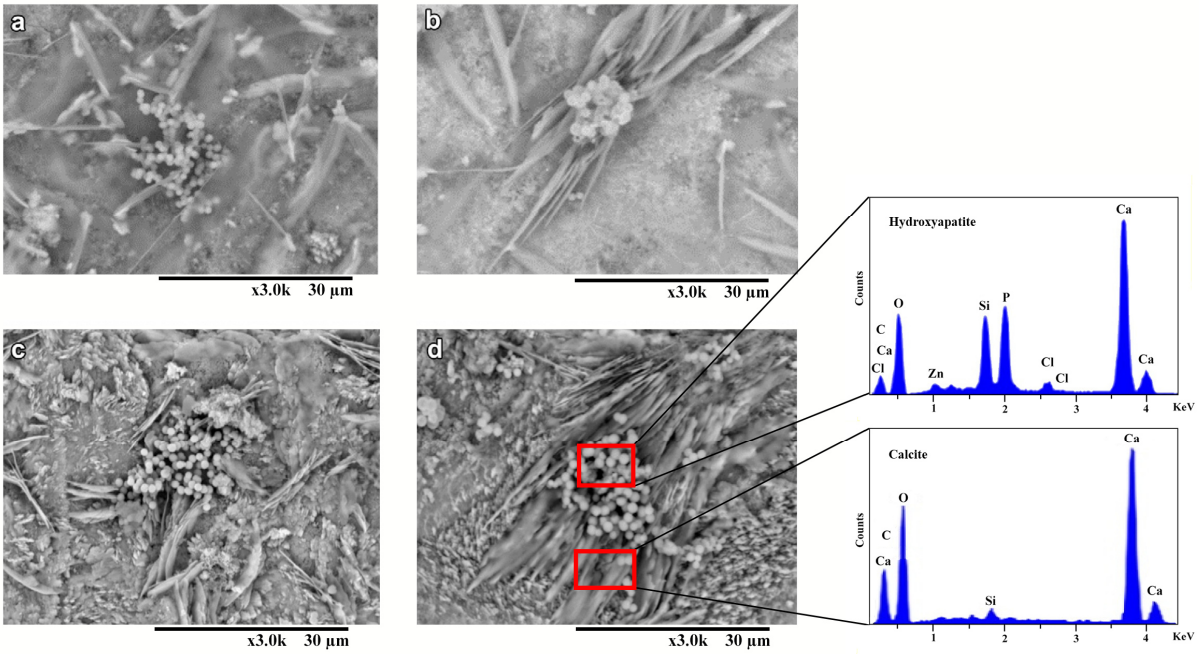

Fig. 2. FESEM micrographs and EDS spectra taken from spherical and flake like crystals precipitated on the surface of 5 mol % Zn containing bioactive glasses, at (a) 14, and (b) 21 days' immersion; and 10 mol % Zn containing bioactive glasses, at (c) 14, and (d) 21 days SBF immersion. Spherical apatite crystals with Ca/P ratios close to 1.67 precipitated on the surface of low Zn-containing glasses at both time intervals. The increase of Zn content to 10 mol % led to the formation of flake-like calcite crystals with much higher Ca/P ratios. (Partially reprinted with the permission from [26])

Mackovic *et al.* [32] reported that calcium carbonate may form on nanoscale bioactive glass powder upon soaking in SBF, whereas no calcite formation was found to occur on micrometric glass particles at any stage of reaction in SBF; interestingly, this suggests the

existence of a “size effect” associated to the in vitro bioactivity assessment. They first synthesized 45S5 glass nanoparticles in different sizes (20-60 nm in diameter) via a flame spray procedure. Then, they pointed out that calcite (in the form of a coarse nano-crystalline precipitate) surpasses hydroxyapatite at the early stage of immersion in SBF. TEM analysis (see Fig. 3) confirmed co-precipitation of calcite and apatite only one day after immersion in SBF. Fig. 3a shows the bright-field TEM micrograph of the as-prepared nanoparticles of the examined bioactive glass. On the basis of selected area electron diffraction (SAED, shown in the inset of Fig. 3a), the agglomerated nanoparticles are completely amorphous. Fig. 3b shows the TEM micrograph and SAED pattern of the glass nanoparticles just after one-day immersion in SBF. In this figure, the coexistence of nanocrystalline coarse particles of calcite and unreacted glass nanoparticles is evident. The blurred diffraction pattern (shown in the inset of Fig. 3b) could be referred to as the presence of unreacted glass nanoparticles. After 7 days’ immersion in SBF, hydroxyapatite also precipitated besides calcite crystals. Fig. 3c indicates the bright-field TEM micrograph and SAED after 7 days of immersion. According to the SAED pattern (inset of Fig. 3c) taken from calcite zone, the coexistence of both nanocrystals and single crystals of calcite is detectable. In fact, calcite nanocrystals surround grown calcite crystals. As shown in Fig. 3d, isolated and compacted agglomerates can also be observed which mainly contain hydroxyapatite crystals (see inset of Fig. 3d) [32].

2.2 Role of porous texture

The porous structure of bioactive glasses is another key factor controlling the rate of glass dissolution and the amount of ions released into the SBF fluids. In a similar study, Mozafari

et al. [28] examined porous sol-gel derived bioactive glasses ($64SiO_2$-$31CaO$-$5P_2O_5$, in mol %) during bioactivity assessment in SBF and highlighted the effect of mesoporous structure on the precipitation of calcium carbonate. They reported the simultaneous formation of both apatite and calcite on the glass surface just at 1 day of soaking in the testing solution [28]. Both phases persisted over time, detectable even at 7 days. The mesoporous glass prepared by Mozafari et al. [28] exhibited a specific surface area of 137.9 m^2g^{-1} and a median mesopore size of 15.4 nm. The significant presence of open porosities along with the cylindrical shape of pores allowed an easy diffusion of calcium ions through the sol-gel derived bioactive glass matrix into the SBF solution. It could be suggested that the textural properties of bioactive glasses are also key players for the co-precipitation of calcium carbonate. Precipitation of calcium carbonate might also occur due to the depletion of PO_4^{3-} ions followed by an increase in the Ca/P ratio and the saturation of the solution with respect to calcium carbonate. With respect to the effect of ion release from the glass structure, apart from the mesoporous structure, the glass particle size may play a critical role in the formation of calcium carbonate [33]. The role of the textural characteristics of bioactive glasses as a function of ion release behavior which directly affects the precipitated phases including calcium carbonate was also underlined by Martinez *et al.* [34]. In general, higher the specific surface area (e.g., tens of m^2/g for sol-gel glasses compared to less than 1 m^2/g for melt-derived glasses [35]), higher the release of Ca^{2+} ions upon soaking.

2.3 Role of glass composition and testing medium

Formation of calcium carbonate during bioactivity assessment of bioactive glasses from very simple binary up to multiple complex compositions have been reported. In this regard, it is

worth pointing out that the degree of reactivity exhibited in the biological environment can be strongly related to the composition of bioactive glasses [36]. Among simple glass compositions, the presence of calcium carbonate on the surface of both experimental and commercial (TheraGlass®) CaO-SiO_2 binary glass powders [34, 37, 38] were assessed as well as wollastonite-containing glass-ceramics [39] that were all soaked in SBF. Similarly, Chen *et al.* [40], reported on the bone bonding capability of sol-gel derived CaO-SiO_2 (30CaO-70SiO_2, in mol %) glass powders. In this regard, the starting glass and its relevant glass-ceramic (sintered at 1000 °C for 2 h) powders were soaked in a SBF solution. At 2 and 4 weeks of soaking, both calcium carbonate and apatite phases were formed as the major and minor crystalline phases in the examined glass, respectively. However, wollastonite remained as a single crystalline phase in the sintered sample at 4 weeks' incubation.

