INTRODUCTION

Bioactive silicate glasses undergo surface reactions when in contact with aqueous media, resulting in ion release and subsequent precipitation of a biomimetic apatite layer. This apatite surface layer is thought to be key to cell adhesion and bone-bonding, while complete degradation in vivo allows for bone tissue regeneration. Studying the reactions of bioactive glasses in a simulated physiological environment is, therefore, key to the development of new bioactive glass compositions with additional functionalities, such as the release of therapeutic ions.

The first step in their long characterization process is typically the immersion of the bioactive glass in an acellular aqueous environment. This allows for the study of ion release and apatite formation (via formation of a preliminary octacalcium phase) under controlled conditions, while also being much less cost-intensive than cell culture studies. Since the development of simulated body fluid (SBF) by Kokubo et al., which aims at mimicking the inorganic composition of blood plasma, SBF has been the medium of choice for many immersion experiments, and different compositions are being used. Other solutions being used include cell culture media of various compositions, artificial saliva, Tris-HCl buffer or physiological sodium chloride solution.
The purpose of each of these solutions is, theoretically, to mimic the physiological environment and its reactions with bioactive glasses closely enough to provide us with information about the suitability of a glass as an implant material. While this is, in practice, not feasible, as a living system is too complex to be mimicked by either acellular immersion studies or even in vitro cell culture studies, these studies allow us to compare the reactions undergone by bioactive glasses, such as variations between glasses belonging to a compositional series. Besides the glass composition, other factors such as the ratio of bioactive glass weight to immersion medium volume are known to have a pronounced impact on the outcome of immersion studies. But also the composition of the immersion medium directly influences the outcome of immersion studies such as the possible precipitation of calcium carbonate in addition to apatite or the time point of apatite formation.

The aim of this paper is, therefore, to provide a review of the influence of immersion medium composition on the ion release and in vitro mineral phase precipitation of bioactive glasses. Owing to the vast number of bioactive glasses developed and investigated to date, and in order to allow for the direct comparison of the effect of different immersion media, this paper focuses on a single composition, that of melt-derived Bioglass 45S5 (in wt%: 45 SiO2, 6 P2O5, 24.5 CaO, 24.5 Na2O, or in mol%: 46.1 SiO2, 2.6 P2O5, 26.9 CaO, 24.4 Na2O).

### 2 IMPACT OF IMMERSION MEDIA COMPOSITION

#### 2.1 Unbuffered solutions

Our body fluids, such as blood or saliva, contain buffers to maintain a stable pH, and this is achieved by bicarbonates and, to some extent, phosphates being present in the fluid. Silicate-based bioactive glasses react with aqueous solutions by an ion exchange, where metal cations (M⁺), connected to the silicate network by non-bridging oxygen bonds (≡Si-O⁻ M⁺), are released from the glass and replaced by protons (H⁺) from the aqueous solution, forming silanol groups (≡Si-OH) and a surface layer which is typically described as a "silica gel." The immersion medium is thus depleted in protons, causing an increase in pH. In vivo, this pH increase is, to some extent, prevented by the body's fluid exchange and buffering system. Solutions used for immersion studies on bioactive glasses, therefore, typically contain buffers, to mimic the body's response. A limited number of studies, however, have been performed in unbuffered media. Cerruti et al evaluated the behavior of 45S5 powder in deionized water. The 45S5 particles used had a size of 2 µm, and 0.3 g of glass was immersed in 200 mL of deionized water. The unbuffered pH increased from 5.8 to above 10.5 within 2 minutes of immersion, where it was then stable until 360 minutes, after which it started to decrease until the end of the 1500-minute study. The authors report ion concentrations for up to 3000 minutes, but for the first 30 minutes of the experiment, they also present the results as the percentage of each ion found in solution relative to the original amount present in the glass. Their data show that despite large changes in pH, the total glass dissolution reached a maximum of 15% during this time period. This included, however, up to 8% of silica at 30 minutes, showing that glass network dissolution occurred, which is not surprising considering this high pH.

Fagerlund et al investigated Bioglass 45S5 dissolution in deionized water based on ISO 719 (ISO 719: Glass—Hydrolitic resistance of glass grains at 98°C—Method of test and classification). According to ISO 719, 2 g of glass granules (particle size range 315 to 500 µm) should be immersed in 50 mL of deionized water and treated at 98°C. The authors included additional temperatures of 20°C, 40°C, and 80°C. Hydrolytic resistance was then evaluated by performing a titration using hydrochloric acid, to determine the amount of hydroxyl (OH⁻) ions in solution, which depends on the amount of modifier ions from the glass exchanged for protons from the solution. In addition to this titration, Fagerlund et al also performed pH measurements and quantified ions in the solution using optical emission spectroscopy (ICP-OES). Based on these data, they calculated activation energies for glass dissolution and concluded that Bioglass dissolution was diffusion-controlled. While modifier ion release from bioactive glasses is typically described as diffusion-controlled, the results by Fagerlund et al also suggest a diffusion mechanism of silicon release, despite significant silicate network dissolution because of high pH values (>10). The diffusion mechanism here may, therefore, not necessarily be the mechanism by which the species (here: soluble silicate species) are released, but possibly the diffusion of water molecules into the glass network.

#### 2.2 Buffered solutions

One of the most commonly used buffered solutions is Tris(hydroxymethyl)aminomethane (Tris) buffer which is frequently used in biological sciences. Tris has a pKₐ at room temperature of 8.07, giving it a pH buffering range between 7.07 and 9.07 when adjusted using hydrochloric acid, for example, and thus making it a suitable system for a range of dissolution experiments that simulate the body's pH. The range of concentrations and pH values studied using Tris and other buffers are summarized in Table 1.

