Incorporation of amino acids within the surface reactive layers of bioactive glass *in vitro*: an XPS study

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Surface reaction layers grown on bioactive glass (Bioglass®), by immersion in either simulated body fluid (SBF) or minimal essential medium (α-MEM) for 2, 5, 32 and 72 h, were analyzed by scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS). Layers grown in α-MEM exhibited delamination when observed in SEM. Low resolution XPS analysis detected nitrogen at the surface of the Bioglass® exposed to α-MEM for 72 h (8 relative at%), whereas insignificant nitrogen was found at the surface of any sample immersed in SBF. XPS depth profiling (argon) showed the presence of nitrogen throughout the depth of the surface layer of the sample incubated in α-MEM for 72 h. Deconvolution of the nitrogen envelope in a high resolution XPS spectrum demonstrated nitrogen characteristic of the amine bonds present in amino acids. Carbon concentration also considerably increased over time with exposure to α-MEM (24–55%), whereas it remained in the 20–25% range in SBF. These results demonstrate that the amino acids contained in the culture medium were incorporated within the growing calcium phosphate rich surface reaction layer of Bioglass®
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1. Introduction

Various techniques have been used to model the calcium-phosphate rich surface layers implicated in the bone-bonding behavior of bioactive glasses (BG), such as Bioglass®. Among these is the incubation of BG in an ionic solution similar to that found in human blood plasma, referred to as simulated body fluid (SBF). In this case an initial amorphous calcium phosphate layer, incorporating carbonate ions, forms a polycrystalline hydroxycarbonate apatite phase (HCA), which is considered (by Fourier transform infrared (FTIR) and FT-Raman analyses) to be similar to bone mineral [1–4].

The problem with such *in vitro* techniques is that the Ca–P layer formation occurs in the absence of organic moieties typical of the *in vivo* environment. Indeed, Kukobo has shown that similar Ca–P layers can be formed on the surfaces of polymers and other glass ceramics exposed to SBF [5]. Thus, the effects of the organic constituents of true body fluid (TBF), which may adsorb prior to, or concomitant with, Ca–P formation, have generally not been taken into account. The purpose of the experiment described herein was to compare the surface reaction layer formed on 45S5 Bioglass® with, and without the presence of organic moieties, by scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS).

2. Materials and methods

45S5 BG discs (a generous gift from USBiomaterials Corporation) of 1 cm diameter and 3 mm thickness were polished on one side using 600 grit paper. These discs were then immersed in 25 ml of either SBF-K9 or α-MEM in order to maintain the surface area: volume ratio at $0.1\ cm^{-1}$. The BG discs were incubated at 37 °C for 2, 5, 32 and 72 h in each of the two solutions. A sample was also immersed in SBF for 4 weeks, to determine whether any nitrogen would incorporate within the surface layer after extended periods. The discs immersed in α-MEM were incubated in a 5% CO_2 atmosphere to prevent major changes in pH.

After each incubation, the samples were washed in ion-exchanged ddH_2O and critical point dried for preparation for scanning electron microscopy (Hitachi 2500).

Another set of samples incubated in both solutions, as well as ''unreacted'' BG discs were analyzed by X-ray photoelectron spectroscopy (Leybold LH Max 200, MgK_α X-ray source at 15 kV and 20 mA emission current). All samples underwent survey scans to detect elements at the surface and low resolution analysis to obtain relative percent compositions of O1s, N1s, Ca2p, C1s, P2p and Si2p. The samples immersed in α-MEM for 3 days were further analyzed by high resolution XPS

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modes, with spectrum calibration achieved by setting the C1s peak for adventitious carbon at 284.6 eV. The individual peaks were deconvoluted using a Gaussian–Lorenzen curve-fitting process.

XPS depth profiling was performed on the 3 day incubated samples by argon sputtering, by using a $4 \times 4\,mm^2$ raster, 10 mA beam current, 3 kV potential and sputter time/cycle ramped up from 1 to 10 min. The sputtering time was later increased to 30 min/cycle to observe changes more rapidly, during which the sputter current was reduced to 3 mA.

3. Results

3.1. SEM

Delamination was observed in both SBF and α-MEM incubated samples. However, scanning electron micrographs revealed a greater degree of HCA layer delamination on the discs incubated in α-MEM compared to those in SBF. This qualitative observation was based on examination of the morphology and size of the detachment areas created in SBF and α-MEM (Fig. 1) respectively. Higher magnification of the delaminated regions showed that the underlying surface had a similar morphology to that of the unreacted Bioglass®.