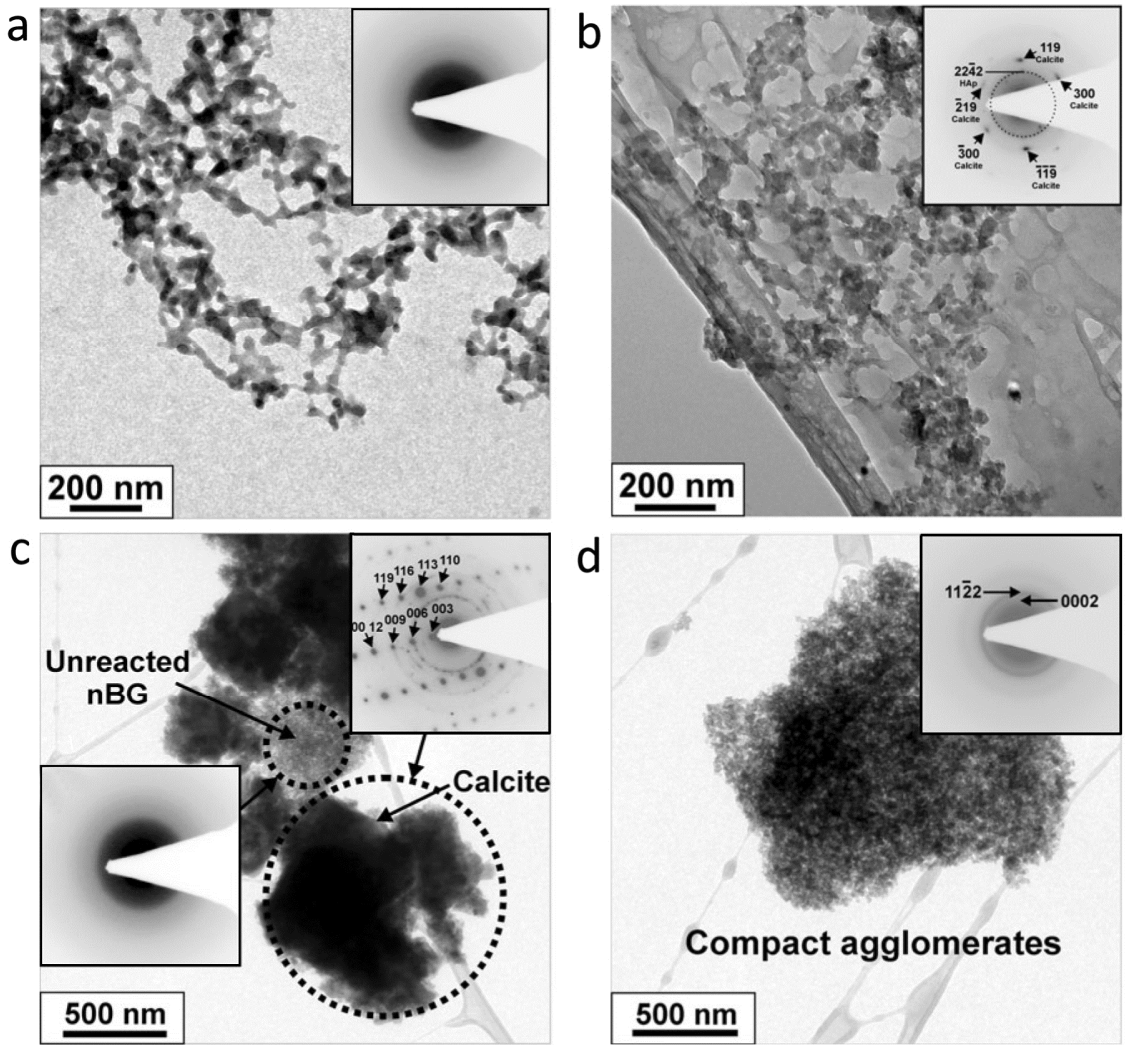

Fig. 3. Bright-field TEM micrographs and selected area electron diffraction (SAED) patterns taken from the examined bioactive glass nanoparticles: (a) as-prepared, (b) after immersion for 1 day in SBF solution. At one-day immersion in SBF, the formation of nanocrystalline and large calcite precipitates besides unreacted glass nanoparticles is detectable. After immersion for 7 days in SBF: (c) a mixture of nanocrystalline large calcite precipitates and single calcite crystals, coexisting with unreacted glass nanoparticles; (d) compacted agglomerates containing hydroxyapatite crystals. (Partially reprinted with the permission from [32])

Formation of calcium carbonate was also observed to occur, during *in vitro* tests in SBF or other testing media, on the surface of multicomponent bioactive glasses including 45S5 and variously-doped glasses [40-43].

In order to highlight the phase transformation occurring on the surface of 45S5 sol-gel derived glass discs in contact with SBF for various time intervals, Seah et al. [41] utilized Raman microscopy mapping coupled with a novel multivariant data analysis algorithm. Raman score images taken from calcite and hydroxyapatite distribution for different time periods is observed in Fig. 4.

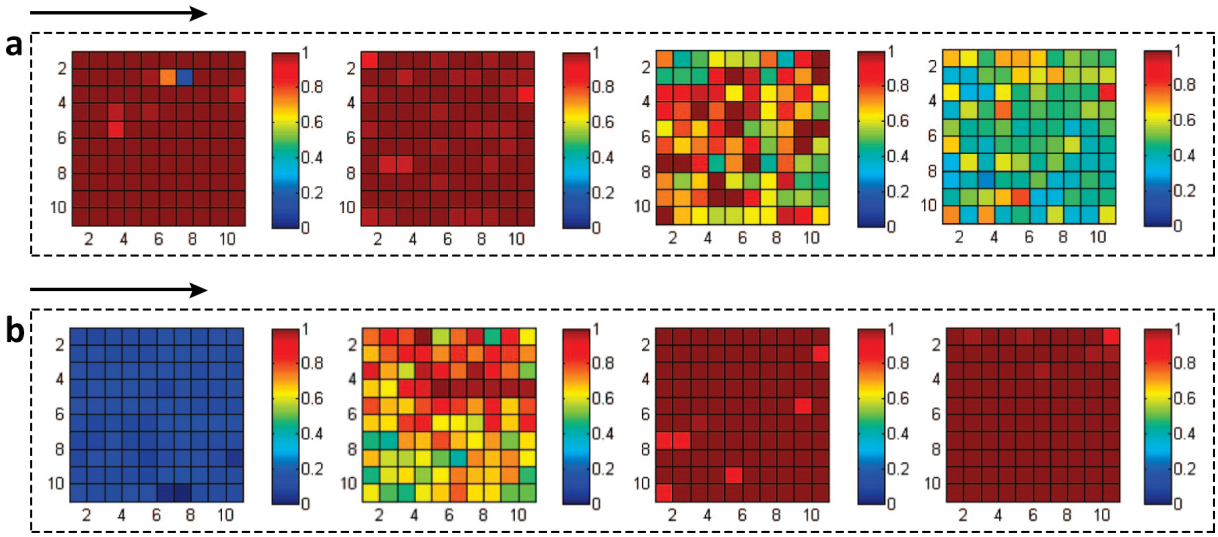

Fig. 4. Raman score images, showing (a) calcite distribution and (b) hydroxyapatite distribution at the center of bioactive glass disks for a seventeen-day evaluation in SBF solution. The axes of score images are in pixels and can be directly correlated to the distance by multiplying each pixel with 20 µm. It is evident that calcite and hydroxyapatite precipitate contradictorily upon immersion in SBF. At the early steps of precipitation, calcite forms homogeneously, whereas hydroxyapatite consistently precipitates at the final steps of immersion. (Partially reprinted with the permission from [41])

