Surface characterization of silicate bioceramics

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The success of an implanted prosthetic material is determined by the early events occurring at the interface between the material and the body. These events depend on many surface properties, with the main ones including the surface’s composition, porosity, roughness, topography, charge, functional groups and exposed area. This review will portray how our understanding of the surface reactivity of silicate bioceramics has emerged and evolved in the past four decades, owing to the adoption of many complementary surface characterization tools. The review is organized in sections dedicated to a specific surface property, each describing how the property influences the body’s response to the material, and the tools that have been adopted to analyse it. The final section introduces the techniques that have yet to be applied extensively to silicate bioceramics, and the information that they could provide.

Keywords: bioceramics; surface characterization; bioglass

1. Introduction

The surface of a prosthetic material is the first region that comes into contact with living tissues after implantation. Within minutes, serum proteins adsorb at the material–body interface, cells adhere and spread, and their activity determines the long-term success or failure of the implant. For over 20 years [1], biomaterial scientists have sought biomaterials with surfaces that interact in a desired way with the tissues that are in contact with them, for example, promoting the formation of a layer of hydroxycarbonate apatite (HCA) if the material is to be joined to bones; or vice versa, inhibiting such a layer if it is to replace cartilage or blood vessels. Both responses can be potentially induced by the same biomaterial, provided its surface is modified with adequate cues.

Of course, the importance of surfaces goes far beyond biomaterials, reaching diverse applications from catalysis to lubrication. Since the last century, chemical physicists have collected a vast array of tools for characterizing material surfaces. Examples include microscopic techniques that allow visualizing reactive defects such as kinks and steps, spectroscopies that provide information on the chemical composition and atomic bonding of the outermost atomic layers, and grazing-angle diffraction setups that discriminate between the crystallographic phases

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at the surface and those in the bulk. Although these techniques can be applied to biomaterials, many of them achieve surface sensitivity by collecting electrons or radiation emitted in ultra-high vacuum (UHV), or by measuring minute electrical fields that can be detected only on atomically flat samples. These constraints have not scared away the biomaterials community—these techniques supply information that in situ measurements or more realistic set-ups cannot. In this review, we will describe both types of surface analytical tools, and we will focus on their application to silicate bioceramics, by which we (and the community) mean silicate-based ceramic, glass–ceramic and glassy materials. In 1971, Hench [2] developed the first silicate bioceramic, bioglass 45S5 (BG), a glass containing 45 per cent SiO$_2$, 24.5 per cent CaO, 24.5 per cent Na$_2$O and 6 per cent P$_2$O$_5$ (weight percentages). BG dissolves once immersed in body fluids, and induces the formation of HCA on its surface, which in turn stimulates collagen integration and bone production. BG is commercially available and used for both dental and orthopaedic applications. In 1982, Kokubo et al. [3] introduced silicate glass–ceramics, which contain MgO, CaO, SiO$_2$ and P$_2$O$_5$, and have been used as bone defect fillers, and for vertebral and iliac crest replacement. Since then, other types of silicate bioceramics have been proposed, which mainly fall into two categories: silica-based bioactive glasses, such as BG, whose composition contains different oxides, and crystalline calcium silicate ceramics, including wollastonite (β-CaSiO$_3$), pseudowollastonite (α-CaSiO$_3$), dicalcium silicate (Ca$_2$SiO$_4$) and tricalcium silicate (Ca$_3$SiO$_5$) [4]. Here, we will discuss the application of surface characterization tools mainly to materials in the first category, although we will also provide examples from the second.

Surface properties determine the response of the body to silicate bioceramics at many stages, from the initial HCA precipitation to subsequent cellular adhesion and spreading. The events that occur at the body–material interface are so crucial that the original classification of bioceramics was based on the evolution of this interface after implantation [5]. Much research has focused on the surface properties responsible for early interfacial events. Although some review articles touch on the techniques used to characterize silicate bioceramic surfaces, there has been no comprehensive review. In fact, recent book chapters dedicated to bioceramics characterization mostly focus on the mechanical properties of these materials [6].

We will organize this review in sections dedicated to a specific surface property; each will begin by introducing how the property influences the body’s response to silicate bioceramics, and then go on to describe the techniques that have been used to analyse it. In the end, we point out that there is still much to do in the field of silicate bioceramic characterization: many recent techniques have yet to be applied, extensively or at all. We will describe what could be learned from each of these techniques.

2. Surface composition

(a) Introduction

Bulk composition is one of the most important parameters determining the bioactivity of silicate bioceramics. For example, melt-derived glasses in the SiO$_2$–CaO–Na$_2$O–P$_2$O$_5$ system are highly bioactive only within a narrow
range of compositions and are not bioactive at all for SiO$_2$ compositions higher than 60 mol\% [7]. The addition of Al ions in the silicate network increases the mechanical properties, but makes the materials bioinert. The surface composition reflects the bulk composition; yet, often the surface is enriched in elements that may determine the bioactivity of a material. For example, the high bioactivity of BG has been attributed to an enriched Na surface layer [8], which strongly enhances the hydrophilicity of the material [9] and speeds up its dissolution in body fluids. Small amorphous clusters of calcium phosphate at the surface of mesoporous bioactive glasses (MBGs) are suspected to be at least partially responsible for the superior bioactivity of these materials [10]. Differences in surface and bulk composition are relevant not only for bioactive glasses, which have an amorphous structure that allows for easy ion migration, but also for silicate bioceramics. For example, substituting Ca with Sr in CaSiO$_3$ increases the local interfacial solid–solution pH upon immersion in body fluids, which in turn can enhance osteoblast activity [11].

(b) Characterization tools

The reader will notice that most of the techniques described in this section are performed in UHV. UHV is necessary to collect the signal (electrons or photons) emitted by the surface layers and to analyse their composition. The analysis of biomaterials in UHV poses serious concerns: on the one hand, the organic, hydrated layer present at their surface may contaminate an instrument operating in UHV; on the other hand, high-energy electron or X-ray beams used as probes may damage the soft components of biological samples [12]. In the last two decades, several techniques were developed to overcome these challenges, thus bringing the field of biological surface analysis from its infancy to a recognized, mature state [13,14].

(i) Energy dispersive spectroscopy

The surface composition of bio ceramics is most commonly analysed with energy dispersive spectroscopy (EDS), also known as energy dispersive X-ray spectrometry (EDX), electron probe microanalysis (EPMA) or energy dispersive analysis of X-rays (EDAX). This technique is not strictly ‘surface’ sensitive because it analyses the X-ray characteristic radiation emitted upon bombarding the sample with electrons, which originates from 0.1 to 10\,\mu m underneath the surface. EDS is commonly performed inside a scanning electron microscope (SEM), which generates elemental distribution maps with nanometre resolution. Since the discovery of bio ceramics, EDS has been used extensively to analyse compositional changes upon implantation or immersion in simulated body fluids (SBFs). In 1977, Hench et al. [15] adopted EDS to observe the formation of a Ca–P-rich layer at the surface of BG after 10 days of implantation; in 1990, Kokubo [16] found a similar layer on hydroxyapatite–wollastonite-coated implants, again using EDS. Since then, this technique has been used to analyse HCA precipitation on almost any bio ceramic developed. The spatial resolution of EDS maps can be tremendously improved if the EDS detector is placed inside a transmission electron microscope (TEM). The combination of TEM and EDS allowed Cerruti et al. [17] to record unprecedented details of HCA formation on BG (figure 1): they observed the separation of Ca–P-rich nanospheres and
silica-rich particles within the first hour of reaction in solutions buffered with tris(hydroxymethyl amino methane) (tris), and they showed that the particles with higher surface area were those composed mainly of calcium phosphate, rather than the silica-rich ones, as previously hypothesized. Using EDS in combination with TEM, Izquierdo-Barba et al. [18] observed for the first time the formation of octacalcium phosphate nanoparticles on an MBG, and their transformation into HCA after a few hours of immersion in SBF. TEM–EDS performed on thin sections of osteoblasts cultured on bioactive sol–gel glasses showed the formation of Ca–P-rich noduli as a result of osteoblast activity, in contact with the Ca–P–Si-rich glass grains [19]. Recently, Aina et al. [20] were able to confirm with TEM–EDS the formation of a fluoroapatite nanostructured phase on fluorine-containing bioactive glasses immersed in both tris and artificial saliva.