3.2. XPS

The survey spectrum of Bioglass® exposed to α-MEM is shown in Fig. 2 and that exposed to SBF in Fig. 3. The elemental compositions of O1s, N1s, Ca2p, C1s, P2p and Si2p are given for α-MEM (Table I) and SBF (Table II). From the samples incubated in SBF, it could be seen that the Ca:P ratios were highest after 2 h immersion (1.19),

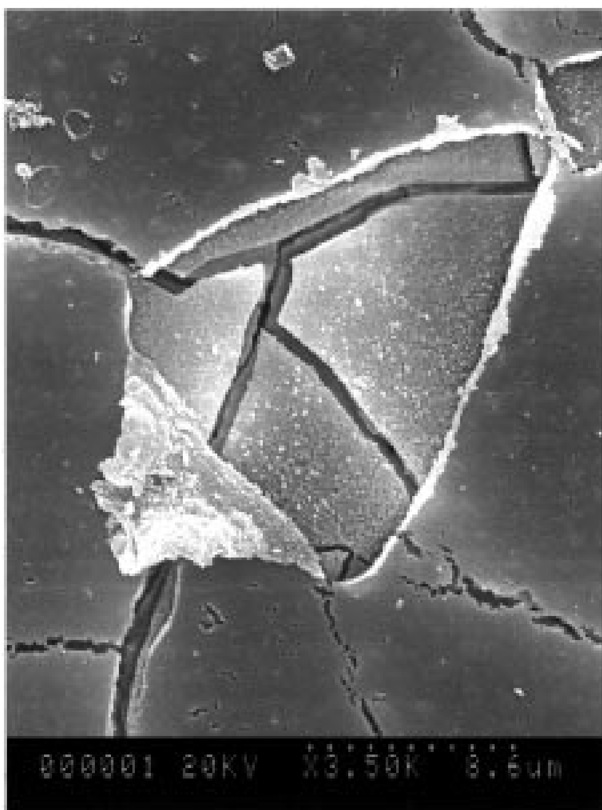

Figure 1 SEM of delaminated Bioglass® surface layer grown in α-MEM. The underlying surface can be clearly seen.

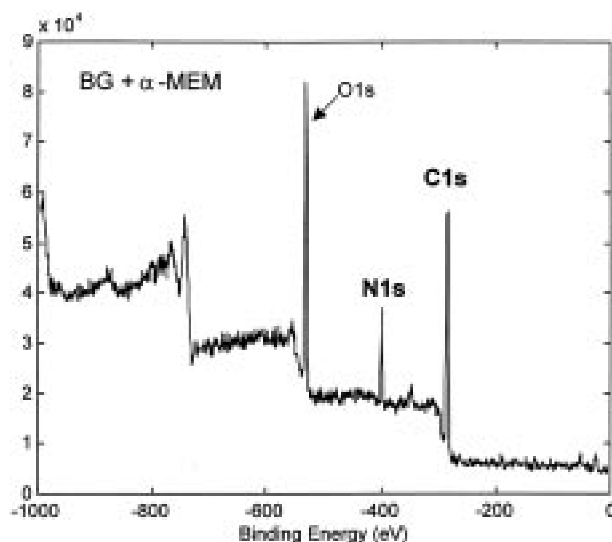

Figure 2 XPS survey spectrum of 72 h immersion in α-MEM. The nitrogen (N1s) peak has been indicated at around 400 eV and carbon (C1s) at around 285 eV.

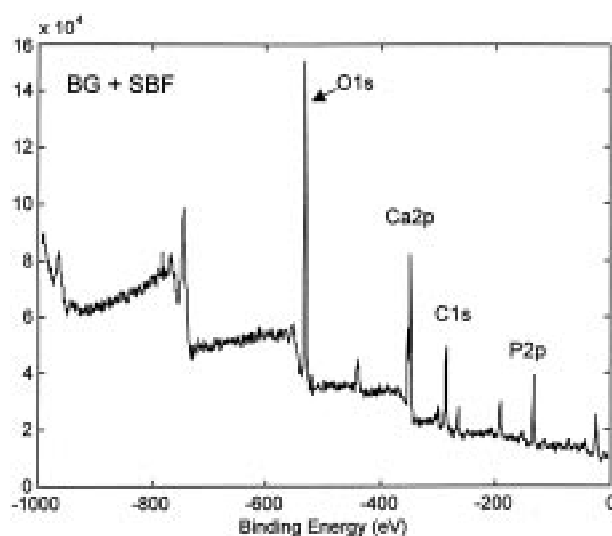

Figure 3 XPS survey spectrum of 72 h immersion in SBF. No peaks corresponding to the amine bonds of organic molecules (nitrogen) were seen.