It could be implied from Fig. 4 that calcite homogeneously distributed at the early days of SBF immersion, showing high concentrations of calcite domains. However, the distribution of calcite became very inhomogeneous at the final steps of immersion, accompanied by decreased concentration. It is believed that during prolonged exposure to SBF, calcium ions can be leached out into the SBF solution and contributed to the formation of calcium phosphate phases in the

medium. Hydroxyapatite showed an adverse trend during the precipitation process. The homogeneous precipitation of hydroxyapatite occurred at the end of the immersion period, probably due to the increased concentration of the leached calcium ions.

Modification of 45S5 bioactive glass with calcium fluoride and its *in vitro* bioactivity assessment in different physiological fluids was reported by some researchers [45, 49-53]. What differs is the type of immersion medium and fluoride content in the glass composition. Lusvardi *et al.* [42] investigated the *in vitro* surface reactivity of some bioactive glasses, based on the composition of 45S5 Bioglass® in which CaF_2 (0-15 mol %) replaced part of CaO and Na_2O, during immersion in Tris buffer solution and commercially-available Dulbecco's modified Eagle's medium (DMEM). The addition of fluoride into the glass structure was assumed to have a significant effect on the surface reactivity in the biological environment; hence, the reactivity of fluoride-containing bioactive glasses was compared with that of the reference 45S5 system. The authors of this study [42] reported a set of reactions involving calcium carbonate formation, which are somehow different from that formerly proposed by Hench for the 45S5 bioactive glass [43]. The recognized crystalline phases were $CaCO_3 \cdot H_2O$ (monohydrocalcite) and $CaCO_3$ (calcium carbonate). In summary, the presence of fluoride caused a higher release of calcium ions in the solution (as already shown in another study [44]); this favored the fast precipitation of $CaCO_3 \cdot H_2O$, which with time converted into stable crystalline $CaCO_3$, as also reported by Jimenez-Lopez et al. [45]. According to Lusvardi et al. [42], if there was a small amount of silica gel on the glass surface, the crystallization of apatite might be retarded *in vitro*, and the high concentration of calcium ions (ten times higher compared to SBF) favored the co-precipitation of calcium carbonate and apatite.

In another study, modified 45S5 glasses (containing 0-32 mol % of CaF_2) were examined for apatite formation ability in cell culture media, up to 7 days [46]. It has been reported that low concentration of fluoride ions enhanced apatite formation, while the higher content of fluoride led to the simultaneous formation of calcite and fluorite (CaF_2). For all studied compositions, the presence of serum protein in the immersion media led to a significant delay in apatite formation. By further addition of CaF_2 to the composition of bioactive glasses, Ca^{2+} ions, acting as the ionic bridges between NBOs, could be replaced by charged specious of CaF^{+} accompanied by the reduced electrostatic bonds strength. Consequently, the glass structure started to be more expanded and disrupted [46, 47].

Brauer *et al.* [48] investigated the surface reactivity of a series of melt-derived bioactive glasses in the multicomponent system of SiO_2-P_2O_5-CaO-Na_2O-CaF_2 (CaF_2 ranging from 0 to 17.76, in mol%). They reported that all the glasses formed a surface apatite-like phase upon soaking in Tris buffer. In this regard, fluoride concentration of the glass was found as a controlling factor, resulting in (i) the preferential formation of fluorite for increasing the fluoride content in the glass structure (due to the lack of available phosphate ions while excess concentrations of fluoride and calcium were available), and (ii) calcium carbonate formation for the fluoride-free composition. Interestingly, the formation of calcite on the surface of fluoride-free glasses was not confirmed in some similar studies working with SBF [48, 49]; this can be explained considering the higher concentration of phosphate ions in the SBF solution compared to that of in Tris solution. In Tris buffer solution, all the available phosphate ions deriving from glass dissolution are consumed for the formation of apatite, and the remaining calcium ions then react with HCO_3^- (from the dissolution of atmospheric CO_2) present in the solution to form

calcium carbonate. On the contrary, the high concentration of phosphate ions in the SBF solution favors the formation of apatite-like phases.

Shah *et al.* [50] examined the dissolution behavior of a fluoride-containing bioactive glass ($47.1SiO_2$-$1P_2O_5$-22CaO-$25.1Na_2O$-$4.8CaF_2$, in mol %) over a week in Eagle's Minimal Essential Medium (MEM), supplemented with either acetate buffer, HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) buffer, HEPS + carbonate, or HEPS + carbonate + fetal bovine serum. The XRD results at 7 days' immersion in the mentioned media confirmed the pronounced formation of calcium carbonate in those samples treated in the carbonate added media alongside very small amounts of apatite.

The exact comparison between the reported data on calcium fluoride modified 45S5 glasses is somehow imprecise due to the different experimental conditions and immersion media. In summary, modification of 45S5 bioactive glasses by calcium fluoride is important in two respects; variation in the content of calcium ions and weakening the glass structural bonds through replacing of oxygens by fluoride ions. Hence, the addition of calcium fluoride to the glass compositions not only increases the content of calcium ions releasing into the surrounding medium but also accelerates the rate of ion release, kinetically. Apparently, the type and content of precipitated phases are also influenced by the type and concentration of ionic species in the immersion media.

It is worthy to note that some classes of glass-ceramic materials could also be categorized as the bioactive materials having superior mechanical properties. In the case of bioactive glass-ceramic materials, lower dissolution and degradation rates are expected compared to their corresponding glasses owing to the slower dissolution rate of the crystalline fraction. Even

though partial crystallization of glass-ceramics does not usually prevent their reactivity in physiological solutions and their amorphous fraction, remaining as the residual glass phase, undergoes dissolution and degradation during soaking in SBF or Tris solutions [51]. Formation of calcite crystals on the surface of a SiO_2-CaO-Na_2O-MgO-K_2O-P_2O_5 based glass-ceramic upon soaking in DMEM was also observed by one of the authors of the present article (F. Baino - unpublished data); at 14 days, these crystals (Fig. 5) exhibited the typical appearance of trigonal calcite crystals [52].