While SBF is supersaturated with respect to apatite, and therefore does not require any release of additional calcium or phosphate ions from the bioactive glass to precipitate
apatite, the same does not hold true for more simple solutions, for example, Tris. The immersion media discussed in this section, which do not contain any physiological cations or anions (with the exception of Cl\(^{-}\) for some solutions), require bioactive glass ion release to make apatite formation possible, and we can, therefore, call this a more “active” participation of the bioactive glass in the apatite forming process.

One of the seminal works for understanding the reaction mechanisms and formation of surface layers for 45S5 in Tris buffer solution was reported by Clark et al\(^{27}\) in 1976. They analyzed compositional changes in the surface layer of 45S5 for up to 60 minutes under static conditions using Auger spectroscopic analysis. Their results demonstrated the formation of surface layers (silica-rich and calcium phosphate, CaP) at very early time points (within about 15 minutes), while the layers were still extremely thin (about 0.5 \(\mu\)m).

Later, work by Cerruti et al\(^{21}\) studied 2 \(\mu\)m particles in 0.05 mol L\(^{-1}\) of Tris at pH 7, 7.4, or 8.8, and in a 1 mol L\(^{-1}\) Tris solution at pH 6.9; all were adjusted with HCl. Their work showed that when the solution was buffered to pH 7 with a concentration of 1 mol L\(^{-1}\), the buffering capacity was sufficient to completely compensate the pH increase induced by the glass dissolution. This work was also the first study to see a drop in pH over the dissolution time. It was suggested that the uptake of carbonate and phosphate ions shifts the equilibrium within the solution toward the product side resulting in a release of H\(^{+}\) ions by the reactions HCO\(_3\)\(^{-}\) \(\rightleftharpoons\) O\(_2\)\(^{2-}\) + H\(^{+}\) and HPO\(_4\)\(^{2-}\) \(\rightleftharpoons\) PO\(_4\)\(^{3-}\) + H\(^{+}\), resulting in a decrease in pH.\(^{21}\) As such a pH decrease is often caused by dissolution of carbon dioxide from the air, it cannot be excluded that CO\(_2\) dissolution caused the observed pH drop here as well.

Cerruti et al\(^{21}\) also compared Bioglass immersion in Tris buffer to immersion in deionized water, discussed above. As expected, the buffering capacity of Tris reduced the pH increase in solutions; however, ion release varied greatly between solutions, with the release in T8 (0.05 mol L\(^{-1}\) Tris, pH 7.4) and T7 (1 mol L\(^{-1}\) Tris, pH 6.9) solutions far surpassing that in deionized water. The ion release profile in deionized water was comparable to that in the T9 solution (0.05 mol L\(^{-1}\) Tris, pH 8.8) across the 3000-minute study.

In a lot of the recent literature, the concentration of Tris used is 0.062 mol L\(^{-1}\),\(^{28-30}\) and it is usually combined with 75 mg of glass in 50 mL of solution. A study by Bingel et al\(^{24}\) investigated the effects of pH on the ionic dissolution of <32 \(\mu\)m-sized particulates of 45S5 at pH 9 \(\pm\) 0.15 and pH 7.35 \(\pm\) 0.15 in Tris-HCl buffer. By using ICP-OES to quantify the ion release, the authors showed the impact that altering the pH can have on the glass's ion release. The shape of the ion release profile was comparable between the two pH values; however, the relative ion concentrations differed dramatically. At pH 7.3, the reaction rate was faster than at pH 9, with nearly 60% of the total sodium present in solution by 24 hours, followed by calcium at 40%, then silica nearing 15%. In contrast, at pH 9, relative sodium concentration in solution was nearly half at around 30%. Calcium concentration, too, was less than at pH 7.3, reaching 17% by 24 hours. This can be explained by lower proton
concentrations at higher pH resulting in less or slower ion exchange.

What is interesting is that the silica species release was greater than the calcium release at pH 9, being around 20%, thus being higher than at lower pH. Silica solubility is strongly dependent on both pH and the salt concentration present, as shown in Figure 1A, and the results described here were probably influenced by both. Also, the amorphous silica gel layer formed during the ion exchange process can be expected to be more soluble at pH values above 8, therefore, at pH 9 more of the silica would be dissolved into solution compared to pH 7.3.31

Phosphate release from bioactive glasses is of interest, as it collates with the readily available calcium ions to form calcium phosphate species, which can be the building blocks for hydroxycarbonate apatite (HCA). Therefore, when the phosphate ion concentration in the solution starts to decrease, it is indicative of a phosphate-rich precipitate forming. This effect is also known to be pH-dependent (Figure 1B),32 and it was reflected in the studies by Bingel et al24 mentioned above. When studying ion release in Tris solutions of different pH, at pH 9 only about 8% of the phosphate from the glass was present in solution within 3 hours before starting to decrease, suggesting precipitation occurring. In contrast at pH 7.3, phosphate concentrations reached 30% at 6 hours before decreasing. At pH 5, discussed in detail in Section 2.5, the phosphate release reached 90% by 15 minutes. These differences were directly related to apatite formation (Figure 2A), as shown by Fourier-transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD). Results showed that at pH 5, P-O-related FTIR vibrations appeared as a broad band between 560 and 590 cm\(^{-1}\) (typically assigned to amorphous CaP) by 15 minutes, while the split band at 560 and 600 cm\(^{-1}\) (indicating P-O bending in crystalline calcium orthophosphate) was clearly evident at 3 hours. At pH 7.3, it took 3 days for the vibrations or diffraction peaks to be evident (Figure 2B,C). In contrast, at pH 9, these changes were not observed throughout the entire length of the study, owing to the lower ion release at this pH as shown by ICP-OES results.