and lowest at 72 h (0.96). These results also showed considerable increase in the relative atomic percent composition of nitrogen in the surface between 32 and 72 h incubation periods in α-MEM (from < 1 to 8%). On the contrary, immersion in SBF for these, and longer periods of up to 4 weeks resulted in negligible amounts of detectable nitrogen. Low levels of silicon (1–2%) were also evident.

Concurrent with the rise in nitrogen levels was the significant increase in carbon (relative atomic percent) at the surfaces of Bioglass® discs exposed to α-MEM, which increased from 31% (at 2 h immersion) to 55% (72 h immersion). In α-MEM immersed samples, the Ca:P ratio was 1.67 at 2 h, although once again, very little silicon was detected. Throughout exposure to α-MEM, the Ca:P ratio remained in the 1.49–1.72 range, which was higher than that of the SBF samples.

High resolution N1s (399.88 eV) peak fitting (Fig. 4) revealed only one type of nitrogen-binding state, corresponding to the amine bond, whereas the broad

TABLE I Surface compositions for samples immersed in α-MEM

	Immersion period in α-MEM 2 h	5 h	32 h	72 h
O1s	43.91	36.33	43.99	24.87
N1s	0.54	1.11	0.46	8.22
Ca2p	10.88	10.10	10.01	1.06
C1s	31.17	43.48	34.56	55.45
P2p	6.53	5.89	6.57	0.71
Si2p	1.41	0.98	1.71	0.36

TABLE II Surface compositions for samples immersed in SBF

	Immersion period in SBF 2 h	5 h	32 h	72 h
O1s	49.78	51.24	50.96	48.54
N1s	0.05	0.3	0.25	0.24
Ca2p	11.51	11.74	11.48	11.41
C1s	24.11	20.87	23.19	25.38
P2p	9.65	11.06	10.83	11.90
Si2p	1.74	1.04	0.77	1.00

peak of C1s was deconvoluted to three peaks at the following binding energies: 283.2, 284.6 and 286.4 eV (Fig. 5).

Depth profiling of the sample showed the presence of large amounts of nitrogen in the surface layer of the sample incubated in α-MEM for 3 days, while calcium and phosphorous signals were negligible (Figs 6 and 7).

It was observed that the nitrogen signal persisted at a steady level throughout the depth profiling process, while carbon concentration increased from approximately 60% to 80% in the same profile.

4. Discussion

Bioglass® has long been known to exhibit the formation of calcium phosphate rich surface reaction layers both *in vivo* [6–8], and when exposed to buffered solutions simulating human blood plasma ionic concentrations *in vitro* [2–5, 9, 10]. Several authors have used such *in vitro* conditions to model the bioactivity of this, and other, bioactive materials [1, 5, 11] and surface analysis techniques such as FTIR have been commonly used to

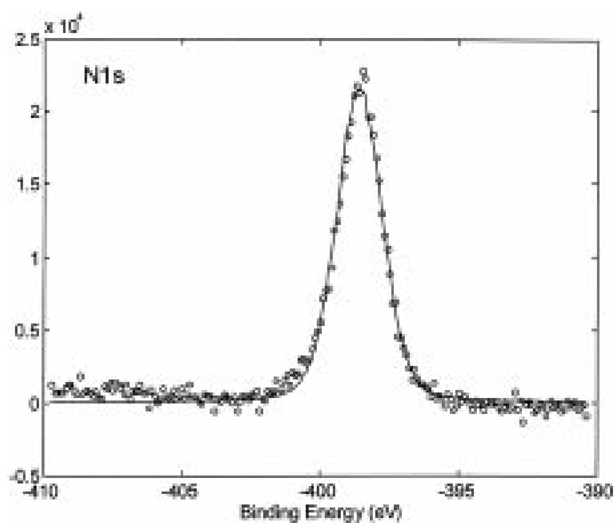

Figure 4 XPS high resolution peak fit of N1s. The deconvoluted peaks have been superimposed on the raw data.