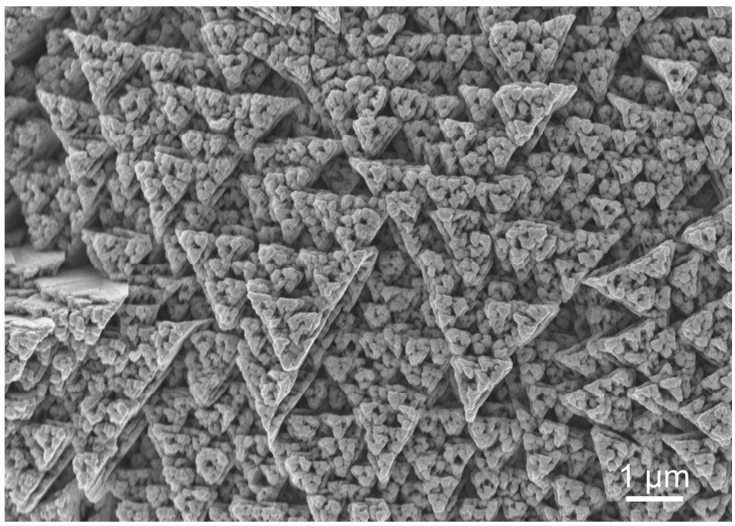

Fig. 5. Crystals of trigonal calcite formed on the surface of SiO_2-CaO-Na_2O-MgO-K_2O-P_2O_5 glass-ceramics soaked in DMEM for 14 days (courtesy of F. Baino - unpublished data).

2.4 Other relevant factors

Apart from glass composition, lack of ionic equilibrium originating from inadequate mixing induces the formation of regions in which glass particles are less available to interact with the SBF solution. As a result, the concentration of calcium ions dissolved from glass particles can be locally changed and led to the calcite precipitation [53].

In summary, the formation of calcium carbonate (mostly as calcite crystals) on the surface of bioactive glasses *in vitro* seems to be governed by a complex interlocking of the glass composition, textural parameters of the material tested, and type of solution in which the samples are soaked.

3. Effects of glass structural features on ion release and dissolution behavior

The increased amount of Ca^{2+} ions, which is the main controlling factor in precipitation of calcium carbonate, is not only influenced by the chemical composition of bioactive glasses or surrounding physiological solutions but also governed by structural features of the exposed glasses including their network connectivity and network compaction [54].

As one of the most popular class of glasses in medicine, silicate-based glasses are mainly composed of silicon centered tetrahedra, where oxygen atoms located at the corner position in each tetrahedron. The oxygens shared between two neighboring tetrahedra to form Si-O-Si bonds are defined as the bridging oxygens (BOs). By incorporation of modifier oxides into the glass composition, glass structural bonds turn to convert from Si-O-Si into $Si\text{-}O^{-}M^{+}$ (M^{+} is the modifier cation) bonds. In this state, each oxygen atom allocates just to one tetrahedron and its electrical charge is balanced by linking to M^{+} ions. These oxygens are described as the non-bridging ones (NBOs) [55].

The structure of bioactive glasses can be evaluated on the basis of two principal factors including network connectivity and network compaction. Network connectivity is equal to the number of BOs per network-forming elements (silicon, phosphorous, or boron) [56, 57]. Network connectivity of bioactive glasses usually varies in the optimized interval of 2-3. More rigid glass

structures with higher network connectivity have always shown less apatite formation capability, owing to the slow rate of ion release and dissolution. On the other hand, lower network connectivity promotes the crystallization tendency originated from highly disrupted glass structures [55].

Network packing (compaction) of the silicate glasses is proportional to their molar volume and oxygen density [58]. Higher the network compaction means more difficult ion release upon soaking. By using modifier cations of varying ionic radii in the glass composition, it is possible to tailor glass dissolution and degradation while keeping constant network connectivity of the glass structures [59]. Brückner *et al.* [54] revealed that utilizing alkali ions of smaller ionic radius (Li for Na or Na for K) could result in a more compact glass network with higher oxygen density, decreasing ion release in contact with Tris solution. Vice versa, alkali ions of larger ionic radius (K for Na or Na for Li), expanded the glass network and facilitated ion release. It is worth mentioning that partially substitution of anions of varying radii may also kinetically control ion release mechanisms.

When contacted with body fluids, a series of sequential reactions occur on the bioactive glass surface [60-62], which are kinetically controlled by the glass structural features. Fig. 6 shows an overview of these sequential surface reactions. In stage I, alkali and alkaline earth ions leave the glass structure through an ion exchange process. These ions are replaced by H^+ or H_3O^+ from the immersion medium. As a result, pH value locally increases, and the Si-O-Si bonds start dissociating. In stage II, silicon ions release into the immersion medium. By formation of silanols (Si-OH groups) in this stage, a silicon riched layer forms on the glass surface. In stage III, by condensation of silanol groups, a layer of silica gel forms on the glass surface. In this stage, ion

exchange between the glass structure and immersion medium is still continued, owing to the open structure of silica gel. Through diffusion of Ca^{2+} and PO_4^{3-} ions from immersion medium as well as glass structure, an amorphous layer of calcium phosphate and calcium carbonate develop over the silica layer, in stage IV. It was shown by Pichon *et al.* [63] that an amorphous calcium carbonate cluster could act as the precursor phase for the upcoming formation of calcite. In stage V, further increase in the thickness of both silica and amorphous (calcium phosphate + calcium carbonate) layers leads to the development of crystallized calcium phosphate (usually as the carbonated hydroxyapatite) and calcium carbonate (usually as the calcite) phases.

It is worth mentioning that silicate based bioactive glasses with low network connectivity can be differently degraded in contact with SBF. In this case, glass dissolution takes place without hydrolysis of Si-O-Si bonds [67] and is mainly governed by the low structural connectivity rather than the ion exchange process of mobile ions, described in stage I.