Björkvik et al33 compared two different Tris formulations. These were obtained by adjusting the pH of a 0.05 mol L\(^{-1}\) Tris solution to pH 7.4 using either lactic acid (Tris-LA) or hydrochloric acid (Tris-HCl), as shown in Table 1. The silica gel layer formed in Tris-LA was thicker than that formed in Tris-HCl, and sodium concentrations were higher in Tris-LA than in Tris-HCl. (Sodium is a good indicator of ion release, as it is much less affected by apatite precipitation than calcium is.) This suggests a more pronounced ion exchange in Tris-LA than in Tris-HCl. Phosphate concentration decreased earlier in Tris-HCl compared to Tris-HAc, indicating faster apatite formation in Tris-HCl. FTIR and XRD results, however, did not

![FIGURE 1](image-url) (A) Relationship between the solubility of amorphous silica and pH at different temperatures (replotted from data presented by Iler\(^{31}\)) and (B) the relationship between calcium concentration and pH for different calcium phosphate species: dicalcium phosphate dihydrate (DCPD, CaHPO\(_4\).2H\(_2\)O), dicalcium phosphate anhydrous (DCPA, CaHPO\(_4\)), octacalcium phosphate (OCP, Ca\(_8\)H\(_2\)(PO\(_4\))\(_6\).5H\(_2\)O), beta-tricalcium phosphate (β-TCP, β-Ca\(_3\)(PO\(_4\))\(_2\)), and hydroxyapatite (HA, Ca\(_{10}\)(PO\(_4\))\(_6\)(OH)\(_2\)) (replotted from data presented by Elliott\(^{32}\))
confirm this for the time points studied (earliest time point 6 hours). Energy-dispersive X-ray spectroscopy (EDX) analysis showed that small but significant amounts of chloride were incorporated into the apatite formed in Tris-HCl, resulting in a partially substituted chloroapatite. Whether this affects precipitation kinetics, however, remains to be investigated. Scanning electron microscopy (SEM) images showed that apatite formation on bioactive glass particles predominantly occurred in protected areas such as cracks or gaps (Figure 3A), suggesting that areas of reduced fluid exchange or perturbation, and thus probably enhanced ionic concentrations, are necessary for apatite precipitation.

Fagerlund et al. performed dynamic dissolution experiments in 0.05 mol L\(^{-1}\) Tris-HCl solution. Here, the Tris solution was fed continuously (flow rate 0.2 mL min\(^{-1}\)) through a flow cell containing the glass particles and then analyzed directly by an ICP-OES, thereby obtaining compositional results on-line and in real-time. While sodium concentrations were above the detection limit, results showed that calcium ions were released fast, with maximum concentrations being reached within the first 350 seconds of the experiment. Afterward, concentrations levelled off but remained high for the remaining time (1000 seconds) of the experiment (see figure 6 for ion release curves from dynamic studies published by Blochberger et al). While concentrations for silicon species and phosphate were lower, the shape of the release curves was comparable for the three elements. pH also showed a similar trend over time, reaching a maximum of about 8.5 within the first 200 seconds of the experiment. These results show that ions are released from the glass very quickly and that these ions include soluble silica species. While apatite precipitation under such dynamic conditions is less likely than under static ones, as the constant flow of fresh Tris buffer makes it more difficult to reach supersaturation, unpublished results show that some apatite can form on the surface of the glass particles under these conditions.

2.3 Simulate body fluids

Bioactive glasses are designed to be used in the human body, therefore, when evaluating glasses for specific biological applications researchers aim to mimic the ionic concentrations of the application environment. A summary of simulated physiological solutions used to study and evaluate bioactive glasses’ behaviors is listed in Table 2. SBF is a commonly used ion-rich solution, which was originally designed to mimic the human body’s blood plasma ionic concentrations. It is highly saturated and uses Tris to buffer the pH to around 7.4. When immersing bioactive glasses in SBF, researchers usually report ionic concentrations (eg, from ICP-OES analysis); however, as with the sodium acetate buffer discussed below, it is usually difficult to quantify sodium concentrations...
release from the glass owing to the high concentrations present in the SBF solution and the, by comparison, much lower amount released from the glass itself.

Work by Helebrant et al. discusses the shortcomings of Kokubo’s SBF solution for mimicking the body’s environment, by highlighting that human blood plasma contains a higher concentration of HCO$_3^-$ ions and less Cl$^-$. Therefore, to understand whether these differences had an effect on 45S5 dissolution, the authors completed a study varying the ratio of Cl$^-$ to HCO$_3^-$ from 131:5 through to 109:27 in 5 mmol L$^{-1}$ increments (Table 2: solutions SBF5 to SBF27). It should be noted that, in this work pH was adjusted between 7.2 and 7.3 instead of the usual 7.4 for SBF testing. One of the most interesting aspects of their work is that the authors studied the stability of the solutions over the length of the experiment study, which enabled them to state that any precipitation found was caused by glass dissolution, rather than by an unstable solution. (Kokubo’s SBF has been reported to be supersaturated with regard to apatite$^5$.