(ii) Auger electron spectroscopy

Auger electron spectroscopy (AES) is a highly surface-sensitive technique that allows analysis of the composition of the outermost monolayers with an atomic
sensitivity of about 0.1 per cent [21]. Auger spectroscopy is based on the analysis of the Auger electrons emitted upon interaction of a material with a beam of either electrons or X-rays with high enough energy (figure 2). Upon irradiation, atoms become ionized and lose a core electron, thus reaching a higher energy, excited state. The relaxation process can occur either by the emission of X-ray radiation or by the decay of a second core electron to fill the vacancy formed, followed by the emission of an electron from a third energy level. The energy of this electron, called the ‘Auger electron’, is characteristic of the emitting atom. Because Auger electrons are emitted with low kinetic energies, only the very top layers (first 5–20 Å) are analysed. Similar to EDS, AES is performed in UHV, and can achieve a spatial resolution of 10 nm. The efficiency of the Auger process is highest for elements with low atomic weight ($Z < 40$), which makes AES particularly suited for the analysis of bioceramics.

In the late 1970s, Hench et al. alternated AES and ion milling to measure compositional depth profiles on BG [22] and other bioactive glasses [23] immersed in SBF, and identified a Ca$-\text{P}$-rich layer formed on top of a silica-rich layer. A few years later, Andersson & Kangasniemi [24] used the same strategy to compare the dissolution of two glasses in solutions buffered with tris only, tris and citric acid, and in SBF. They found that the thickness of the silica-rich layer was largest in the solution containing both tris and citric acid, probably because citric acid can form complexes with Ca$^{2+}$ ions and leach them out from the glasses. Interestingly, since then AES was rarely used to characterize bioactive glasses; however, in the early 2000s, Liu et al. [25,26] applied AES to study wollastonite. They showed that the mechanism of HCA formation was similar to that observed for bioactive glasses: a silica-rich layer formed after 30 min of immersion, and was covered by a Ca$-\text{P}$-rich layer after 12 h. The surface of these materials was enriched in Ca before P (figure 3).

(iii) X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) determines the elemental composition of the surface of a sample by analysing the kinetic energy of core electrons emitted upon irradiation of the material with X-rays (‘photoelectrons’; figure 4). The kinetic energy of the photoelectrons depends on the energy with which
Figure 3. Auger depth profile acquired on wollastonite coatings soaked in SBF for 12h. The phosphorus concentration decreases, whereas calcium increases with depth, indicating that calcium accumulates on the silica-rich layer prior to phosphorus. Reproduced with permission from Liu et al. [26].

Figure 4. Schematic of the emission of a 1s photoelectron. (Online version in colour.)

they were bound to the emitting atom, thus allowing identification. XPS is as surface sensitive as AES, and an even higher surface sensitivity can be achieved by collecting the photoelectrons emitted at shallow angles from the surface [27]. As with AES, XPS requires UHV.

The application of XPS to bioceramics is relatively recent. Serra et al. [28] used XPS to confirm the presence of different amounts of non-bridging oxygen atoms (NBOs; i.e. oxygen atoms that do not bridge between two silicate tetrahedra forming the glass network) in bioactive glasses with different compositions. Mahmood & Davies [29] performed XPS depth profiles, similar to those described earlier for AES, on samples of BG immersed in amino acid-containing media, and showed that the amino acids adsorb continuously on its surface while the Ca—P-rich layer is formed. Pérez-Pariente et al. [30] presented a study of the effect of Mg in sol–gel bioactive glasses; among other techniques, they used
XPS to show a lower amount of Ca at the surface of all the glasses compared with bulk, and a higher amount of phosphate on the Mg-containing glasses. The association of phosphate with Mg instead of Ca at the surface of the Mg-containing glasses seemed to be responsible for their lower bioactivity. Cerruti et al. [9] used XPS to prove that surface Na atoms are responsible for the strong hydrophilicity of BG. Karakoti et al. introduced nanoceria in bioactive glass scaffolds to increase collagen production by human mesenchymal stem cells (HMSCs). More collagen was produced by HMSCs seeded on scaffolds containing nanoceria synthesized in water rather than in dextran. The authors analysed the particles with high-resolution XPS, and related their biological effect to a higher Ce$^{3+}$–Ce$^{4+}$ ratio found on the ceria nanoparticles synthesized in water [31]. Mladenovic et al. showed compositional changes at the solid–liquid interface of BG and two hydroxyapatite-based bone grafts (Bio-Oss and Algipore) in cell culture medium by measuring XPS spectra of fast-frozen samples [32,33]. This technique showed several interesting results: more Cl$^{-}$ than Na$^{+}$ ions were adsorbed on fast-frozen BG, thus indicating that the BG surface was positively charged; a similar result was shown for Algipore, whereas Bio-Oss particles turned out to be negatively charged. The positive surface charge was correlated with the larger amount of organic material adsorbed and with the lower amount of calcium phosphate precipitated on BG and Algipore compared with Bio-Oss. Also, high-resolution XPS spectra showed that the amino acids adsorbed on BG and Algipore were partially deprotonated, which was hypothesized to be related to the bioactivity of these materials.

(iv) Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is intrinsically a bulk technique. Given its sensitivity to the environment surrounding the central atom analysed, solid state $^{29}$Si and $^{31}$P magic angle spinning (MAS) NMR have been used extensively to characterize the number of NBOs in silicate and phosphate species present in bioactive glasses before and after immersion in SBFs, or the coordination of alkaline ions in silicate bioactive glasses. Some level of surface sensitivity with NMR can be obtained by analysing small particles, which have a high surface-to-bulk ratio, and using NMR techniques that measure interactions between nuclei of different elements. For example, using $^1$H $\rightarrow$ $^{31}$P cross polarization (CP) and $^{31}$P homonuclear double-quantum NMR, Lin et al. [34] were able to show the formation of an amorphous P-containing layer on microscopic beads of bioactive glasses during the first 3h of immersion in SBF (figure 5). This was proved to be a surface layer, strongly interacting with water molecules and HPO$_4^{2-}$ ions. The layer partially transformed into HCA after 6h of immersion; however, even after 22h, the authors could still detect an amorphous phosphate layer, which indicates how surface sensitive this technique is. Recently, Leonova et al. [35] performed $^1$H $\rightarrow$ $^{31}$P and $^1$H $\rightarrow$ $^{29}$Si CP-NMR experiments on ordered MBGs. They showed that silanol groups and physisorbed water present at the pore walls were always associated with Q$^2_{Ca}$, i.e. silicate tetrahedral species containing two NBOs in interaction with Ca$^{2+}$ cations. Also, the $^1$H signal relative to adsorbed water was broader on glasses containing more Ca$^{2+}$; these observations indicated that the pore surfaces were enriched with Ca$^{2+}$. Finally, CP-NMR showed that surface
Ca\textsuperscript{2+} was associated with phosphate groups, which pointed at the formation of nano-sized clusters dispersed along the pore walls. These clusters were hypothesized to be responsible for the high bioactivity of mesoporous-ordered bioactive glasses.