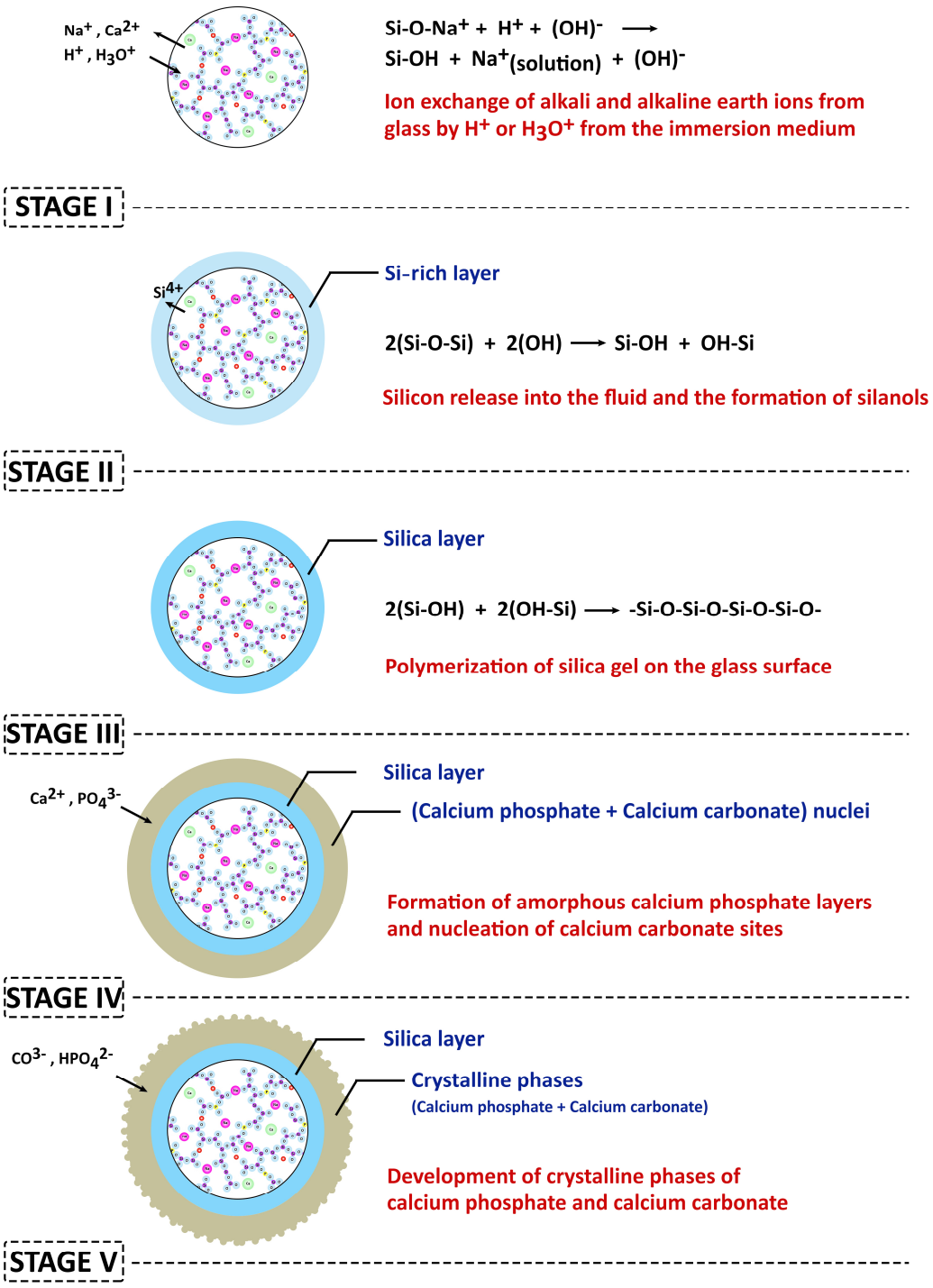

Fig. 6. A schematic overview of different stages of bioactive glass dissolution in contact with physiological fluids and its surface sequential reactions leading to biomineralization and formation of crystalline phases (calcium phosphate and calcium carbonate).

4. Effects of SBF composition on the precipitation of calcium carbonate

SBF, which has a composition close to that of human plasma, is the preferred solution used to perform *in vitro* bioactivity tests [2], with the aim of understanding whether a given biomaterial can form a surface apatite layer or not [24]. The ability of *in vitro* tests using SBF to predict what happens *in vivo*, i.e., the actual bone-bonding ability of biomaterials, has been repeatedly advocated and castigated and this controversy still lingers on. The interested reader is addressed to a couple of key publications on this specific topic [2, 3], which is not the matter of the present work. Given the importance and wide use of SBF as a testing medium for biomaterials, it is indeed interesting to analyze the role of its composition in affecting the formation of apatite vs. calcium carbonate on the surface of bioactive glasses upon *in vitro* soaking.

A detailed history of SBF conceptualization and development was reported by Kokubo and Takadama [2]. The original SBF proposed by Kokubo *et al.* in 1990 [64] lacked the SO_4^{2-} ions contained in human blood plasma, which was corrected later in a refined formulation [65]. Since then, the corrected SBF has been commonly used as "conventional" SBF. Cho *et al.* [66] reported a detailed recipe for the preparation of this SBF, that was still richer in Cl^- ions and poorer in HCO_3^- ions compared to that of human blood plasma. In 2003, Oyane *et al.* [67] tried to correct this difference by preparing a revised SBF (r-SBF) in which the concentrations of Cl^- and HCO_3^- ions decreased and increased, respectively, to the levels of human blood plasma. However, calcium carbonate has a strong tendency to precipitate from r-SBF because it is supersaturated with respect to both apatite and calcium carbonate [68]. Specifically, Oyane *et al.* [67] prepared

four kinds of solutions with different ion concentrations for comparative purposes (i.e., c-SBF, r-SBF, i-SBF, and m-SBF), as shown in Table 1.

Table 1. Normal ion concentration of the SBFs, in comparison with those of human blood plasma in total and dissociated amounts[69, 70].

	Concentration/ mM							
	Human blood plasma		Original SBF	c-SBF	r-SBF	i-SBF	m-SBF	(n-SBF)
	Total	Dissociated						
Na^+	142.0	142.0	142.0	142.0	142.0	142.0	142.0	142.0
K^+	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Mg^{2+}	1.5	1.0	1.5	1.5	1.5	1.0	1.5	1.5
Ca^{2+}	2.5	1.3	2.5	2.5	2.5	1.6	2.5	2.5
Cl^-	103.0	103.0	148.8	147.8	103.0	103.0	103.0	103.0
HCO_3^-	27.0	27.0	4.2	4.2	27.0	27.0	10.0	4.2
HPO_4^{2-}	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
SO_4^{2-}	0.5	0.5	0	0.5	0.5	0.5	0.5	0.5

The c-SBF was the "conventional" SBF, having concentrations of ions equal to those of human blood plasma, except for Cl^- and HCO_3^- that were higher and lower, respectively, than the physiological ones. The r-SBF was designed to have the concentrations of all ionic species (including Cl^- and HCO_3^-) equal to those of human blood plasma. The i-SBF, abbreviated from ionized SBF, was designed to have concentrations of dissociated ions equal to those found in

human blood plasma. Finally, the m-SBF, abbreviated from modified SBF, was designed to have concentrations of ions equal to those of human blood plasma, except for HCO_3^- (its concentration decreased to the level of saturation with respect to calcium carbonate), as shown in Table 2 [71-74]. The ionic activity products (IP) of each solution for both apatite and calcium carbonate were calculated according to a set of equations reported in [67], and are listed in Table 2.

Table 2. Negative logarithms of ionic activity products (IP) of the SBFs with respect to hydroxyapatite and calcium carbonate [67].

Phase	-log (IP)			
	c-SBF	r-SBF	i-SBF	m-SBF
Hydroxyapatite	94.3*	94.6*	96.5*	94.1*
Calcium carbonate	9.0***	8.2*	8.4*	8.6**

*The –log (IP) for hydroxyapatite and calcium carbonate are 117.0 and 8.6, respectively. Notations of *, **, and *** indicate supersaturation, saturation, and undersaturation, respectively, with respect to the corresponding phases.*

The data reported in Table 2 revealed that all the solutions prepared by Oyane *et al.* [67] were highly supersaturated with respect to hydroxyapatite. The ionic activity products of the solutions with respect to hydroxyapatite increased in the following order i-SBF < r-SBF < c-SBF < m-SBF. The lower ionic activity product of i-SBF compared to that of r-SBF was due to the lower concentration of Ca^{2+} in the former. The lower ionic activity products of r-SBF compared to c-SBF was due to the higher concentration of HCO_3^- ions in the former. Furthermore, HCO_3^- decreased the actual concentration of Ca^{2+} in the solution by forming ion pairs with Ca^{2+}.

