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Changes in the elemental composition of Bioglass during its surface development in the presence or absence of proteins

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The present study examined the elemental distribution with depth from the surface of Bioglass 45S5 immersed in simulated body fluid (SBF) in the presence or absence of proteins derived from foetal calf serum for 960 h using energy dispersive spectroscopy (EDS).The changes in elemental composition of the surface of Bioglass were also analysed with EDS for different immersion times (10 min to 960 h) before and after immersion in SBF with or without proteins. The presence of proteins consistently altered the elemental composition of Bioglass with depth from the surface, although the development of a silica rich layer was similar in both the presence or absence of proteins. In the presence of proteins the accumulation of calcium phosphate at the surface of Bioglass was slower, formed a narrower layer and had lower concentrations of calcium and phosphate than the calcium phosphate rich layer that developed in SBF alone.

1. Introduction

Since the discovery of Bioglass in the 1970s [1] interest has grown in bioactive, or surface-active biomaterials and a range of materials such as the Bioglasses [2], bioactive glass-ceramics [3,4] and dense hydroxyapatite [5] have all been found to be bioactive. When implanted into the body as bone substitutes, the surfaces of these materials undergo a number of reactions and thereby establish a strong bond between bone and the implanted biomaterial surface [6]. It is suggested that this bond not only mechanically stabilizes the implant, but also protects the implant from further deterioration with time [7].

It is well established that following implantation, the surface of Bioglass is modified and a calcium phosphate rich layer formed in combination with a silica rich layer on the surface [8]. This calcium phosphate rich layer is suggested to be responsible for the bone bonding behaviour [9].

The surface modification of Bioglass that occur *in vitro* have been studied most commonly using exposure to Tris-buffered solutions [10], or simulated body fluid (SBF) containing ion balances described as comparable with those found *in vivo* [11]. Whilst these studies have been informative and allowed a mechanism of surface development of Bioglass to be proposed, it is our concern that they do not take into account the presence of proteins present in blood following implantation and therefore do not represent the environment likely to be encountered by Bioglass *in vivo*. It is accepted that when blood comes into contact with an artificial surface the very first event is a physicochemical interaction with H_2O molecules followed by plasma protein adsorption within seconds [12]. Indeed during the mineralization of matrix during bone formation, a range of proteins influence initial sites of mineral deposition, the orientation of crystals and their size [13]. These factors clearly indicate the importance of proteins in determining biological events or processes, particularly following implantation into bone. Indeed we have shown that the presence of proteins in SBF changes the surface development of Bioglass *in vitro*, both in terms of reaction rate and crystallinity [14]. The present study investigates how the presence of proteins alters the elemental composition of Bioglass with respect to depth from the surface which should provide further information regarding the likely surface modifications of Bioglass that occurs *in vivo*.

2. Materials and methods

A Bioglass 45S5 rod was prepared according to the following procedure: Reagent grade powders of 32.9% SiO_2, 4.4% P_2O_5, 30.7% Na_2CO_3 and 32.0% $CaCO_3$ in wt % were weighed in a dry glove box and mixed for 5 h in a sealed polyethylene bottle. The mixture was melted in a covered platinum crucible at 1300° C for 20 h before casting into a pre-heated 15 mm diameter, 200 mm long graphite mould. The Bioglass rod obtained was then heat treated at 450° C for 4 h, cooled and cut into discs 1.5 mm thick using a diamond saw (Isomet, Buehler, USA).

The Bioglass discs were stuck to polishing stubs using dental sticky wax (Kemdent, UK) and first ground with a graded series of silicon carbide papers,

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then polished with 6 μm diamond paste finishing with 1 μm diamond paste (Struers, Denmark). DP-blue, an ethanol based lubricant (Struers, Denmark) was used when polishing to minimize aqueous contact and therefore minimize or prevent surface modification before immersion experiments were initiated. The polished Bioglass discs were cleaned in an ultrasonic bath containing xylene for 3 min before washing with two changes of xylene to remove any sticky wax, then degreased in acetone, cleaned in an ultrasonic bath with methanol (99.9%) for 10 min and washed twice in ethanol (99.5%) before air drying in a laminar air flow cabinet (HF-4, ICN Biomedicals Ltd, UK). Freshly polished and cleaned Bioglass discs were sterilized using dry heat at 160° C for 1 h immediately prior to experiments.