3. Surface functionalities

(a) Introduction

Surface functional groups strongly influence the bioactivity of silicate bioceramics: they can act as nucleation sites for HCA, and they affect the interactions with water or other biologically relevant molecules. Understanding these interactions is crucial to achieving an atomistic description of bioactivity, and is currently the subject of much research, both experimental and computational. For example, surface silanols were hypothesized to be nucleation sites for HCA [16], which could explain why HCA precipitated, even on pure silica. However, both early experimental results [36] and later computational models [8] disputed this hypothesis. Tilocca & Cormack’s [8] molecular dynamics (MD) simulations showed that silanol concentration is only very marginally higher on BG compared with bioinert glasses, and cannot be sufficient to explain BG’s much higher bioactivity. As an alternative to silanols, early work by Pereira & Hench [36] and later \textit{ab initio} cluster simulations paired with NMR spectroscopy by
Sahai and co-workers hypothesized three-membered siloxane rings as nucleation sites for HCA on both wollastonite [37] and bioactive glasses [38]. However, Tiloca & Cormack’s [8] MD models showed that the concentration of these small rings was higher on bioinert glasses than on BG. These apparently contradictory results may indicate that small siloxane rings influence HCA nucleation only after the immersion of silicate bioceramics in body fluids, while their initial concentration may not be a direct indication of bioactivity [8]. Surface coordinatively unsaturated cations (cus) are cations possessing lone electron pairs not involved in interactions with other atoms or molecules, and are often important sites of catalytic activity. Cerruti et al. [9] showed that while surface cus Na\(^+\) cations are responsible for the high hydrophilicity of BG and for the formation of a layer of surface sodium carbonate, the interactions with water and CO\(_2\) are determined by surface cus Ca\(^{2+}\) cations in SiO\(_2\)–CaO–P\(_2\)O\(_5\) sol–gel glasses [39,40]. Recent MD computational models by Tiloca & Cormack [41] confirmed that surface cations in close proximity to silicon atoms are strong sites for water interaction.

Finally, surface functionalities are crucial if one wants to modify silicate bioceramics [42]. Biological cues (proteins, peptides, etc.) can be bound to surface functional groups to control interactions with cells, and achieve a desirable response when the material is implanted.

(b) Characterization tools

While silicate bioceramics are relatively recent materials, the surface functionalities of catalysts have been studied in detail for almost a century. Castner & Ratner [13] recently made a direct comparison between surface functionalities in biomaterials and in catalysts; they pointed out that biomaterial performances could be improved if the reactions occurring at their surfaces were studied at a molecular level, similarly to what is routinely performed on catalysts. Cerruti et al. [39,43] pioneered this approach on sol–gel bioactive glasses, and studied their surface reactivity with probe molecule-based spectroscopic and calorimetric techniques typically used to study catalysts. Experimental work in this field is paralleled by multi-scale computational models, which can provide an atomistic description of the average phenomena measured experimentally [5,8,37,38,41,44–47]. Even though not specific on surfaces, excellent reviews of computational models of the structure and reactivity of bioactive glasses have been published recently [48,49].

(i) Infrared spectroscopy

Infrared (IR) spectroscopy monitors functional group vibrations that are excited by the absorption of IR radiation at specific frequencies. The surface sensitivity of IR spectroscopy depends on its configuration. An IR spectrum measured in transmission on KBr-diluted pellets shows mostly bulk vibrations. Greater surface sensitivity can be achieved by recording spectra in reflection mode—either specular reflection on smooth samples (IR reflection spectroscopy; IRRS), or scattered reflectance from powders or rough samples (diffuse reflectance IR spectroscopy; DRIFT). In the early 1970s, Hench and co-workers applied IRRS to study silicate glasses [50], and pointed out that the surface sensitivity of IRRS for silicate-based materials is of the order of 0.5 \(\mu\)m. With this expertise, Hench
and co-workers used IRRS in 1980 to study the reactivity of bioglasses in tris-buffered solutions [23]. Since then, DRIFT and IRRS have been extensively used to monitor the transformations of silicate bioceramics in body fluids. These techniques can discriminate between Si–O containing bridging oxygens and NBOs, which makes it possible to monitor cation release and the formation of a silica-rich layer. Also, bands relative to P–O stretching can be broad or show clearly separated peaks, thus allowing one to discriminate between amorphous and crystalline calcium phosphate layers.

Similar bands can be studied with attenuated total reflectance (ATR) IR spectroscopy. ATR measures are performed by bouncing IR waves through a crystal with high index of refraction, which does not absorb IR radiation (figure 6). IR radiation striking the internal interface of the crystal at an angle higher than the so-called ‘critical angle’ is totally reflected. A standing evanescent wave travels on the opposite side of the interface up to a depth of fractions to a few micrometres, thus sampling the material of interest, provided it is in intimate contact with the crystal. A good discussion of surface sensitivity of Fourier transform (FT)-IR ATR applied to (polymeric) biomaterials can be found in Barbucci et al. [51]. Because ATR is sensitive to only a thin layer of material, spectra of samples immersed in water can be recorded with this configuration. This is a great advantage over other configurations, in which the signal coming from water completely obfuscates any other signal. FT-IR ATR allows analysis of changes occurring on materials immersed in aqueous solutions in real time, such as protein adsorption [52]. Cerruti et al. [53] used FT-IR ATR to study changes occurring on small BG particles during the very first few minutes after immersion in tris-buffered solutions. They showed that cations are released from the glass at the same time as silica and Ca–P-rich layers are formed on the glass surface, rather than in successive steps as originally hypothesized (figure 7). ATR-FTIR microscopy combines spectroscopic analysis with a spatial resolution of 1–2 μm; Marelli et al. [54] recently used this technique to map the location of BG in collagen scaffolds, and monitor its transformation upon immersion in SBF. Similar maps were measured earlier on BG/poly (DL-lactic acid) composite scaffolds [55].