**TABLE 2** Summary of the ionic concentrations of different simulated body fluids tested on 45S5 (all values in mM)

| Ionic solutions | Na$^+$  | K$^-$  | Mg$^{2+}$ | Ca$^+$  | Cl$^-$  | HCO$_3^-$ | HPO$_4^{2-}$ | SO$_4^{2-}$ | pH  |
|-----------------|---------|--------|-----------|---------|---------|-----------|-------------|------------|-----|
| Human blood plasma$^{37}$ | 142.0   | 3.6-6.5 | 1.0       | 2.1-2.6 | 95.0-107.0 | 27.0       | 0.65-1.45   | 1.0         | 7.2 |
| SBF6           | 142.0   | 5.0    | 1.5       | 2.5     | 148.8   | 4.2        | 1.0         | 0.0         | 7.4 |
| K7$^{39}$      | 142.0   | 5.0    | 0.0       | 1.6     | 144.0   | 4.2        | 1.0         | 0.0         | 7.2 |
| K8$^{39}$      | 142.0   | 5.0    | 0.7       | 1.6     | 145.4   | 4.2        | 1.0         | 0.0         | 7.2 |
| K9$^{39}$      | 142.0   | 5.0    | 1.5       | 2.5     | 148.8   | 4.2        | 1.0         | 0.0         | 7.2 |
| SBF5$^{37}$    | 142.0   | 5.0    | 2.5       | 1.0     | 131.0   | 5.0        | 1.0         | 1.0         | 7.2-7.3 |
| SBF10$^{37}$   | 142.0   | 5.0    | 2.5       | 1.0     | 126.0   | 10.0       | 1.0         | 1.0         | 7.2-7.3 |
| SBF15$^{37}$   | 142.0   | 5.0    | 2.5       | 1.0     | 121.0   | 15.0       | 1.0         | 1.0         | 7.2-7.3 |
| SBF20$^{37}$   | 142.0   | 5.0    | 2.5       | 1.0     | 116.0   | 20.0       | 1.0         | 1.0         | 7.2-7.3 |
| SBF27$^{37}$   | 142.0   | 5.0    | 2.5       | 1.0     | 109.0   | 27.0       | 1.0         | 0.4         | 7.4 |
| TE$^{38}$      | 152.0   | 5.0    | 1.5       | 2.5     | 135.0   | 27.0       | 1.0         | 0.0         | 6.5 |
| AS$^{10a}$     | 11.2    | 5.4    | 0.0       | 4.0     | 21.3    | 0.0        | 2.2         | 0.0         | 6.5 |

$^a$Artificial saliva (AS) also includes mucin.
making the solution thermodynamically unstable and resulting, sooner or later, in spontaneous apatite precipitation, regardless of any material present). Here, the authors calculated the relative supersaturation with regard to various calcium phosphates in the original immersion fluid as well as with regard to hydroxyapatite (HA) over immersion time. They also measured ion concentration over time both in the presence and absence of glass 45S5. Ion concentrations remained within the error limits if no Bioglass was present, indicating that no spontaneous precipitation occurred within 14 days. During the immersion of 45S5, results showed the typical decrease in phosphate concentration owing to apatite precipitation; however, no correlation was observed between the decrease in the phosphate concentration in solution and apatite crystallinity in the XRD results at 14 days. Calcium concentrations did not show any decrease over time, as phosphate was the limiting factor for apatite formation, while calcium release from the glass compensated for calcium consumed during apatite formation. Because of the solutions containing varying amounts of carbonate, it can be expected that this affected the amount of carbonate substitution in the apatite formed. Unfortunately, no FTIR was performed to detect carbonate-related bands, and the broad peaks in XRD make it difficult to distinguish between HA and HCA. Interestingly, XRD results suggest that apatite was formed (at 14 days) in the solutions with the lowest (SBF5 and SBF10) and the highest (SBF27) carbonate content, while no precipitation was observed in the two intermediate solutions. The authors explained this with precipitation being pH-dependent. The authors' overall conclusions were that for a more accurate prediction of bioactive glass dissolution and behavior in SBF, the ionic ratio of Cl\(^-\) to HCO\(_3^-\) should fall at 127:20 (SBF27) instead of 148.8:4.2 as used by the Kokubo method.

Martin et al. investigated the phosphate surface layer formation on polished Bioglass plates in SBF using small-angle X-ray scattering (SAXS), which allows for analyzing thin surface layers. Reaction times in SBF were varied between 1 and 72 hours, using unreacted samples as controls. Incident angles were varied between 0.2 and 1.6\(^\circ\), thereby varying the penetration depth. Results showed the presence of an amorphous CaP layer at early stages, while at 3 days, large Bragg peaks dominate the diffraction patterns, indicating the presence of HA. The authors were further able to show that HA crystallization occurred on the surface of the amorphous CaP layer, that is, between amorphous CaP and SBF, rather than directly on the bioactive glass surface.

Work by Filgueiras et al investigated the effect of additional ionic species during the dissolution of 45S5, by substituting Mg\(^{2+}\) for Ca\(^{2+}\) in SBF, as shown in Table 2. Their work showed that the conversion from amorphous calcium phosphate to crystalline HCA occurred more quickly in SBF solution K7 (containing Ca\(^{2+}\) only but no Mg\(^{2+}\)) than in Tris (90 minutes vs 120 minutes). The inclusion of Mg\(^{2+}\) ions retarded the formation of the amorphous calcium phosphate layer and its consequent conversion to HCA to 150 minutes for K8 and 360 minutes for K9 solution (both of which contained both Ca\(^{2+}\) and Mg\(^{2+}\), albeit in differing concentrations). While it has been shown that Mg\(^{2+}\) can be incorporated into the apatite lattice, the amount that can be accommodated by the apatite crystal structure is small, owing to its smaller ionic radius compared to Ca\(^{2+}\), and Mg\(^{2+}\) ions are also known to inhibit apatite crystallization.