With respect to calcium carbonate, c-SBF was undersaturated, r-SBF and i-SBF were supersaturated, and m-SBF was saturated. The ionic activity products of the solutions with respect to calcium carbonate increased in the order of c-SBF < m-SBF < i-SBF < r-SBF. The lower ionic activity products of c-SBF compared to m-SBF, and the lower ionic activity products of m-SBF compared to i-SBF were due to the lower HCO_3^- concentration in the formers. The lower ionic activity products of i-SBF compared to r-SBF was due to the lower concentration of Ca^{2+} in the former.

The obtained results indicated that c-SBF and m-SBF had the potential to form hydroxyapatite but no potential to form calcium carbonate, whereas r-SBF and i-SBF had the potential to form both hydroxyapatite and calcium carbonate. The stability of the solutions in terms of cluster formation was also different [68]. Calcium carbonate particles with a hydrodynamic diameter of 10-30 nm were observed to form in r-SBF and i-SBF after storage for 1 day, but no clusters were detected in c-SBF and m-SBF even after 7 days [75]. The observed calcium carbonate was supposed to be a calcite cluster because the solubility of calcite is lower than any other calcium carbonate [76-79], and this hypothesis was confirmed by XRD results [68].

As far as the mechanism of co-formation of hydroxyapatite and calcium carbonate, it has been reported that phosphate ions may form ion pairs with Ca^{2+} [71], as shown in the following reactions (1) and (2) along with the equilibrium constants:

$$Ca^{2+} + H_2PO_4^- \leftrightarrows CaH_2PO_4^+ \qquad K=10^{0.9138} \qquad (1)$$

$$Ca^{2+} + HPO_4^{2-} \leftrightarrows CaHPO_4\,(aq.) \qquad K=10^{2.387} \qquad (2)$$

where phosphate ions are involved in the equilibrium reactions (3) to (5) [65, 80],

$$H_3PO_4 \leftrightarrows H^+ + H_2PO_4^- \qquad K=10^{-2.196} \qquad (3)$$

$$H_2PO_4^- \leftrightarrows H^+ + HPO_4^{2-} \qquad K=10^{-7.185} \qquad (4)$$

$$HPO_4^{2-} \leftrightarrows H^+ + PO_4^{3-} \qquad K=10^{-12.19} \qquad (5)$$

When HPO_4^{2-} ions were excluded from r-SBF, the actual Ca^{2+} concentration in the solution increased. As a result, the increased Ca^{2+} concentration accelerates the nucleation rate of calcite by increasing the ionic activity product of the solution for calcite, as illustrated in the reaction (6):

$$Ca^{2+} + CO_3^{2-} \leftrightarrows CaCO_3 \qquad K=10^{3.38} \qquad (6)$$

It was also thought that the calcium carbonate clusters grew spontaneously by consuming calcium and carbonate ions from the surrounding fluid because both r-SBF and i-SBF were inherently supersaturated with respect to calcite.

In 2003, c-SBF was proposed to the Technical Committee ISO/TC150 of International Organization for Standardization as a potential "standard" solution for *in vitro* bioactivity assessment of biomaterials. Takadama *et al.* [81] proposed a newly improved solution (n-SBF) in which the concentration of Cl^- ions decreased to the level of human blood plasma, while the concentration of HCO_3^- ions was kept equal to that of the c-SBF. It was proved that the c-SBF did not differ from n-SBF in terms of stability and reproducibility, and thus c-SBF became the

commonly-used SBF adopted for *in vitro* tests worldwide as recommended by the relevant ISO standard [24]. A refinement of this protocol was recently proposed by Macon *et al.* [38] in order to allow more reliable testing of bioactive materials – especially bioactive glasses – with a high surface area. The idea was to base the test on the mass-to-liquid ratio instead of fixing the surface-to-volume ratio; the results of an early round-robin study were promising, and the scientific discussion on this topic is currently in progress.

5. Effect of SBF volume on the formation of calcium carbonate

The formation of calcium carbonate on the surface of bioactive glasses might also be related to the volume of SBF used for *in vitro* testing. Zhang *et al.* [39] reported that calcium carbonate could be easily formed and detected on the surface of MgO-CaO-SiO_2-P_2O_5 based bioactive glass-ceramics if SBF was not refreshed during the *in vitro* tests. The formation of calcium carbonate was thought to be the product of the reaction between Ca^{2+} (partly from the original SBF and partly released from the glass) and CO_3^{2-} ions (from the original SBF). It can also be suggested that carbon dioxide can dissolve in the immersion medium, especially when the test is conducted in an incubator (the CO_2 concentration, about 5%, matched with physiologic conditions to maintain a constant pH). This reaction is strongly dependent on the concentration of Ca^{2+} in SBF. In fact, upon immersion in SBF, Ca^{2+} dissolution from the bioactive glass occurs through an ion exchange reaction, which increases the calcium concentration in the solution thereby promoting the precipitation of calcium carbonate. In another study, Rybarikova *et al.* [82] reported that the formation of calcium carbonate could be totally restrained if the SBF-to-glass ratio was great enough, but detailed and systematic information on it was not provided.

An interesting quantitative study was reported by Lukito *et al.* [37], who assessed the *in vitro* bioactivity of a $70SiO_2$-30CaO (in wt.%) bioactive glass using various concentrations of glass powder in SBF (10, 5, 3.3, 2.5 and 2 mg/ml). An apatite-like crystalline phase was formed within the first 6 h of immersion in SBF regardless of the glass concentration, due to the high concentration of PO_4^{3-} in the original solutions. Furthermore, the elemental analysis revealed no traces of carbon on the surfaces of all samples, indicating that calcium carbonate was formed in neither crystalline nor amorphous states at this short-term time point.

At 6 h of soaking, except for the SBF solution with the lowest concentration of glass (2 mg/mol), the other four solutions were supersaturated for calcium carbonate as calcite phase, which further shown to precipitate on the surface of the bioactive glasses when the soaking time prolonged to 1 day. This phenomenon was quantitatively explained by calculating the negative logarithms of ionic products (-log (IP)) of Ca^{2+} and CO_3^{2-} at 6 h of soaking in SBF (Table 3). It was shown that the maximum value of –log (IP) for calcium carbonate formation in SBF was 8.6 for the solution with. 2 mg/ml of glass, whereas the -log(IP) values of all the other four solutions were smaller than 8.6.

Comparison between these data and the results reported by Oyane *et al.* [67] allows the conclusion that the solutions with higher glass concentrations were supersaturated for calcium carbonate, having a strong tendency to form crystalline calcium carbonate on the surfaces of the bioactive glasses over prolonged immersion periods.

Table 3. -log(IP) of Ca^{2+} and CO_3^{2-} for various SBF solutions at 6 h of soaking [37].

	The amount ratio of bioactive glasses to SBF solution				
	10 mg/ml	5 mg/ml	3.3 mg/ml	2.5 mg/ml	2 mg/ml
-log(IP)	8.04	8.14	8.30	8.40	8.61

For the bioactive glasses soaked at higher concentrations in SBF, the XRD patterns showed the peak corresponding to apatite to disappear – which was found at 6 h – and the appearance of a sharp peak identifying nanocrystalline calcite ((104) reflection) (Fig. 7). In contrast, when the glass concentration in SBF decreased to 2 mg/ml, the peak corresponding to hydroxyapatite ((211) plane) became more apparent, and there was no evidence of nanocrystalline calcium carbonate phase on the surface of the bioactive glass.