The highest surface sensitivity to analyse functional groups can be achieved by measuring IR spectra in vacuum while monitoring the adsorption of probe molecules from the gas or vapour phase. This technique was pioneered in the mid-1970s by Morrow & Cody [56] on silica substrates, and since then, it has been used extensively to study active sites of heterogeneous catalysts, and the intermediates of reaction formed on their surfaces. This method involves preparing very thin pellets of the sample of interest, and measuring the spectra in transmission mode;
the signal from bulk groups saturates the detector, while surface group vibrations can be analysed. Spectra are recorded in vacuum, and shifts in peak positions observed upon the adsorption of probe molecules indicate the availability of surface functional groups. Cerruti et al. [39,40,43] used this technique to analyse surface groups of SiO$_2$–CaO–P$_2$O$_5$ sol–gel bioactive glasses, and observed that Ca and P were responsible for the formation of much more active surface sites than silanols. They identified these sites as surface $\text{cuss}$ Ca$^{2+}$ cations and Si–O–P labile bridges; $\text{cuss}$ Ca$^{2+}$ were especially involved in the strong coordination of water, acetonitrile (a Lewis acid of medium strength) and CO$_2$. The importance of surface $\text{cuss}$ Ca$^{2+}$ was later confirmed by Bolis et al. [57] who studied methanol adsorption. Cerruti et al. [9] measured transmission IR spectra in vacuum on thin pellets of BG too, and showed that the strength of H bonds between surface hydroxyl groups decreased after reaction in tris-buffered solutions. They related this observation to the strong coordination of water on $\text{cuss}$ Na$^+$ cations on BG; this strong water coordination was recently connected with the high bioactivity of BG [8].

(ii) Raman spectroscopy

Similar to IR, Raman spectroscopy monitors functional groups by analysing their vibrations; however, the sample is excited not with IR radiation, but with a laser emitting in the ultraviolet–visible range, and the differences in vibrational

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**Figure 7.** Attenuated total reflectance spectra of 2$\upmu$m BG particles (4502) immersed in tris for: (a) 5 min, (b) 2h, (c) 6h, (d) 24h and (e) 48h. Inset: (1) 4502 immersed for 1h in tris, (2) tris solution 0.05M at pH 8 and (3) spectrum (1)–(2). The decrease of the band at 940 cm$^{-1}$ and the increase of the band at 1200 cm$^{-1}$ occurring at the same time indicate that cations leach out while the silica gel layer is formed on BG. Reproduced with permission from Cerruti et al. [53].
states before and after the excitation are analysed. Raman spectra are commonly recorded inside a microscope; thus, the surface sensitivity is determined by the numerical aperture (NA) of the objective used and the wavelength of the excitation laser ($\lambda$) according to the following equation:

$$h = \frac{4\lambda}{NA^2}.$$ 

For example, a laser at 785 nm (commonly used to analyse biological samples) and an objective with $NA = 0.8$ provide a depth of analysis of 4.9 $\mu$m [58]. Higher surface sensitivity can be achieved with lasers at higher frequency and higher NA objectives; however, higher frequency lasers often cause fluorescence in biological samples, and may damage them.

Because different vibrational modes are ‘active’ in Raman and IR spectroscopy, the information obtained with the two techniques is complementary. For example, Raman can show more clearly than IR the amount of bridging and NBOs in bioactive glasses as a function of their silica content [59], as well as three- and four-membered silica rings, which IR cannot detect [60]. Raman has been used to analyse apatite formation and the transformations occurring upon immersion in SBF both on bioceramics and on their composites with polymeric materials in the form of scaffolds for tissue engineering.

The full potential of Raman microscopy can be exploited by combining Raman maps with statistical data analysis. A beautiful example of this combination has been recently shown by Seah et al. [61], who used band-target energy minimization to analyse the Raman maps collected on BG pellets immersed in SBF for up to 17 days. They showed the formation and the spatial distribution of calcium carbonate, and proved that amorphous calcium phosphate formed in the early stages of reaction later transformed into octacalcium phosphate and finally HCA after 12 days of immersion (figure 8).
The greatest advantage of Raman over all the techniques presented so far is that Raman allows one to easily analyse samples immersed in water because water vibrations are virtually inactive in Raman (different from IR). Bonino et al. used Raman spectroscopy to analyse the transformations of a sol–gel bioactive glass immersed in tris buffer, thus avoiding issues of contamination that otherwise arise when the samples are removed from a solution and dried [62]. Jell et al. [63] used Raman microscopy to detect the activity of foetal osteoblasts (FOBs) in contact with media containing the dissolution products of BG particles. Using multi-variate statistical analysis, they were able to show that RNA levels in FOBs cultured on BG-conditioned media were lower than that measured in media that did not contain the ions dissolved from BG. This was an indication of a higher differentiation of FOBs in BG-conditioned media, and confirmed previous observations that osteoblast genes were upregulated if the cells were in contact with dissolution products from bioactive glasses [64]. Gough et al. [65] used Raman to study in situ mineralization in the presence of osteoblasts on BG surfaces, and concluded that this technique was more sensitive than alizarin staining in the detection of HCA.

(iii) X-ray photoelectron spectroscopy

As described in §2b(iii), XPS is a surface-sensitive technique that analyses the elemental composition of a sample by measuring the kinetic energy of photoelectrons emitted from the sample. However, because this energy is influenced not only by the emitting atom, but also by the atoms surrounding it, one can use XPS also to analyse the valence state of surface elements, or the presence of surface functional groups, by acquiring spectra with high-energy resolution. Few researchers exploited this capability of XPS in the context of silicate bioceramic characterization. Vallet-Regí et al. [66] attributed one of the components of the O peak measured on bioactive glasses immersed in SBF for 7 days to surface P–OH groups, and stated that such groups were formed because of the lower solution pH achieved in their experimental conditions, which better mimicked the in vivo ones. Verné et al. [67] analysed the high-resolution Si and O peaks of K- and Mg-containing silicate bioceramics, and showed that both a silica gel and surface carbonates formed on these materials upon immersion in SBF. Cerruti et al. [9] studied the evolution of the C, Si, P and O peaks in spectra collected on BG immersed in tris. The evolution of the C peak was particularly interesting: covalently bound surface carbonate species showed up at first, and after a week they were transformed into ionic carbonates, even though they were still different from the carbonate species observed in HCA.

Surface modification of bioceramics was studied using XPS by two groups of researchers. Chen et al. [68] confirmed the successful functionalization of BG scaffolds with amino groups at first, and then glutaraldehyde by looking at changes in the elemental surface composition with XPS. This functionalization was not shown to influence the rate of HCA precipitation on these materials. Verné et al. [42] used treated amino-modified glasses in solutions containing either a model protein (carnitine) or bone morphogenic protein (BMP). They analysed high-resolution XPS spectra both to understand which preliminary treatment led to the highest amount of surface hydroxyl groups, which were necessary for the amination, and to prove that surface amination was necessary to graft carnitine and BMP.

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4. Surface area and porosity

(a) Introduction

Surface area and porosity play an important role in determining the bioactivity of a material and its interactions with the body once implanted: a bioceramic with larger surface area will dissolve faster, and offer a larger number of active sites for HCA nucleation, collagen adsorption and interactions with osteoblasts and fibroblasts. In 1994, Greenspan et al. [69] showed that HCA precipitated faster on smaller BG particles, and Cerruti et al. [70] confirmed it more recently for very small particles (2 μm). Both studies showed that the HCA layer formed on the smaller particles was thinner than on the larger ones. One can understand the effect of surface area by comparing melt-derived and sol–gel glasses [5]; the former have surface areas lower than 2 m² g⁻¹, whereas the latter have surface areas in the range 100–300 m² g⁻¹, and while melt-derived glasses are not bioactive if they contain more than 60 mol% of silica, sol–gel glasses containing up to 90 mol% silica are bioactive. Sol–gel bioactive glasses can induce HCA nucleation even faster than BG.