The SBF solution was prepared by dissolving 1.5 mM $MgCl_2 \cdot 6H_2O$, 2.5 mM $CaCl_2$, 0.5 mM Na_2SO_4, 1 mM $K_2HPO_4 \cdot 3H_2O$, 4.2 mM $NaHCO_3$, 136.8 mM NaCl and 3 mM KCl in double-distilled water as described previously [15]. SBF was buffered at pH 7.25 with trishydroxymethyl-aminomethane ($(CH_2OH)_3CNH_2$) and HCl before sterilization in an autoclave at 115° C for 30 min prior to use.

The Bioglass discs were immersed in 40 ml sterile SBF solution, either with or without the addition of 10% foetal calf serum (FCS) (Imperial Laboratory Ltd, UK) as the source of proteins and incubated for periods of time ranging from 10 min to 960 h in an incubator (IG150, Jouan, France) at a constant temperature of 37 °C in an atmosphere of 5% CO_2. Following incubation the Bioglass discs were rinsed in 99.5% ethanol twice and air dried before embedding in a polyester resin (Metset SW, Buehler, UK) in an orientation that enabled sectioning of the discs perpendicular to their surface. Following sectioning the samples were polished and examined using a JEOL JSM 5300LV (Jeol, UK) scanning electron microscope (SEM) with an accelerating voltage of 20 kV. The elemental composition was analysed at 1 μm spaced points from the surface on both the Bioglass discs immersed in SBF and those immersed in SBF with 10% FCS using Energy Dispersive Spectroscopy (EDS) (Princeton Gamm-Tech, System 4plus, USA). The EDS analysis was carried out in terms of the X-ray peak strengths generated by the electron beam for each of the elements, Ca, P, Si and Na, which were then normalized against a control unreacted sample of Bioglass of known composition for each of the four elements. The relative mole percentage (mol%) of each element could then be calculated for the unknown reacted samples. The elemental composition of the surface of the Bioglass discs before and after immersion for different time periods in SBF solution with or without proteins were also analysed by examining their surfaces using EDS.

3. Results

3.1. Bioglass surface modification in SBF

The backscattered SEM images demonstrated four clearly demarcated zones in terms of contrast from the cross sectional surface of the Bioglass which had been