![FIGURE 4](image-url) (A) FTIR spectra and (B) X-ray diffraction patterns of Bioglass 45S5 (<38 \(\mu\)m) immersed in either Tris or simulated body fluid (SBF) (SBF10)\(^{37}\) at 75 mg in 50 mL and 37\(^\circ\)C; initial pH = 7.4\(^{42}\)
These results explain observations in a recent study\textsuperscript{42} that apatite formation of 45S5 powder (<38 µm) in SBF (SBF10 after Helebrant\textsuperscript{37}) was delayed compared to apatite formation in Tris (0.062 mol L\textsuperscript{-1}), with first clear apatite features appearing in FTIR and XRD at 24 hours in Tris and 72 hours in SBF (Figure 4). In addition, the apatite formed in Tris seemed to possess a higher crystallinity, as bands in FTIR and peaks in XRD were more defined than those of apatite formed in SBF. In another study\textsuperscript{43} the opposite was observed, with in situ Raman spectroscopy indicating that apatite formed on polished glass discs in SBF (after Kokubo\textsuperscript{6}) showed a higher crystallinity than that formed in Tris (0.05 mol L\textsuperscript{-1}). These discrepancies may, however, be related to differences in experimental design and methods.

Maçon et al\textsuperscript{16} published the results of a round-robin study, comparing the results of immersion studies in Kokubo’s SBF either following an ISO standard (ISO 23317: Implants for surgery—In vitro evaluation for the apatite-forming ability of implant materials) or the authors’ own proposed method, termed “TC04 method.” ISO 23317 proposes the use of a fixed glass surface area to solution volume ratio, while the TC04 method uses a fixed weight to volume ratio of 75 mg glass immersed in a 50 mL of SBF solution. Samples were held at 37°C and semi-dynamic conditions, that is, agitation during immersion at 120 rpm in an orbital shaker, were chosen. Results obtained were reproducible between the different participating research facilities, suggesting that using the proposed TC04 method will enable a comparison between results from different laboratories. The influence of particle size and variation between ISO standard and TC04 method will be discussed in Section 3.

Zhang et al\textsuperscript{44} investigated the differences in pH changes and layer formation of Bioglass 45S5 (500 to 800 µm particle size) when exposed to Kokubo’s SBF under either static (0.95 g glass in 40 mL of SBF) or dynamic (0.95 g glass, flow rate 33 mL min\textsuperscript{-1}). Experiments were performed for up to 48 hours. pH increased under both conditions; however, the pH increase under dynamic conditions was more gradual, while the pH increase under static conditions happened faster and also showed a pronounced spike, reaching a maximum at 4 hours. In addition, pH varied greatly with the location within the particle bed under static conditions, while no such gradient was observed under dynamic conditions. Glass particles showed surface layer formation under both conditions, as shown by scanning electron microscopy. Under static conditions, layers were thicker and included a distinct CaP surface layer; however, layer thickness was rather uneven. In contrast, layers formed under dynamic conditions were thinner and CaP was only observed in a surface layer mixed with silica, but layers had a more uniform appearance.

### 2.4 Cell culture media and other solutions containing proteins

A range of complex solutions are used for in vitro cell culture experiments and often these are also used in acellular immersion experiments for bioactive glasses. Table 3 gives an overview of compositions discussed here.

Work by Sepulveda et al\textsuperscript{45} studied the dissolution characteristics of melt-derived 45S5 powders of three particle size ranges and compared the effects during immersion in SBF to α-MEM (Minimum Essential Medium) culture medium. Their solution consisted of 400 mL of α-MEM, 1.6 mL of penicillin/streptomycin, and 40 mL of fetal calf serum, at a ratio of 50 mL of solution to 0.5 g of glass. Their results showed that the pH in the cell culture medium increased between 8.5 and 9.5, depending on the particle size, by 24 hours.

| Solution name in publication or authors | Solution constituents | pH | Mass of glass (mg) | Volume of test solution (mL) |
|----------------------------------------|-----------------------|----|---------------------|-----------------------------|
| Sepulveda et al\textsuperscript{45}    | 400 mL of α-MEM, 1.6 mL of penicillin streptomycin, 40 mL of fetal calf serum | 7.4 | 500 | 50 |
| Lu et al\textsuperscript{48}           | TE solution (see Table 2), 0.05 mol L\textsuperscript{-1} Tris, 1 mg human plasma fibronectin | 7.4 | 1 | 1 |
| Shah et al\textsuperscript{9}          | A-MEM, Carbonate-free MEM, 20 mL, sodium acetate buffer solution | 4.8 | 75 | 50 |
|                                        | H-MEM, Carbonate-free MEM, 20 mL, 1 mol L\textsuperscript{-1} HEPES buffer solution | 6.6 | 75 | 50 |
|                                        | HC-MEM, Carbonate-supplemented MEM, 20 mL, 1 mol L\textsuperscript{-1} HEPES buffer solution | 7.4 | 75 | 50 |
|                                        | HS-MEM, Carbonate-supplemented MEM, 20 mL, 1 mol L\textsuperscript{-1} HEPES buffer solution, 100 mL of fetal bovine serum | 7.3 | 75 | 50 |
In contrast, pH in SBF remained between 7.5 and 8.25 at the comparative time points. Changes seen in the ionic release rates and concentration for the cell culture medium were consistently slower and lower than that for SBF, except for silicon for the 5-20 µm particle size range. Evaluation of surface changes using FTIR, XRD, and SEM confirmed these changes, showing a reduction in HA formation in the culture medium solution. This finding was associated with the proteins present in cell culture media but absent in SBF. It was thought that owing to the proteins’ charge they were attracted to the glasses negative surface charges, forming a coating. This coating then inhibits the degradation of the glass by forming a barrier between the aqueous media and glass itself. This barrier, however, is permeable, allowing for the dissolution of silica and other ionic species, just at a retarded rate. In contrast, in serum-free media, once an apatite layer is formed it impedes the degradation of the silica, resulting in lower overall silica release.