Decoupling the effects of surface area, pore size and composition on bioactivity is quite hard. For example, Salinas et al. [71] showed that larger amounts of P₂O₅ in SiO₂–CaO–P₂O₅ sol–gel bioactive glasses increased at the same time the porosity, the surface area and the rate of crystallization of HCA, while glasses with no P₂O₅ had a lower surface area, but induced a faster formation of an amorphous Ca–P layer on their surface. These differences must be ascribed to differences in solubility and in the amount and type of nanostructures present on these materials, as a later high-resolution TEM (HR-TEM) study by Vallet-Regí et al. [72] clearly indicated. Similar difficulties were encountered by Pérez-Pariente et al. [30], who showed that an increase in Mg in glass composition induced both an increase in surface area and a decrease in HCA growth rate on these materials. The authors explained this result by observing that the Mg-containing samples did not show any Ca–P domains on their surfaces, and hypothesized that these clusters were nucleation sites for HCA. In 2004, Horcajada et al. [73] showed no HCA precipitation even on the extremely porous silica MCM-41 (pore volume approx. 0.98 cm³ g⁻¹), which has a surface area higher than 1000 m² g⁻¹. The authors originally attributed this result to a lack of defects on the surface of this material; however, it is possible that the non-interconnected channel structure present in MCM-41 may be responsible for a lack of precipitation of HCA, as the group later hypothesized by comparing HCA precipitation on MCM-41 and on MCM-48, another highly porous silica with a three-dimensional instead of two-dimensional pore structure [74]. This hypothesis would be confirmed by a more recent work by Yan et al. [75], who synthesized highly ordered MBGs by sol–gel with the help of lock copolymers. The authors showed that MBGs with the same pore volume and pore size showed different bioactivity depending on their pore structure: a three-dimensional pore structure induced higher bioactivity than a highly ordered hexagonal pore arrangement with one-dimensional channels.

Together with surface area, pore volume and pore interconnectivity, pore size is an important determinant of bioactivity. A few studies attempted to decouple the effect of pore size from the other parameters. Chen et al. [76] found higher bioactivity for sol–gel glasses synthesized in the presence of lactic acid...
as a sacrificial agent; these glasses had a narrow either unimodal or bimodal
distribution of pore sizes, with the average diameter being around 2 nm. Also,
while nano-sized pores are important to obtain high surface area in sol–gel
bioceramics, the presence of large pores is crucial if the bioceramics are to
be used as scaffolds for tissue engineering; pores in the range 100–300 μm are
necessary to seed osteoblasts and allow vascularization of the scaffolds once
implanted [77]. The optimization study performed by Jones et al. [78] confirmed
that by decreasing the volume of nano-sized pores and therefore the surface area
of the scaffolds, the rate of HCA formation decreased.

(b) Characterization tools

One can quantitatively evaluate surface area and pore volume in a material
using N₂ adsorption at 77 K and Hg intrusion. In addition to these two techniques,
TEM, although inherently a bulk technique, can allow visualizing micropores
diameter less than 2 nm) and mesopores (diameter between 2 and 50 nm),
whereas macropores (diameter greater than 50 nm) are easily observed with SEM
and X-ray microtomography (microCT). In this subsection, we will describe the
first two techniques, and then briefly introduce how TEM, SEM and microCT
have been used to evaluate surface area and porosity in silicate bioceramics.

(i) N₂ adsorption and Hg intrusion porosimetry

Surface area, micropore and mesopore volume and size can be evaluated by
measuring the amount of N₂ gas adsorbed on and desorbed from samples at
77 K [79], and by processing the data using different models. The Brunauer–
Emmett–Teller (BET) model provides an estimate of the surface area; the
Barrett–Joyner–Halenda (BJH) model allows the evaluation of mesopore volume
and average size by analysing the desorption branch of isotherms showing a
hysteresis loop; the so-called ‘t-plot’ based on early doses of adsorbed N₂ allows
the evaluation of micropore volume [79]. While N₂ adsorption isotherms provide
meaningful results for samples that have surface areas of at least 5 m² g⁻¹, Kr
must be used as a probe gas for samples with lower surface areas [17]. N₂
adsorption has been used extensively to evaluate surface area and porosity of
sol–gel bioceramics and on melt-derived glasses, which develop a high surface
area upon immersion in SBF. For example, Cerruti et al. [17] showed that BG
particles approximately 90 μm and 2 μm large, with surface areas of 0.2 and
2 m² g⁻¹, respectively, developed surface areas of 170 and 250 m² g⁻¹, respectively,
after only 6 h of reaction in tris (figure 9). While the starting particles were not
porous, after a few hours, they were transformed into highly porous materials
containing both micro and mesopores; after 2 days of reaction, the micropores
were completely occluded by the deposition of the HCA layer, but the mesopore
volume was unaltered for both samples [17].

Macropore volume can be evaluated by Hg intrusion porosimetry. Even
though this technique has been used to evaluate the porosity on mesoporous
materials [71,80], it provides accurate results on samples containing mostly
macropores. Thus, it is especially useful to evaluate the porosity of scaffolds for
tissue engineering applications, containing a high volume of pores with a diameter
of at least 100 μm.

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Figure 9. Specific surface area variation measured by N$_2$ adsorption during immersion in tris of BG samples with starting particle size of 2 $\mu$m (circles) and 90 $\mu$m (squares). Reproduced with permission from Cerruti et al. [17].

(ii) Visualization techniques

N$_2$ adsorption and Hg intrusion porosimetry provide average values for surface area and porosity; HR-TEM, SEM and microCT can be used to understand pore shape and spatial evolution of surface area or porosity. Lin et al. [81] clearly showed that the assumption that mesopores are on average cylindrical, which is made by the BJH model to evaluate mesopore size from adsorption isotherms, is incorrect: their TEM images of sol–gel silica–calcium oxide glasses showed pores with highly irregular shape, produced by interstitial spaces between secondary particles formed during the sol–gel process. A similar combination of N$_2$ adsorption and TEM was adopted by the same group to evaluate the effect of the addition of trimethylethoxysilane on the porosity of sol–gel glasses [82]. Cerruti et al. combined TEM/EDS and N$_2$ adsorption to evaluate the evolution of surface area and morphology of BG particles reacted in tris. They showed that the Ca–P-rich phase obtained upon dissolution and reprecipitation was more porous than the silica-rich phase, and should therefore be held responsible for the observed increase in surface area [17].

Macropore volume, size and interconnectivity in bioceramic scaffolds are visualized with SEM [78] and microCT. Computational analysis allows the extraction of quantitative data such as average pore size and volume from microCT images [83]. Jones et al. [84] recently showed that pore size evaluated by microCT does not always match Hg intrusion porosimetry estimates, and attributed this discrepancy to the mathematical models used to interpret Hg porosimetry data. High-quality microCT images were obtained by
Renghini et al. [85] using synchrotron radiation as the X-ray source. By combining microCT and computational image analysis, they were able to visualize the formation of different phases on bioactive glass–ceramic scaffolds immersed in tris and SBF (figure 10). They could achieve this result without the help of any other characterization technique, simply based on the structural evolution shown by microCT. They also showed a bimodal pore size distribution upon immersion in both tris and SBF, including both larger (100–500 μm) and smaller (1–100 μm) macropores.