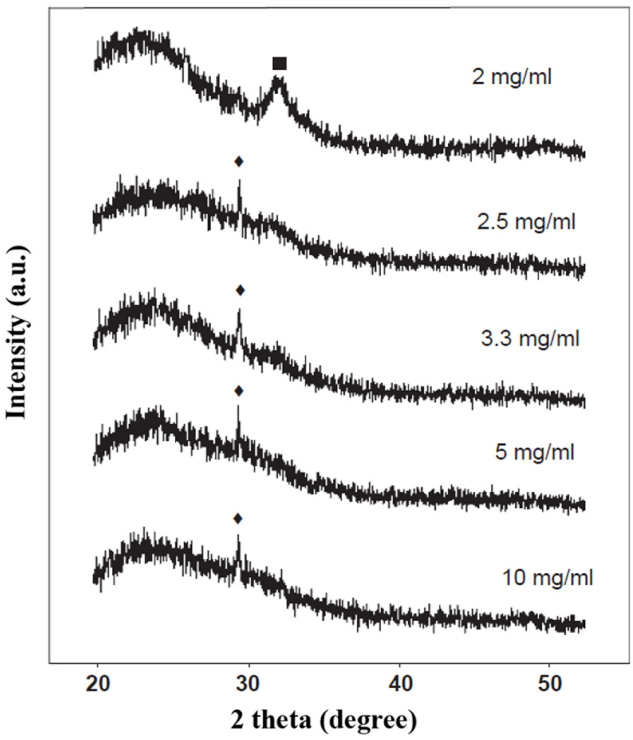

Fig. 7. XRD patterns of $70SiO_2$-30CaO (in wt %) bioactive glasses at one-day soaking in various amounts of SBF solutions (10, 5, 3.3, 2.5 and 2 mg/ml). In the first hours of immersion, due to the high concentration of phosphate groups in the original SBF solutions, hydroxyapatite crystals formed on the surfaces of all samples, regardless of glass/SBF ratio. However, at the first day, except for the sample in 2 mg/mol, the other samples were supersaturated for calcite. In other words, calcite crystals could not form on the surface of samples in 2 mg/ml of SBF solution, (■: HA, ♦: calcite). (Reprinted with the permission from [37].)

Lukito *et al.* [37] suggested two possible mass-transfer processes for Ca^{2+} when bioactive glasses are immersed in SBF solutions. The first one involves the ion exchange of Ca^{2+} ions from bioactive glasses with H_3O^+ ions in SBF, which would increase the concentration of Ca^{2+} in the solution, and the second one is the formation of an apatite-like phase on the surface of bioactive glasses, which would consume Ca^{2+} and give a decrease in the concentration of Ca^{2+}. In comparison with the Ca^{2+} consumption by the formation of the apatite phase, ion exchange between Ca^{2+} and H_3O^+ dominated in the initial soaking hours due to the high calcium

concentration in the bioactive glass samples, thereby leading to an increase in the concentration of Ca^{2+} in the SBF. For longer soaking times (up to 1 day), the concentrations of PO_4^{3-} in all five SBF solutions were assessed to be less than 0.01 mM, which was much lower than that of the original SBF. Most of PO_4^{3-} ions in SBF were consumed to produce hydroxyapatite, the formation rate of which progressively decreased, due to the low concentration of PO_4^{3-} (<0.01 mM) in the solutions or even stopped, although there were still enough Ca^{2+} ions in the SBF. As a final result, the formation of crystalline calcium carbonate from the reaction between Ca^{2+} and CO_3^{2-} became more likely, depending on the remaining concentration of Ca^{2+} in the SBF solutions [83-87].

It should be noted that, usually, lower bioactive glass powder concentrations are used for *in vitro* tests in SBF than those employed by Lukito *et al.* [37] in order to avoid supersaturation with respect to calcite; specifically, a glass powder-to-liquid ratio of 1.5 mg/ml has been recommended by Macon *et al.* in a basic research dealing with *in vitro* testing methodologies [38].

6. Concluding remarks: summary, challenges and opportunities

The available literature gives evidence that calcium carbonate may form on the surface of bioactive glasses upon soaking *in vitro*. This paper provides a comprehensive phenomenological picture of the mechanism of calcium carbonate formation on the surface of bioactive glasses during bioactivity assessment. Specifically, the chemical reasons why and the experimental conditions under which calcium carbonate may form are analyzed and discussed on the basis of available results and observations from relevant literature. In this regard, the key factors seem to be the high concentration of calcium and carbonate ions in the testing solution and the high

glass-to-liquid ratio (i.e., when, generally speaking, low volumes of testing solution are used). High concentration of Ca^{2+} in the testing solution can be an inherent characteristic of the original medium used [42, 88] or the result of glass dissolution over time [25, 28, 34, 37-39, 42, 48]. There have always been a higher dissolution rate when glasses exhibit a nanoporous texture associated to a high specific surface area available for ion-exchange reactions (e.g., sol-gel bioactive glasses with the specific surface area above 100 m^2/g) [28, 38].

Given this general picture, an important methodological aspect deserves to be considered, i.e., the key role played by the type of testing medium. The formation of calcium carbonate on the surface of bioactive glasses soaked in DMEM is not surprising, as this medium contains a high concentration of HCO_3^- ions (ten times higher compared to SBF), which favors the precipitation of calcite [42]. A study by Rohanova *et al.* [88] clearly indicated that DMEM is not suitable for *in vitro* bioactivity tests, as it does not allow apatite formation and, furthermore, requires the necessity to maintain in sterile conditions during the test. On the contrary, the well-known Kokubo's SBF – which has a composition similar to that of human plasma and is commonly used for *in vitro* bioactivity testing, as also recommended by the relevant ISO standard [24] – is undersaturated with respect to calcium carbonate. Thus, it should have no potential to form this phase *in vitro* while having the potential to form apatite [67].

Keeping this important consideration in mind, we can also point out that, in some studies, the formation of calcium carbonate on the surface of bioactive glasses *in vitro* was actually observed as a result of having used a non-standard SBF (e.g., "old" formulations [67]) and/or a smaller volume of SBF than the one prescribed by currently-recognized protocols [37]. Therefore, in these cases, it is difficult to understand whether calcium carbonate formation *in*

vitro occurs as a "genuine" effect of glass dissolution/reactivity in SBF or is due to non-standard/incorrect experimental procedures. Therefore, the adoption of testing procedures in close accordance to ISO standard or well-recognized protocols is mandatory to obtain a reliable and robust comparison among the results achieved by different research teams. This statement may sound quite obvious, but the comparison in the literature is often difficult as many research groups still use different protocols for testing *in vitro* behavior of biomaterials. This issue was strongly pointed out in a publication by the Technical Committee 4 (TC04) of the International Commission on Glass (ICG) [38].

Taken together and critically interpreted, the results from in vitro tests that have been discussed in this review seem to suggest that calcium carbonate cannot definitely form on "conventional" bioactive glasses (i.e., glass solid pieces or micrometric melt-derived particles) soaked in SBF unless the experiments are incorrectly performed. This typically occurs if a too large solid-to-liquid ratio – or in other words, a too small volume of SBF – is used: as a result, the excessive amount of calcium ions released from the glass into the solution may lead to calcite formation. The relevant ISO standard [ref] prescribes that bioactive glass solid pieces (e.g. discs or tiles) are immersed in SBF using a fixed volume-to-surface ratio; on the contrary, a mass-to-volume ratio of 1.5 mg/mL was proposed by Macon et al. [38] when bioactivity tests in SBF are performed on glass powders. In both cases, calcite should not form provided that the volume of SBF is appropriately selected.