Other studies comparing 45S5 performance, with and without serum, or at alternative pH values are documented in the literature. Work at pH 7.4 and 7.3 with and without 10% of heat-inactivated fetal bovine serum concluded similar results to those in the study by Sepúlveda, showing that the presence of proteins on the glass particle surfaces reduces the rate of ionic exchange and HA precipitation.

In a study by Lu et al the effect of protein adsorption on 45S5 ion release and apatite precipitation was analyzed using a single protein, human plasma fibronectin. Commercial 45S5 particles (<10 µm) were immersed in an electrolyte solution (TE, Table 2) either with or without fibronectin. Their work found that the presence of fibronectin delayed the formation of a calcium phosphate surface layer. Zeta potential measurements suggested that the presence of fibronectin resulted in a more positive surface charge compared to the protein-free control. The authors concluded that protein adsorption on the glass may alter the kinetics of calcium phosphate formation directly at the glass surface, possibly also caused by competitive binding of calcium ions by the fibronectin molecule.

Bioactive glasses have been used for dental applications; therefore, it is of interest to have a solution to simulate oral environments. An example of such a solution used in literature is artificial saliva (AS: Table 2). While a large variety of AS compositions is used, many of which being commercially available, the one used by Aina et al had lower concentrations of Ca^{2+}, Na^+, Cl^−, and HPO_4^{2−}, with higher K^+ and no Mg^{2+}, HCO_3^−, and SO_4^{2−}; it also contained 2 g of mucin. Mucin is a glycosylated protein, produced by epithelial tissues in most animals. It is composed of about 75% carbohydrates and 25% amino acids and is able to bind with calcium ions at low concentrations through a carbohydrate moiety. At high calcium concentrations, its behavior changes, bonding through the amide group instead via a protein moiety. The authors compared the dissolution of 45S5 powders in AS with their previous study in Tris; however, conclusions are limited owing to different particle sizes reported in the two studies. In their AS work they used a particle size of <26 µm, while in their Tris work it was around 2 µm. Their work reported a greater pH rise in AS compared to Tris of 7.8 to 8.5 vs 6.5 to 9.5, respectively, within 2 hours. Ion release and the formation of crystalline calcium phosphate (mostly as HA) were faster in Tris than in AS. The authors hypothesized that this could be caused by the silica layer acting as a nucleating agent for the formation of a CaP species in Tris which was more similar to HA. However, it is difficult to draw conclusions as these differences may simply originate from differences in surface area for the two different particle sizes used in the tests. Transmission electron microscopy (TEM) images combined with EDX analysis showed that at 1 hour of immersion in AS (Figure 3B) or Tris buffer solution, CaP aggregates had already formed on the surface of Bioglass 4S5S particles. At 1 week of immersion (Figure 3C), the bioactive glass particles had reacted completely, consisting of a silica gel and CaP exclusively.

### 2.5 Acidic solutions

Björkvik et al studied the behavior of 45S5 in acidic solutions containing either a weak acid (lactic acid, LA; \( pK_a = 3.8 \)) or a strong acid (hydrochloric acid, HCl). SEM images show progressing surface layer formation during immersion (Figure 5C). What makes this study particularly interesting is that the authors compared solutions having either the same pH \( (pH = 2; c(LA) = 0.4 \text{ mol L}^{-1}, c(HCl) = 0.01 \text{ mol L}^{-1}) \) or the same acid concentration \( (c = 0.04 \text{ mol L}^{-1}; pH(LA) = 2.6, pH(HCl) = 1.4) \). Results (Figure 5) show that not only the initial pH is relevant, but that also the total acid concentration and, particularly, the buffering capacity in the solution affect solution pH, mass loss, and layer formation. LA, as a weak acid, is only partially dissociated in solution, leaving a significant amount of undissociated LA molecules available to donate protons and react after some reaction with the glass has occurred. HCl, in contrast, is completely dissociated in dilute concentrations, meaning the pH reflects the total proton availability in solution, without any further “reservoir” being available once protons have been consumed. As a result, when comparing the two solutions at the same initial pH, ion release (except Si species) was much higher in LA than in HCl, owing to a larger total amount of protons (including the undissociated ones present in LA molecules) available for ion exchange reactions. This is also reflected in a higher total weight loss (Figure 5A) and a much thicker silica gel layer formed in LA (Figure 5D,E). As LA can form a buffer, pH reached a maximum of about 3.8 (Figure 5A), corresponding to its \( pK_a \), while the pH in the HCl solution increased to over 8 at day 3. As a result, a CaP layer is formed in the HCl
solution but not in LA (Figure 5D,E), as the pH remained too low.

When comparing LA and HCl solutions of the same total acid concentration, ion release and weight loss were comparable in both solutions (Figure 5B). The shape of the pH curves varied between the two solutions, forming a plateau at about 3.8 for LA, corresponding to the lactic acid/lactate buffer formed during the reaction. The thickness of the silica gel layers formed was comparable, too, owing to comparable ion release. A CaP layer was formed on top of it in both solutions. However, while the CaP layer formed in LA was thin but dense, the one formed in HCl was not only thicker but also covered in a layer of flake-shaped crystals (Figure 5F), possibly indicating the formation of octacalcium phosphate rather than an apatite.