5. Surface charge, roughness, topography and wettability

(a) Introduction

Proteins adsorb on a biomaterial within minutes of its implantation in the body; this protein layer determines later responses, such as the formation of a fibrin-based insulating capsule around the material, or the arrival of osteoblasts and the deposition of collagen and HCA to initiate bone formation [86]. The amount and type of proteins adsorbed at the biomaterial–body interface are therefore crucial determinants of the in vivo success of an implant, and are influenced by different surface parameters, including surface charge. Bioceramics and bioglasses have negatively charged surfaces at physiological pH owing to the presence of silicate ions and silanol groups [87]. Even though both cell walls and many serum proteins are negatively charged, protein adsorption and osteoblast adhesion is high on bioceramics. Lu et al. [88] postulated that negatively charged BG surfaces were able to attract the overall negatively charged fibronectin by interacting with the heparin-binding domains of this protein, which are positively charged at physiological pH. Others hypothesized that negatively charged surfaces increase osteoconduction in vivo by attracting positively charged Ca$^{2+}$ ions, which in turn enhances fibrin adsorption and osteoblast migration [89].

Roughness and topography also influence cell adsorption [90]. Indeed, the reaction of cells to different surface landscapes, with features both in the micro and in the nanometre range, has been the object of many studies for decades. While these experiments can be carefully performed on polymeric and insoluble materials including hydroxyapatite, the effect of surface roughness on silicate biomaterials is harder to evaluate because an increase in roughness also
induces changes in other parameters, such as surface composition or rate of
dissolution in body fluids. In fact, Zhang et al. [91] recently showed that soluble
factors released by pseudowollastonite grains were more important than surface
roughness in determining osteoblast response. Italä et al. [92] showed a higher
attachment of osteoblast-like human cells MG-63 on micro-roughened bioactive
glasses compared with smooth ones; however, they also noted that this response
might have been related to the faster formation of a silica gel layer on the rough
glasses. Gough et al. [65] showed more mineralized nodules formed if osteoblasts
were cultured on rough BG surfaces than on smooth ones; even though the
authors at the time could not explain the exact reason for this, they noted a
faster precipitation of HCA on rough BG. In fact, a study from the same group
showed that ionic release rather than surface topography affected cell response
on tape-cast and sintered bioactive glasses: very fast dissolution induced the
highest level of apoptosis, possibly because of too high pH or concentration of Na
or Si [93].

Both surface roughness and charge drastically influence surface wettability [94].
This parameter can therefore be used as an additional indicator of the interactions
between biomaterial surfaces and proteins and cells. Amaral et al. [87] observed
that BG and BG composite ceramics had larger wettability than HCA and
tricalcium phosphates; Nishizawa et al. [95] studied the combined effect of surface
charge and wettability on calcium phosphate bioceramics, and pointed out that
on the most hydrophilic samples, cell adhesiveness could be related to surface
charge rather than wettability.

(b) Characterization tools

(i) Surface charge

Surface charge is evaluated by measuring the so-called ‘zeta potential’. This
potential is generated by the charges bound to the surface of a
particle and the counterions strongly interacting with it. The zeta potential is
evaluated by measuring the electrophoretic mobility of the particle, according to
Smoluchowski’s equation [96],

\[ \nu_e = \frac{E\varepsilon \zeta}{4\pi\eta}, \]

where \( \zeta \) is the zeta potential and \( \nu_e \) is the velocity of the particle moving in
an electric field of intensity \( E \), in a solution of viscosity \( \eta \) and permittivity \( \varepsilon \).
Silicate bioceramics always show negative zeta potentials, indicative of a negative
surface charge [87,97]. El-Ghannam et al. [98] were able to achieve more negative
zeta potentials on BG by treating it at temperatures ranging between 550 and
700°C—they related this decrease to the separation of a phosphate-rich glassy
phase, possibly more negatively charged. Interestingly, they also showed a lower
amount of serum proteins adsorbed on surfaces with more negative zeta potential.
This is in contrast with other findings showing that more proteins adsorbed on
bioceramic surfaces with more negative zeta potential [87]. These contradictory
results simply confirm that protein adsorption on silicate bioceramics cannot
be described with only one parameter. Lu et al. studied the evolution of zeta
potential on BG particles immersed in solutions simulating plasma electrolytes,
with and without the addition of fibronectin. They observed a change from
negative to positive zeta potential when an amorphous calcium phosphate layer was formed on bioglass [99], and they noted that this change was delayed in the presence of fibronectin [88]. The authors attributed this delay to the much more negative surface charge obtained during the initial dissolution steps in the presence of fibronectin.

(ii) Surface roughness and topography

Surface topography can be visualized with nano-scale resolution with SEM. Quantitative data concerning average surface roughness of silicate biomaterials have been obtained with three different techniques: (i) mechanical stylus profilometry [100], which measures the displacement of the tip of a stylus tracing across the surface, and allows resolution of features in the 10 nm to 1 mm height range; (ii) optical interferometry [65,91], which measures surface profiles by analyzing the interference of white light radiation with the sample surface; this technique does not require contact of a tip with the sample, thus avoiding sample contamination and scratching, and allows resolution of features as short as 1 Å; (iii) atomic force microscopy (AFM), which determines surface topography by measuring the forces acting between the material surface and a microcantilever kept at a short distance from it. AFM measures are most often performed in ‘tapping’ mode on silicate bioceramics: the cantilever is oscillated at a frequency close to its resonance frequency, and a feedback loop keeps the oscillating frequency (or amplitude) constant by changing the height of the tip of the cantilever relative to the sample surface. AFM can resolve differences in height lower than 1 Å, and can evaluate the average roughness of a surface by scanning over micron-size areas. One of the main advantages of AFM over SEM is that it allows the measurement of surface roughness and topography of a biomaterial in situ, in aqueous solutions. Leonor et al. [101] followed the evolution of the surface topography of partially crystallized BG discs immersed in SBF, and observed that HCA formed by nucleation of a large number of Ca–P-rich islands that fused together after about 4 h (figure 11). This was accompanied by a decrease in surface roughness observed immediately upon immersion in SBF. Similar in situ AFM experiments were performed by Leonelli et al. [102] on Ce-doped bioglass.

(iii) Surface wettability

Surface hydrophilicity (equivalent to ‘wettability’ in aqueous environments) is evaluated by measuring the contact angle between a microdroplet of water and the surface of a material. Lower contact angles (i.e. more spread-out droplets) indicate a more hydrophilic surface. Contact angle studies on silicate bioceramics are mostly performed in ‘static’ mode, i.e. by measuring the contact angle of the droplet after it has been deposited on the material surface. These measures were performed both to relate surface properties to protein adsorption [87,95], and to quickly evaluate the efficiency of surface treatments. For example, Verné et al. [42] used this technique to assess which method introduced the largest amount of surface hydroxyl groups on bioactive glasses, hence increasing their hydrophilicity, as well as the efficacy of a silanization treatment, which vice versa decreased glass hydrophilicity.
Figure 11. AFM images showing the formation of Ca–P layer on the surface of BG in situ. Reproduced with permission from Leonor et al. [101].