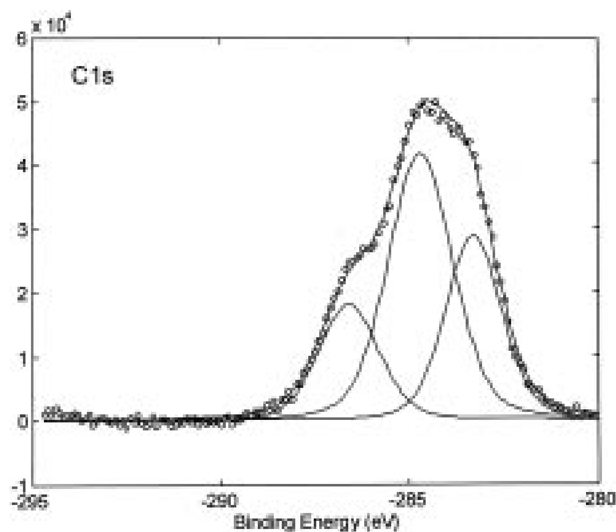

Figure 5 XPS high resolution of C1s. The deconvoluted peaks have been superimposed on the raw data. The broad secondary peak of carbon is characteristic of multiple bonds associated with C1s.

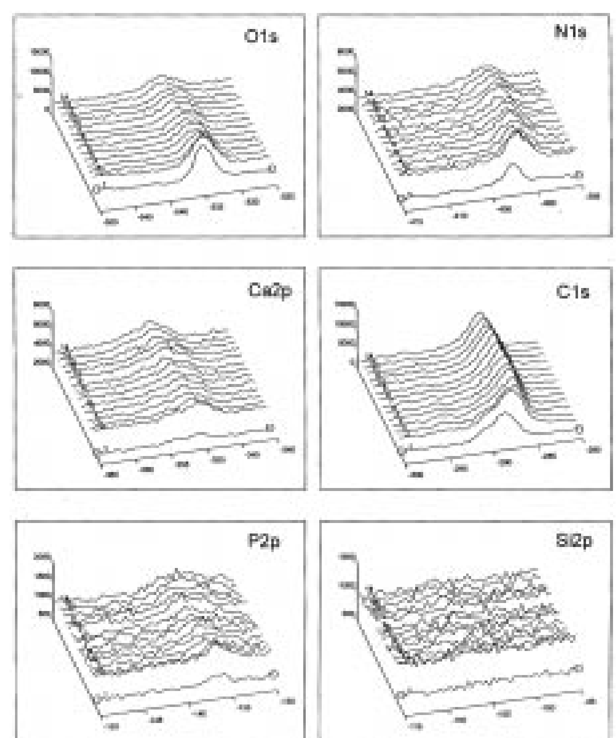

Figure 6 3-D depth profile of Bioglass® immersed in α-MEM. The spectrum for each element is shown separately.

characterize the surface [1–4]. Apart from FTIR studies showing retardation of surface layer reaction kinetics [12], little has been reported pertaining to the effects of biological entities on the evolving layer, especially those effects which relate to the composition of the layer in the presence of organic biological molecules. In our experiments, the analyses were performed comparing the standard SBF-K9 and tissue culture medium, α-MEM. The latter was chosen because it is a common, buffered, cell culture fluid which contains amino acids, thereby initially reducing the complexity associated with analyzing adsorption of serum components.

Observation of scanning electron micrographs showed differences in the stability of the HCA after exposure to SBF and α-MEM. In the case of Bioglass® exposed to