This study by Björkvik et al. is particularly relevant as lactic acid is formed during the degradation of poly(lactic acid) (PA), a degradable polymer used clinically as a resorbable implant material. Bioactive and other silicate glasses have been used as fibers or particles to reinforce PLA, and the study by Björkvik et al. gives insight into possible reactions between 45S5 and PLA degradation products, which may occur in vivo once the PLA matrix starts degrading.

For studies at low pH, a combination of sodium acetate and acetic acid has been used as a buffered solution. Acetic acid’s $pK_a$ of 4.76 makes it suitable for experiments at low pH; however, owing to the contribution of sodium in the solution itself, sodium release from the glass is usually not reported. A study by Bingel et al. investigated the effects of pH on ion release and apatite formation of fine 45S5 powder (<32 µm) at an initial pH of 5 ± 0.05 in an 0.1 mol L$^{-1}$ acetic acid/sodium acetate buffer, comparing it to 0.062 mol L$^{-1}$ Tris-HCl at pH 7.35 ± 0.15 (Figure 2A). As expected, ion release was much higher at pH 5, owing
to more protons being available for ion exchange. At pH 5, ion release occurred at the earliest time point, with 90% of calcium and phosphorous ions being found in solution by 15 minutes. At 3 hours, about 90% of Ca and 80% of P were present in solution. At pH 7.3, the reaction rate was slower, with 40% of Ca and 30% of P being detected in solution at 3 hours. This was reflected in the onset of apatite formation. FTIR showed the typical split band at 560 and 600 cm\(^{-1}\) at pH 5 as early as at 3 hours (with an amorphous CaP being possibly present by 15 minutes), while it was possibly present at pH 7.4 at 6 hours, with a clear split band being present at one day. XRD confirmed the CaP layer formed to be apatite.

Blochberger et al\(^{36}\) performed similar immersion experiments, except that the pH of their 0.1 mol L\(^{-1}\) acetic acid/sodium acetate (HAc/NaAc) buffer was adjusted to pH 4 only. While the results observed for ion release were comparable to those by Bingel et al\(^{24}\), the pH increase in acetate buffer was not high enough (remaining below pH 4.5) to allow for significant apatite formation. As a consequence, apatite signals in FTIR and XRD were very low. The authors also performed dynamic ion release studies (Figure 6) using the same setup as used by Fagerlund et al\(^{35}\) mentioned above. Ions in solution were normalized to the total amount of ions present in the untreated glass, and ion release was studied over a period of 1500 seconds. Sodium concentrations could not be determined, as concentrations were above the detection limit of the ICP-OES. Calcium concentrations, again, were higher in acetate buffer (pH\(_0\) = 4, up to about 7 L\(^{-1}\)) than in Tris-HCl (pH\(_0\) = 7.4, up to about 4 L\(^{-1}\)). Silicon concentrations were higher at pH 7.4 (up to about 1 L\(^{-1}\)) compared to pH 4 (around 0.2 L\(^{-1}\)). However, most noticeable was the difference in phosphate concentrations, with concentrations reaching over 30 L\(^{-1}\) at pH 4 compared to just above 1 L\(^{-1}\) at pH 7.4. While CaP formation can be expected to be less under dynamic conditions than under normal static (or semi-static, i.e., shaking) conditions owing to the continuous flow making it less likely to reach supersaturation, it cannot be entirely

**FIGURE 6** Ion release from Bioglass 45S5 in (A,B) Tris-HCl or (C,D) HAc/NaAc buffer under (A,C) dynamic or (B,D) static conditions\(^{36}\) [Color figure can be viewed at wileyonlinelibrary.com]
excluded. It is, therefore, possible that phosphate concentrations at pH 7.4 were lower as some had been consumed during CaP formation, while this had not occurred at pH 4.

Shah et al. performed dissolution experiments on 45S5 in a low pH culture medium. The medium was based on Eagle's Minimum Essential Medium with Earle's salts, but pH was adjusted to 4.80 using a sodium acetate buffer solution. ICP-OES results showed phosphate depletion by day 3 and FTIR showed a sharp P-O stretching phosphate band at 1020 cm⁻¹; however, apatite signals in XRD were weakly pronounced even at day 7. As the pH in the body can become acidic under certain conditions such as inflammation and bacterial growth, acidic conditions are known to prolong inflammation and delay healing, reinforcing the evidence that at high glass loadings the dissolution behavior directly correlates with the observed silicon concentrations, which for the fine 45S5 powders increased from 0.9 to 70 ppm within the first 1.5 hours of the study. The coarsest particle fraction only reached 25 ppm by the equivalent time point. Similar trends were reported for calcium dissolution and reduction in phosphorous concentration.

The work by Maçon et al. showed the influence of glass particle size and relative surface area. They compared 45S5 in two different particle size ranges (Bioglass, BG: particle size 45-90 µm; NovaBone, NB: 90-710 µm). The dissolution was studied at a ratio of 75 mg in 50 mL of SBF. While the ion release data shown in the publication do not allow us to distinguish between the ion concentrations for BG and NB, FTIR and XRD results clearly show that BG formed apatite slightly faster than NB, with phosphate bands at 560 and 600 cm⁻¹ being more pronounced for BG at 24 hours. This is most likely caused by the smaller particle size and larger relative surface area of BG, allowing for faster ion exchange and reaching supersaturation faster than NB.