6. Missing techniques

As we have seen, surface features of silicate bioceramics have been characterized with many techniques. Table 1 summarizes the salient features of those we discussed so far. Even though the list is long, the expert surface scientist would notice the absence of some tools that are commonly used to analyse surface features. In this section, we will briefly describe these ‘missing techniques’, i.e. those that have not been used, extensively or at all, to analyse surface features of silicate bioceramics. The goal is to show the insights that these techniques can provide, and to inspire researchers studying silicate bioceramics to add them to their arsenal of surface-sensitive characterization tools.

(a) Time-of-flight secondary ion mass spectrometry

Time-of-flight secondary ion mass spectrometry (TOF-SIMS) determines surface composition by analysing the secondary ions ejected from the surface of a
Table 1. Salient features of the techniques discussed in this review.

<table>
<thead>
<tr>
<th>technique</th>
<th>information</th>
<th>surface sensitivity</th>
<th>prerequisites/limitations</th>
<th>advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDX</td>
<td>composition</td>
<td>0.1–10 μm</td>
<td>UHV</td>
<td>nanometre spatial resolution</td>
</tr>
<tr>
<td>AES</td>
<td>composition</td>
<td>5–20 Å</td>
<td>UHV</td>
<td>depth profiles possible</td>
</tr>
<tr>
<td>XPS</td>
<td>composition and functional groups</td>
<td>10–30 Å</td>
<td>UHV</td>
<td>depth profiles possible</td>
</tr>
<tr>
<td>NMR</td>
<td>composition, coordination</td>
<td>bulk; can be surface sensitive with special techniques</td>
<td>enrichment with NMR-active atoms may be required</td>
<td>fine details about atomic environment, element specific</td>
</tr>
<tr>
<td>FT-IR spectroscopy</td>
<td>functional groups</td>
<td>0.5 μm to nanometre depending on method</td>
<td>water obfuscates the signals in some spectral regions</td>
<td>fast, in situ possible, microscopy possible</td>
</tr>
<tr>
<td>Raman spectroscopy</td>
<td>functional groups</td>
<td>2–10 μm</td>
<td>fluorescence may hinder Raman signals</td>
<td>water does not interfere, microscopy</td>
</tr>
<tr>
<td>N₂ adsorption and Hg intrusion</td>
<td>porosity and surface area</td>
<td>bulk + surface</td>
<td>mathematical models needed to analyse data</td>
<td>micro, meso and macropores can be analysed</td>
</tr>
<tr>
<td>microCT</td>
<td>porosity</td>
<td>bulk</td>
<td>1–10 μm spatial resolution</td>
<td>visualize pore structure</td>
</tr>
<tr>
<td>HR-TEM</td>
<td>crystallinity, composition, porosity</td>
<td>bulk + surface</td>
<td>UHV</td>
<td>atomic resolution, local order and phase identification</td>
</tr>
<tr>
<td>SEM</td>
<td>roughness, morphology</td>
<td>surface visualization</td>
<td>normally UHV</td>
<td>approx. 1 nm spatial resolution</td>
</tr>
<tr>
<td>AFM</td>
<td>roughness, morphology</td>
<td>surface visualization</td>
<td>relatively slow, artefacts possible</td>
<td>&lt;1 Å height resolution, in situ possible</td>
</tr>
<tr>
<td>mechanical profilometry</td>
<td>roughness</td>
<td>n.a.</td>
<td>scratching, 10 nm height resolution</td>
<td>up to 1 nm features can be analysed</td>
</tr>
<tr>
<td>optical profilometry</td>
<td>roughness</td>
<td>n.a.</td>
<td>lower resolution than AFM</td>
<td>inexpensive, easy to perform</td>
</tr>
<tr>
<td>zeta potential</td>
<td>surface charge</td>
<td>few angstrom from surface</td>
<td>mathematical model to analyse data</td>
<td>unique information provided</td>
</tr>
<tr>
<td>contact angle</td>
<td>wettability</td>
<td>n.a.</td>
<td>average measure over a large area</td>
<td>fast, preliminary information</td>
</tr>
</tbody>
</table>
sample bombarded with a source of primary ions (typically Ga or Cs). This is one of the most sensitive techniques available, allowing the detection of traces (ppm) of elements sputtered from atom-thick surface layers; additionally, composition depth profiles can be obtained, as well as maps with micron or even nano-scale feature resolution (nanoSIMS). Although TOF-SIMS has been used since the 1990s to characterize surface features of silicate-based glasses and hydroxyapatite, to the best of our knowledge, this technique has been used only once in the silicate bioceramic literature—as a support tool to analyse compositional inhomogeneity in sol–gel SiO2–CaO bioactive glasses [103]. However, TOF-SIMS has proved to generate incredibly detailed information when applied to biomaterials. For example, by combining TOF-SIMS with statistical data analysis, Tyler et al. [104] studied proteins adsorbed on biomaterials. The authors were able to visualize proteins adsorbed on polymeric substrates by taking advantage of the high surface sensitivity, chemical specificity and spatial resolution of this technique. More recently, Baio et al. [105] used this technique to study fine details about protein orientation upon surface immobilization. By exploiting the nano-scale spatial resolution of nanoSIMS, Azari et al. [106] followed intracellular hydroxyapatite precipitation, and showed that S-rich proteins were associated with the mineral deposits. Given these recent results, we believe that the application of TOF-SIMS to silicate bioceramics could elucidate important details about their reactivity both in vitro and in vivo.

(b) Particle-induced X-ray emission

Particle-induced X-ray emission (PIXE) was developed in the 1970s by Johansson [107]; it allows for trace (ppm) element detection by analysing the core X-rays emitted upon hitting a sample with a beam of charged particles (usually protons) with energy in the MeV range. The surface sensitivity of this technique varies depending on the energy of the particle beam used, the sample and the angle at which the X-rays are collected. Even though not inherently surface sensitive, the extremely high sensitivity of this technique makes it very interesting for the analysis of trace elements. Also, because the particle beam can be focused on single spots, the sample composition can be mapped with micrometre-level resolution. These features make PIXE promising for the analysis of biological samples [107]. We have found one very good example of the application of PIXE to silicate bioceramics, which shows the potential of this technique: Jallot et al. [108] recently generated PIXE maps of SiO2–CaO scaffolds before and after immersion in Dulbecco-modified Eagle’s medium, and were able to show that the Ca-P-rich layer formed at the pore walls contained trace amounts of Mg (figure 12). Such an observation has not been reported before, and is quite important because Mg influences bone formation in vivo.