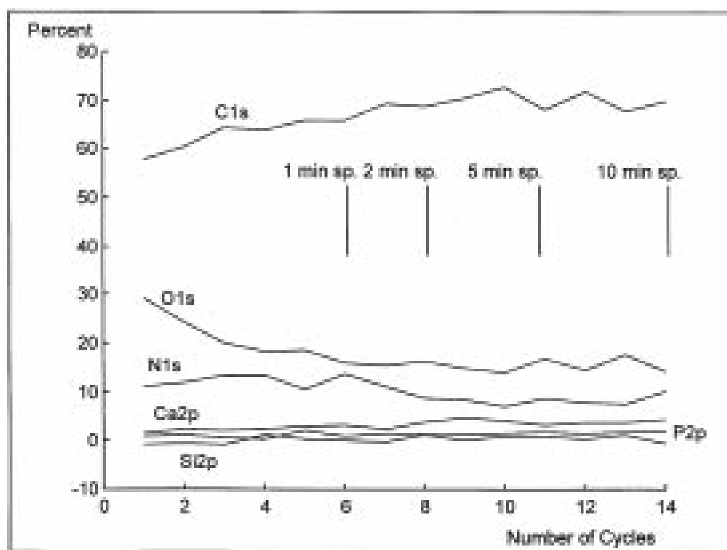

Figure 7 2-D depth profile of Bioglass® immersed in α-MEM. The bars indicate sputtering time/cycle.

culture medium, the surface layer was unable to resist the stresses of critical point drying as well as the samples exposed to SBF, which maintained a more stable HCA layer throughout the process.

The very low levels of silicon, after immersion in both solutions, indicated that the calcium phosphate-rich layer was covering the silica gel. In the SBF exposed samples however, the decrease in calcium compared to phosphate ions indicated compositionally dynamic levels of calcium and phosphate ions, a result that complemented the aforementioned FTIR studies, which have demonstrated changing peaks characteristic of the components of the HCA layer. In addition, there appeared to be little change in the surface compositions of the other ions measured at the surface of Bioglass® immersed in SBF which suggested that, except for calcium and phosphate ion variation, the surface layer was relatively stable by 2 h of exposure to SBF.

In α-MEM incubated samples, the Ca:P ratio was 1.67 after 2 h, although once again very little silicon was detected, which could indicate the likely formation of an early Ca–P rich layer.

The hypothesis that some of the Ca^{2+} ions were perhaps being replaced by organic components was further substantiated by the increase in nitrogen detected at the surface by low resolution XPS, almost certainly due to the organic components of α-MEM incorporated within the surface, since the nitrogen present in the α-MEM system was only that contained in the amino acids, DNA and RNA present in the medium. The binding energy of the nitrogen peak was found via high-resolution XPS to be 399.88 eV. This is the characteristic chemical binding energy of the amine bond of cysteine, an amino acid contained in α-MEM, when the effects of adjacent bonds are taken into account. The nitrogen detected in the SBF incubated samples, which was present at levels which precluded high resolution deconvolution spectra, was most likely due to Tris, $NH_2C(CH_2OH)_3$, in the SBF.

More specific bonding information was obtained by deconvolution of the high-resolution C1s spectrum. The broad secondary peak of carbon is characteristic of multiple bonds associated with C1s, a phenomenon which is common in the presence of nitrogen. This complements the low-resolution XPS information, which showed the relative atomic percent of C1s at 55% for the sample incubated in α-MEM for 3 days. This increase in carbon atoms, as detected by low-resolution XPS, was also likely due to the increase in amino acids at the surface. It was also seen from the deconvoluted high-resolution C1s spectrum however, that carbonate was not abundant enough to be detected. The reference standard binding energy for carbonate has been determined to be 287.8 eV [13], which did not correspond to the deconvoluted C1s peaks.

From the steady nitrogen levels obtained from the depth profile, it could be seen that amino acids had attached to the calcium phosphate-rich layer even at the early stages of Bioglass® surface reaction layer formation. The very low amounts of calcium and phosphorous detected in the depth profile suggested that the amino acids may have adsorbed onto and thereby coated the calcium phosphate. This phenomenon was observed through the entire depth of the profiled layer, suggesting that the organic moieties in α-MEM had a tendency to adsorb onto the Ca–P as the layer grew.

From the lack of any substantial signal for silicon, even after long sputtering times, it could be seen that the sputtering process had not fully penetrated the overlying calcium phosphate-rich layer into the silica gel.

5. Conclusion

The different modes of XPS clearly indicate the adsorption and retention of biological moieties at the surface of, and within the surface reaction layer of Bioglass®. We have also demonstrated that immersion in SBF is not an appropriate model of *in vivo* growth of such surface reactive layers on bioactive materials, since the effects of organic constituents of body fluid, which alter the composition, structure and stability of the surface layers, are not taken into account.

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