Maçon et al. also compared different glass to SBF volume ratios, by comparing the method described in ISO 23317, using a fixed glass surface area to solution volume ratio, and their TC04 method, which uses a fixed weight to volume ratio (details see Section 2.3). Using the ISO method resulted in different mass to volume ratios for different samples (4.17 mg of BG or 2.78 mg of NB per 100 mL of SBF), depending on the glass particle size and measured surface area, while in the TC04 method a fixed ratio of 150 mg in 100 mL was used. Particularly for compositions with a large surface area (eg, sol-gel glasses), the ISO approach leads to such low mass of glass to be used that the experiments cannot be performed. The reason is that the ISO standard was designed for solid samples of regular geometric shape, not for powders or porous materials. The much lower weight to volume ratio compared to the TC04 method also resulted in a later onset of apatite formation at 72 hours for BG, compared to 24 hours using the TC04 method. The authors, therefore, recommend the use of a fixed weight/volume ratio for immersion studies of bioactive glasses. It is important to note, however, that substitutions in the glass composition may make it necessary to adjust the weight used during immersion studies. When replacing an element with another of lower (eg, replacing sodium with lithium) or higher atomic weight (replacing calcium with strontium) on a molar base, the molar weight of the glass changes. If a constant mass of glass (eg, 75 mg) is used for the experiments, the molar amount changes with composition, which has been shown to affect pH and, thus, ion release during immersion experiments.

3 | IMPACT OF GLASS CONCENTRATION AND SURFACE AREA

Early work by Jones et al. studied the effect of glass concentrations between 0.001 g mL⁻¹ and 0.015 g mL⁻¹ on dissolution behavior in SBF. The particles studied in this work were 5 µm in size; ion release was characterized, and the rate at which HA was formed was assessed by FTIR and XRD. At 2 hours, all concentrations of glass presented the P-O vibration in FTIR and when analyzed via XRD only the 0.002 g mL⁻¹ concentration showed the apatite main reflex at 31°2θ, synonymous with HA formation. However, as the concentration increased from 0.005 to 0.015 g mL⁻¹ a competing calcite peak (29°) formed instead. As the study progressed to 22 hours of dissolution, more phosphate vibrations were evident in the FTIR spectra for the 0.001 and 0.002 g mL⁻¹ concentrations studied. At higher glass concentrations, just one single band corresponding to amorphous calcium phosphate was observed instead of multiple P-O vibrations, reinforcing the evidence that at high glass loadings HA formation is inhibited.

Sepulveda et al. studied the dissolution of melt-derived 45S5 powders of three sizes, fine (5-20 µm), medium (9-300 µm), and coarse (90-710 µm) in SBF. Their work showed that the dissolution behavior directly correlated with the particle size tested, with the greatest dissolution occurring for the finest particle fractions. The change in pH related to particle size fraction was evident: for the coarsest particle size range, the pH did not increase above 7.6, whereas for the fine volume fraction, it peaked around 8.3. This change in pH had a subsequent effect on the observed silicon concentrations, which for the fine 45S5 powders increased from 0.9 to 70 ppm within the first 1.5 hours of the study. The coarsest particle fraction only reached 25 ppm by the equivalent time point. Similar trends were reported for calcium dissolution and reduction in phosphorous concentration.
Many immersion studies on 45S5 are performed on sintered porous scaffolds.\(^6^9\) Owing to the pronounced crystallization tendency of 45S5,\(^6^0\) these scaffolds tend to be partially to fully crystalline. Amorphous and crystalline phases differ in their solubility, resulting in the heterogeneous dissolution of the material.\(^6^1\) As the relative amounts of amorphous and crystalline phases present are rarely quantified, comparing the results is challenging.

Recently, Rohanová et al.\(^6^2,6^3\) performed a series of in vitro tests on scaffolds derived from sintering (and, thus, partial crystallization) of Bioglass 45S5. These scaffolds were composed of crystalline (77.4 wt%) and glassy phases (22.6 wt%). The crystalline phase consisted mainly of combite (Na\(_2\)O-2 CaO-3 SiO\(_2\)). About 0.05 g of scaffold was immersed under static, static-dynamic (SBF solution was daily exchanged) or dynamic conditions (solution flow rate 48 mL d\(^{-1}\)) in SBF. SBF was prepared according to ISO 23317, which is buffered using Tris buffer. This was compared to scaffolds immersed in (a) Tris buffer, (b) deionized water, and (c) non-buffered SBF.\(^6^2\) Results showed that Tris buffer accelerated scaffold dissolution, with Ca\(^{2+}\) concentrations being twice their original value in SBF during the first 8 hours of immersion. Moreover, Tris buffer enhanced HA formation, while in Tris-free SBF amorphous CaP was formed only. Similar results were obtained when using HEPES\(^6^3\) (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) or MOPS\(^6^4\) (3-(N-morpholino)propanesulfonic acid) buffer instead of Tris.

These results suggest that the type of buffer used has a significant impact on the outcome of bioactive glass immersion studies. While the results published so far focused on partially crystalline samples, which are known to behave differently from amorphous 45S5, it might still be necessary to reconsider the type of buffers used for simulating a physiological environment during immersion tests.

### 5 | CONCLUSIONS

When evaluating ion release, pH changes, and apatite precipitation during immersion experiments using bioactive glasses, there is a wide variety of solutions available that can be used. However, depending on the solution composition, pH, and buffering capacity, the results are likely to vary. In addition, bioactive glass particle size and solution volume/glass surface area ratio will affect the resulting ion concentration in solution, and, thus, the rate at which apatite is formed. It is, therefore, important to consider these effects when planning experiments or interpreting results.

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