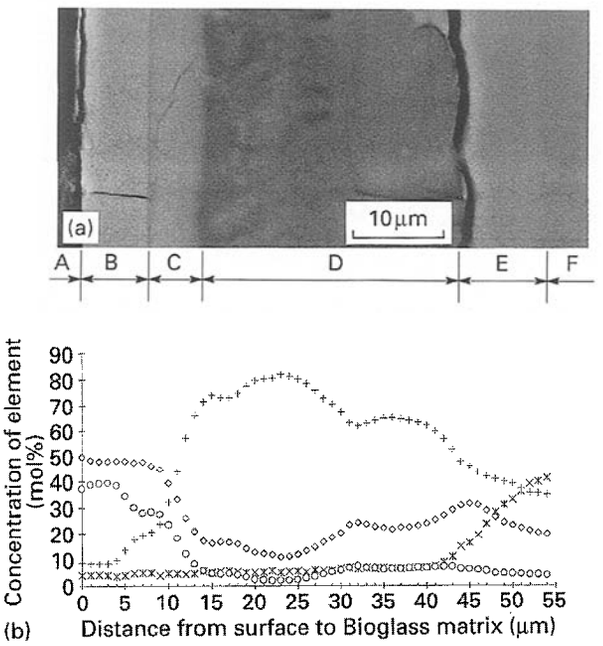

Figure 1 a) Backscattered SEM micrograph showing the different reaction layers (labelled A–F) which were identified following immersion of Bioglass 45S5 in SBF for 960 h in combination with EDS analysis shown in b). A - embedding resin, B - calcium phosphate rich layer, C - intermediate layer of variable composition, D - silica rich layer, E - intermediate layer, F - Bioglass matrix. Note the crack which formed between the silica rich layer (D) and the intermediate layer beneath (E) which may have occurred during dehydration of the samples in ethanol and caused shrinkage. Bar width = 10 μm. b) Graph representing the elemental distribution with depth from the surface of a cross section of Bioglass 45S5 immersed in SBF for 960 h as determined using EDS. The scale of the x axis of the graph is the same as the micrograph in a) and the same zones A-F apply. *, Na; +, Si; ○ , P; ◇, Ca.

immersed in SBF for 960 h and one less clearly visible zone, shown in Fig. 1a. Combining the SEM and EDS information at the same scale as in Fig. 1 allowed the clear demarcation of five zones, B-F (A represents the embedding resin) within the Bioglass surface development.

Plotting the mole percentage of Ca, P, Si and Na present in Bioglass samples against depth from the surface (Fig. 1b) for discs immersed in SBF for 960 h illustrated the previously described development of a calcium phosphate rich layer at the surface and a silica rich layer beneath [9]. The calcium phosphate rich layer (represented by B in Fig. 1) extended approximately 7 μm below the Bioglass surface, backscattered the most electrons and consisted of approximately 50 mol% Ca and 40 mol% P. The silica rich layer (represented by the region D in Fig. 1) occurred approximately 14 μm beneath the surface and extended 30 μm deeper, was the layer which backscattered fewest electrons and had a silica content of 55–80 mol%.

The total reaction layer of Bioglass samples was approximately 53 μm thick and between the calcium phosphate rich layer (B) and the silica rich layer (D) was an intermediate layer (C) which was clearly delineated in the backscattered SEM image but of variable composition according to the EDS data. There was a similar intermediate layer which was apparent from the EDS analysis between the bulk of the Bioglass (F)

and the silica rich layer (D), which although clearly demarcated from the silica rich layer in the backscattered SEM image was not easily distinguished from the bulk of the Bioglass.

3.2. Bioglass surface modification in SBF with 10% FCS

Immersion of Bioglass discs in SBF with 10% FCS for 960 h altered the appearance of the backscattered SEM images and the EDS analysis compared with Bioglass discs reacted in the absence of proteins. The total reaction layer was considerably narrower (approximately 36 μm wide) than occurred in the absence of FCS and the backscattered SEM images showed only four demarcated reaction zones (Fig. 2a). The reaction zones consisted of a silica rich layer with a decreasing content of Ca and P shown as zone C (approximately 3 μm wide), a silica rich layer (zone D approximately 18 μm wide), an intermediate layer (zone E approximately 15 μm wide) between the silica rich layer and the Bioglass matrix (zone F). The presence of 10% FCS in SBF solution either inhibited or did not provide suitable conditions for a calcium phosphate rich layer to form on the surface of Bioglass. The silica rich layer (D) was considerably narrower and was also located much closer to the Bioglass surface than occurred without the presence of proteins in the SBF.