On the contrary, the formation of calcium carbonate may be even favored compared to hydroxyapatite when glass particles with ultrahigh surface area are tested. This means that, if mesoporous or nano-sized bioactive glass particles are immersed in SBF, the commonly-

adopted mass-to-volume ratio of 1.5 mg/mL is not appropriate to reveal the bioactive properties (i.e., the apatite-forming capability) of the material, thereby leading to an underestimation of the bioactivity or even false negative results. Early evidence of these limitations was clearly reported in a couple of exemplary studies by Macon et al. [38] and Maclovic et al. [32], who observed the formation of calcite on the surface of mesoporous sol-gel or 45S5 nano-sized glass particles, respectively, after 1 day in SBF because of the excessive amount of calcium ions released into the solution. This involves a higher Ca/P ratio and a pH shift in the SBF, and such a mechanism eventually favors the precipitation of calcite. In our opinion, a future refinement of in vitro bioactivity testing protocols should indeed involve the rethinking of the "optimal" volume of SBF (well below 1.5 mg/mL) to use with mesoporous and ultrafine glass particles.

At final, a very delicate aspect deserves to be mentioned at the end of this article. The relationship between *in vitro* (using SBF) and *in vivo* results has been a matter of strong debates in the last decade. Kokubo and Takadama [2] reported convincing evidence that the examination of apatite formation on a given material in SBF is useful to predict its bioactivity *in vivo* (i.e., bone-bonding ability). On the other hand, Bohner and Lamaitre [3] clearly demonstrated that this approach is questionable due to some important limitations and there is still room for improvements. Currently, most scientists recognize the importance of *in vitro* tests in SBF to obtain a preliminary indication on materials bioactivity, albeit being aware that *in vitro* conditions can only approximately match those *in vivo* and an ideal SBF does not exist that exactly mimic the *in vivo* environment. Therefore, an important question emerges on whether and how the formation of calcium carbonate *in vitro* may be predictive of what actually happens

in vivo. To the best of our knowledge, no evidence of calcium carbonate formation at the bioactive glass/host bone interface *in vivo* has been reported to date in the literature, while new bone formation has been indeed observed in tight contact with the glass in many studies [89]. This possibility, however, cannot be completely excluded at this stage of knowledge especially if bioactive glasses are implanted with an ultrahigh surface area associated to high release of calcium ions after being put in contact with biological fluids. Future studies could be addressed to investigate the glass/host bone interface in the short-term to assess if the formation of calcium carbonate deposits actually occur at the early post-implantation stages. Should this occur, it would not be undesirable, also considering that calcium carbonate can act as a precursor for the formation of bone-like hydroxyapatite nanostructures [ref]. Furthermore, calcium carbonate is a well-known biocompatible material that is clinically used for many years in clinical practice. Bone bonding ability of calcite is somewhat comparable to that of hydroxyapatite. It is biodegradable and biocompatible and has been used in bone cement as the filler and in the field of guided bone regeneration [90].

Fujita *et al.* [91] reported that calcium carbonate in the form of crystalline calcite bonded directly to bone; the failure load of the bone-bonding (4.11 kg) was lower than that of apatite-wollastonite glass-ceramics (7.43 kg) and HAp (6.28 kg), but was comparable to that of Ceravital®-type glass-ceramics (4.28 kg) (see Fig. 8a, b, and c) [92]. At present, coralline calcium carbonate is marketed under the tradename of Biocoral® and widely implanted in the form of granules and porous blocks for the treatment of infrabony defects in dentistry, spine surgery and orbital floor repair (see Fig. 8d, e, and f) [93, 94]. Furthermore, we should consider that human bone mineral, apart from the predominant apatite phase, also contains about 10 wt.% of calcium

carbonate [22]. Therefore, co-formation of calcium carbonate along with apatite *in vivo* could constitute proof of an accurate biomimetic behavior.

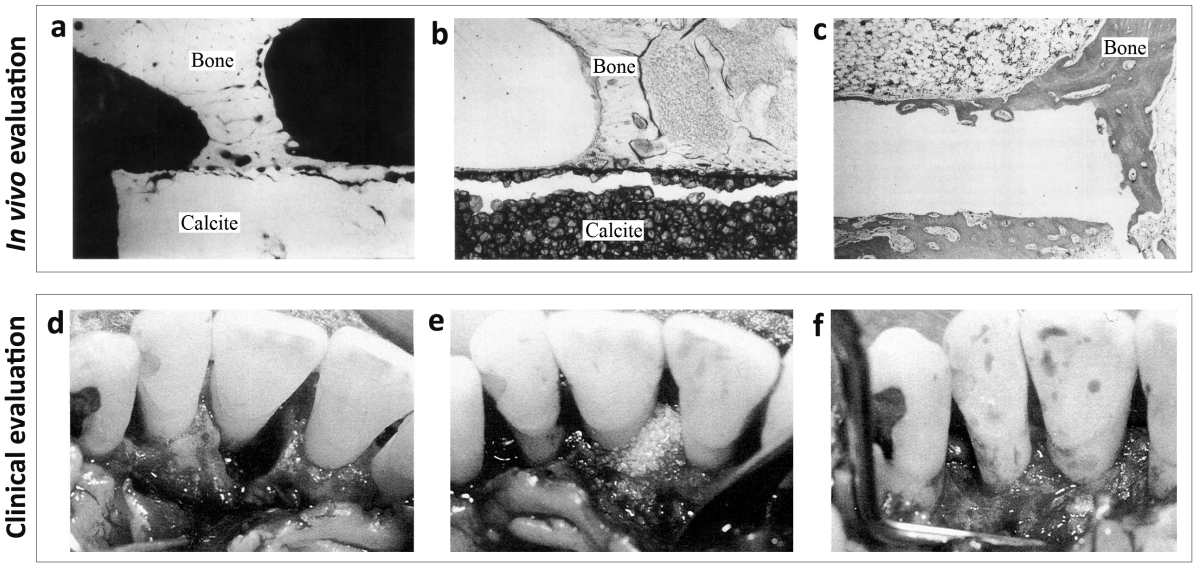

Fig. 8. *In vivo* evaluation: Plates of calcite ($CaCO_3$) were implanted in rabbit models (a) Contact microradiogram at 8 weeks after implantation, (b) Giemsa surface stain at 8 weeks after implantation, (c) Hematoxylin-eosin stain at 8 weeks after implantation (original magnification X40). They indicated that calcite is a biodegradable material that bonds to bone without a surface apatite layer. Clinical evaluation: Adjacent defects on teeth #8 (A resorbable coralline calcium carbonate graft material grafted) and #7 (control debridement). The original defect is depicted in (d), the calcium carbonate graft in place is shown in (e), and the re-entry appearance showing virtually complete defect fill on the experimental and very little clinical change in the control is depicted (f). The authors stated that the results were similar to those with other synthetic and natural bone replacement graft materials. However, the ease of handling this class of materials, their resorbability, and their potential for improved bone regeneration may be of clinical advantage. (Reprinted with the permission from [91] and [95]).

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