(c) Synchrotron-based techniques

Even though many synchrotron-based techniques analyse bulk features, it is worth mentioning them in this section because the information they provide can often be related to surface phenomena. Synchrotron-based X-ray diffraction (XRD), for example, can show details about both crystalline phases and local atomic arrangement that conventional XRD cannot pick up, because of the higher
Figure 12. Elemental maps of a SiO₂–CaO glass scaffold after 2 days of interaction with biological fluids (125 × 125 μm²). Reproduced with permission from Jallot et al. [108]. (Online version in colour.)

flux and lower wavelength of exciting X-rays used. Jones & Skipper applied this technique to analyse the transformation of silicate bioceramics in SBF [84,109]; they showed Ca²⁺ ions being continuously released and re-deposited on the material surface, and the formation of an octacalcium phosphate layer, followed by an amorphous layer that only after several hours crystallized into HCA (figure 13). These results thus showed that surface reactions on these materials are more complex than originally hypothesized. Two other synchrotron-based techniques, extended X-ray absorption fine structure spectroscopy (EXAFS) and X-ray absorption near-edge structure (XANES), can provide information on local atomic arrangements, which can often be associated with surface phenomena. With these techniques, Skipper et al. [110] were able to show changes in Ca²⁺ coordination and the formation of an amorphous Ca–P-rich layer on sol–gel bioactive glasses that was not observable with conventional XRD. Small angle X-ray scattering (SAXS) is a highly surface-sensitive synchrotron-based technique that measures diffraction patterns obtained by hitting the sample with X-ray
Figure 13. Real space pair correlation function \( D(r) \) data showing changes in interatomic distances for \((\text{CaO})_{0.3}(\text{SiO}_2)_{0.7}\) samples treated in SBF: from top to bottom, 7 days, 72h, 25h, 10h, 5h, 1h, 30 min, unreacted. In all cases, the correlation functions have been normalized to the Si–O first-neighbour peak as it appears in the unreacted sample. The changes in peaks labelled a–g refer to changes in Ca–O, Ca–O–Ca, P–O and P–O–P distances. Reproduced with permission from Skipper et al. [109].

beams at shallow (less than 2\(^\circ\)) angles of incidence. Martin et al. used SAXS to investigate the transformation of BG surface upon immersion in SBF for 1h up to 3 days. They showed that an amorphous phosphate layer transformed into crystalline HCA at the BG–SBF interface, and that the crystalline HCA layer partially dissolved between 8 and 24h of reaction [111]. SAXS was used in combination with wide angle X-ray scattering by FitzGerald et al. [112] to monitor \textit{in situ} the evolution of both porosity and mineral growth on foamed sol–gel bioactive glasses immersed in SBF. This study evidenced the formation of both tricalcium phosphate and HCA and a parallel decrease in the average mesopore size, thus confirming the importance of mesopores to initiate calcium phosphate mineralization on sol–gel glasses. The results obtained by these authors in the last few years have shown the great potential of synchrotron-based techniques to study silicate bioceramics, especially amorphous ones, where local atomic arrangement is more important than long-range order.

\((d)\) \textit{Isothermal microcalorimetry}

Isothermal microcalorimetry (IMC) measures heat flows in the microwatt range produced or consumed at a constant temperature. The calorimetric events can be related to adsorption of gases or liquid on solid samples, dissolution, hydration, phase changes, or any other physical or chemical processes that release or consume heat. This quantitative and dynamic information can be obtained on any kind of samples, and does not require labelling. Several groups
have applied IMC to study biomaterials; for example, researchers have tested the stability of ultra-high molecular weight polyethylene by checking the heat evolved in simulated shelf storage and body implantation conditions [113]. Other IMC studies have monitored setting of dental adhesives, including calcium silicate [114]. Additionally, IMC was used to monitor granulocyte and macrophage response to different biomaterials [113]. Still, very few studies have explored the application of this technique to silicate bioceramics. To the best of our knowledge, the only papers published on the subject are those from Cerruti et al. and two very recent papers from Doostmohammadi et al. [115,116]. Cerruti et al. [39,43] monitored the heat of interaction between sol–gel bioactive glasses and vapours of water and acetonitrile, a Lewis acid of medium strength. They combined IMC and FT-IR spectroscopy to understand the availability of surface functionalities on these materials, and found that the greatest amounts of heat evolved were related to the interaction between probe molecules and surface cus cations or Si–O–P bridges (figure 14). Doostmohammadi et al. [115] compared the heat of hydration of BG and HCA, and found a much higher heat for BG. However, they noticed that the heat they measured on BG was much lower than that measured on calcium silicate, which could explain why no inflammation and necrosis are observed when BG is implanted. In a short paper from a conference proceeding, Doostmohammadi et al. [116] reported heat of interaction between BG and yeast and chondrocytes; they observed a delay in cell growth, as monitored by released heat, only in the presence of large (greater than 6.6 mg ml\(^{-1}\)) BG concentrations. These results, as well as the work performed on other biomaterials, clearly show that IMC can quantify biologically relevant processes occurring at interfaces. These numbers could supplement merely qualitative arguments often used in the field of silicate bioceramics.

(e) **Sum frequency generation vibrational spectroscopy**

Sum frequency generation vibrational spectroscopy (SFS) measures vibrational spectra of molecules at interfaces. It is based on the nonlinear optic effect of sum frequency generation (SFG), i.e. the fact that the light emitted by a sample hit by two laser beams, one in the visible and one in the IR, has a frequency equal to the sum of the frequencies of the two lasers; the intensity of the emitted light changes if the IR laser is in resonance with a vibrational mode of the molecules adsorbed at the sample surface. Thus, by changing the frequency of the IR laser, a vibrational spectrum of the adsorbed molecules is obtained [117]. SFS spectra were first recorded in the late 1980s, making it the most recent technique described so far. Because of its selection rules, SFS can probe only the vibrations of ordered molecules in contact with a surface. Thus, the resulting signal is surface specific, and it is not obfuscated by water. Also, by analysing SFS spectra collected with different incident beam polarizations, one can understand the orientation of the molecules adsorbed on the surface. Because of all these features, SFS is an ideal tool to analyse molecules at biointerfaces in contact with aqueous solutions. The orientation of model peptides and fibrinogen on both hydrophilic and hydrophobic surfaces was investigated with SFS (see Weidner et al. [118] and references therein). Presently, there are no SFS studies of molecules adsorbed on silicate bioceramics; one of the reasons for this lack may be that SFS requires flat substrates. However, the degree of flatness required is lower
Figure 14. Differential adsorption heat as a function of water amounts adsorbed on bioglass samples activated at 573 K: 58S (mol% composition: 60 SiO₂, 36 CaO, 4 P₂O₅): primary (filled squares) and secondary (open squares) adsorption; 77S (mol% composition: 80 SiO₂, 16 CaO, 4 P₂O₅): primary (filled circles) and secondary (open circles) adsorption. The horizontal dotted line trace corresponds to the latent molar heat of condensation of water (44 kJ mol⁻¹). The differences between primary and secondary adsorptions indicate irreversible adsorption of water molecules on cus Ca²⁺ cations. Reproduced with permission from Cerruti et al. [39].

than for IRRS, which was used in early studies on BG-polished surfaces [50]. We anticipate that SFS applied to silicate bioceramics will provide experimental evidence that will validate or disprove existing computational models of amino acids and oligopeptides adsorbed at their surfaces.

7. Conclusions

A rich and multi-faceted picture of the surface reactivity of silicate biomaterials has emerged and evolved in the past four decades, owing to the adoption of many complementary surface characterization tools. Still, we are far from being able to monitor all the complex phenomena occurring at the interface between biomaterials and body tissues. The nature of the surface sites responsible for HCA precipitation and for interacting with biomolecules is still controversial; the effects of surface charge on cell behaviour are convoluted with those of roughness and topography, and may be understood only with a combined in situ analysis with different techniques. Advances in surface characterization tools, especially those that allow for in situ analysis in aqueous environments and in vivo, will certainly refine our understanding of silicate bioceramic bioactivity, thus enabling us to improve these materials and the quality of life of the people they serve.

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