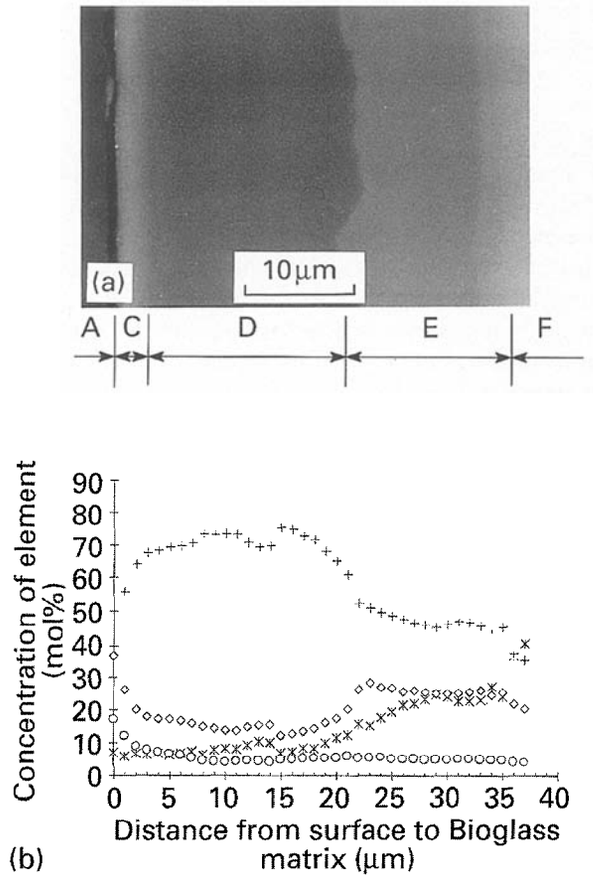

Figure 2 a) Backscattered SEM micrograph showing the different reaction layers (as in Fig. 1 labelled A–F except B is not present) which were identified following immersion of Bioglass 45S5 in SBF with 10% FCS for 960 h. A - embedding resin, C - silica rich intermediate layer containing Ca and P, D - silica rich layer, E - intermediate layer, F - Bioglass matrix. Bar width = 10 μm. b) Graph representing the elemental distribution with depth from the surface of a cross section of Bioglass 45S5 immersed in SBF with 10% FCS for 960 h as determined using EDS. The scale of the x axis of the graph is the same as the micrograph in a) and the same zones A-F apply. Key as for Fig. 1.

3.3. Bioglass surface development in SBF with time

Plotting the percentage of Ca, P, Si and Na measured using EDS at the surface of Bioglass discs incubated in SBF against the immersion time showed how the surface of Bioglass developed (Fig. 3). Initially there was an increasing concentration of Si at the surface up to 10 h which was mirrored by a decrease in Na over the same time period which appeared to represent the formation of a silica rich layer at the expense of Na. Between 10 and 20 h a dramatic change occurred at the Bioglass surface, with a marked increase in Ca and P being matched by a sharp decrease in Si and appeared to represent the formation of a calcium phosphate rich layer. Both Ca and P accounted for over 40 mol% each at the Bioglass surface after 40 h, whilst Si and Na were decreased to less than 10 mol% and continued to decrease with longer immersion times. Ultimately both Ca and P represented 50 mol% each at the Bioglass surface after 960 h.

3.4. Bioglass surface development in SBF containing 10% FCS with time

Plotting the percentage of Ca, P, Si and Na measured using EDS at the surface of Bioglass discs incubated in SBF with 10% FCS against the immersion time (Fig. 4) showed that the presence of proteins altered the rate of reaction at the Bioglass surface compared with the reaction that took place in SBF alone. Si was again concentrated at the Bioglass surface as occurred in the absence of proteins but did not reach its maximum until 20 h after immersion (compared with 5 h in SBF alone). There was also a slow decrease in Ca concentration up to 10 h (compared with 2 h in SBF alone) before a slow increase in concentration preceding the marked rise in Ca content at the surface 40 h (the marked rise in Ca occurred after 10 h in SBF alone). The accumulation of P at the Bioglass surface

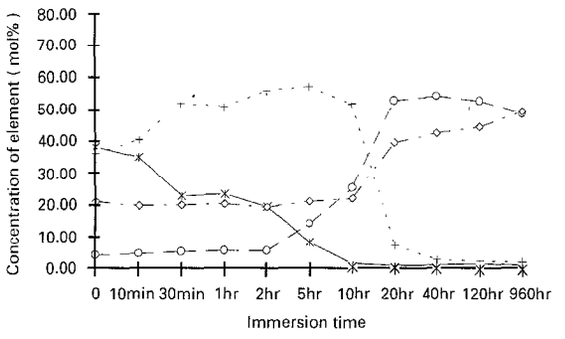

Figure 3 A graph to show the changes in the elemental composition at the surface of Bioglass 45S5 before and after immersion in SBF for different times from 10 min to 960 h. The silica rich layer formed in the first 30 min and subsided after 20 h when the calcium phosphate rich layer then predominated. Key as for Fig. 1.

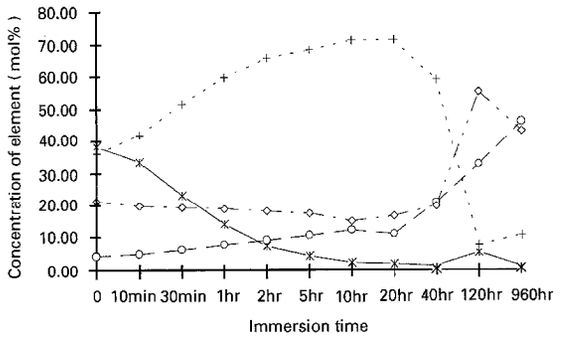

Figure 4 A graph to show the changes in the elemental composition at the surface of Bioglass 45S5 before and after immersion in SBF with 10% FCS for different times from 10 min to 960 h. The silica rich layer formed over the first 20 h before subsiding and Ca and P increased after 120 h. Key as for Fig. 1.

was also delayed by proteins with a clear increase in concentration occurring after 20 h whilst in the absence of proteins this increase in P concentration was seen after only 2 h. Thus proteins delayed the accumulation of a Ca and P layer at the surface of Bioglass until 120 h after immersion, whilst a calcium phosphate rich layer had been found to occur after 20 h in the absence of proteins. The decrease in Na at the surface seemed to follow a similar pattern either in the presence or absence of proteins, although both Si and Na were still present on the surface of Bioglass when reacted in SBF with FCS, whilst absent from the surface when reacted in SBF alone.

4. Discussion

The results presented here clearly indicate that the presence of proteins derived from FCS influence the *in vitro* surface development or modification of Bioglass when immersed in SBF. The presence of proteins appears to modify Bioglass in three particular ways, in terms of the reaction rate, the concentration of different elements both at the surface and with depth from the surface and the arrangement and thicknesses of reaction layers or zones at or near the Bioglass surface. Despite the differences that were identified in the presence or absence of proteins, a silica rich layer always developed and the reactions that took place in the presence of SBF alone appeared to confirm previous findings regarding the surface development of Bioglass [8, 9]. However there did appear to be compositional or phase variations in the silica rich layer (D) within the Bioglass sample immersed in SBF alone (Fig. 1a), which were not observed when proteins were present (Fig. 2a), which is further evidence that proteins are influencing the kinetics of the reactions occurring on and in the Bioglass.

Precisely how the proteins added to SBF were exerting their effects on the development of the Bioglass surface was not investigated in the present study, although the lack of a calcium phosphate rich layer may have resulted from certain proteins and macromolecules acting as inhibitors of calcium phosphate crystallization, which has been reported to occur in supersaturated solutions [16] which SBF is with respect to calcium and phosphate. It was suggested [17] that the transformation of amorphous calcium phosphate to hydroxyapatite at normal physiological concentrations is strongly inhibited by albumin. The protein albumin constitutes 50–60% of the total serum proteins, is negatively charged at physiological pH and has been reported to be the dominant inhibitor of hydroxyapatite formation *in vivo* although other serum constituents can also exhibit inhibitory effects [18]. Two mechanisms may have contributed to the inhibition of hydroxyapatite formation on the Bioglass surfaces in the presence of proteins in the present study. Firstly albumin and other serum constituents may have rapidly adsorbed onto the Bioglass surface forming a proteinaceous layer and coating the surface of reaction products such as the silica rich layer, or possibly the amorphous calcium phosphate rich layer. This adsorbed protein layer may then have prevented or reduced further exchange of ions at and across the surface or interface, which would obviously decrease the reaction rate as was observed. However, there was little evidence of any calcium phosphate rich layer at the surface of Bioglass up to 120 h and even at this stage it was less marked in mol% than seen in SBF alone, therefore the likelihood of the calcium phosphate layer being affected by the presence of proteins (Fig. 2a) was small. Inhibitory proteins may also have interfered with the nucleation of hydroxyapatite and subsequent crystallization.

5. Conclusions

The presence of proteins in SBF alters the rate of reactions that occur at the surface and within Bioglass, the elemental composition of Bioglass both at the surface and with depth from the surface and the arrangement of reaction layers or zones that develop at or near the surface of Bioglass. However certain aspects of Bioglass surface development remain similar in either the presence or absence of proteins, particularly the development of a silica rich layer. It appears the proteins have a more profound impact on the chemical reactions during the surface development of Bioglass than solely inhibition of crystallization.

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References

1. L. HENCH, R. J. SPLINTER, W. C. ALLEN and T. K. GREENLEE, *Biomed. Mater. Res. Symp.* **2** (1972) 117.
2. L. HENCH and J. WILSON, in "Silicon Biochemistry" (Wiley, Chichester, 1986 (Ciba Foundation Symposium 121)), p. 231.
3. T. KASUGA, K. NAKAGAWA, M. YOSHIDA, and E. MIYADE, *J. Mater. Sci.* **22** (1987) 3721.
4. T. KITSUGI, T. YAMAMURO, T. NAKAMURA, S. KOTANI, T. KOKUBO and H. TAKEUCHI, *Biomaterials* **14** (1993) 216.
5. M. JARCHO, C. H. BOLEN, M. B. THOMAS, J. BOBICK, J. F. KAY and R. H. DOREMUS, *J. Mater. Sci.* **11** (1976) 2027.
6. R. D. RAWLINGS, *Clin. Mater.* **14** (1993) 155.
7. L. HENCH, *Science* **208** (1980) 826.

8. T. KOKUBO, *J. Non-Cryst. Solids* **120** (1990) 138.
9. L. HENCH, *J. Amer. Ceram. Soc.* **74** (1991) 1487.
10. C. Y. KIM, A. E. CLARK and L .L. HENCH, *J. Non-Cryst. Solids* **113** (1989) 195.
11. T. KOKUBO, H. KUSHITANI, S. SKKA, T. KITSUGI and T. YAMAMURO, *J. Biomed. Mater. Res.* **24** (1990) 721.
12. R. E. BAIER and R. C. DUTTON, *ibid.*, **3** (1969) 191.
13. A. L. BOSKEY, *Clin. Orthop.* **5** (1981) 225.
14. J. MEI, R. M. SHELTON, M. AINDOW and P. M. MARQUIS, In *Third Euro-Ceramics* **3**, Proceedings of 3rd European Ceramic Society meeting, Madrid, Spain, September 1993, edited by P. DURAN and J. F. FERNANDEZ (Faebza Editrice Iberica, 1993) p. 167.
15. T. KOKUBO, H. KUSHITANI, C. OHTSUKI and S. SAKKA, *J. Mater. Sci.: Mater. Med.* **3** (1992) 79.
16. G. H. NANCOLLAS and S. J. ZAWACHI, *Conn. Tiss. Res.* **21** (1989) 239.
17. J. L. MEYER and H. FLEISCH, *Mineral and Electrolyte Metabolism* **10** (1984) 249.
18. R. I. MARTIN and P. W. BROWN, *J. Mater. Sci.: Mater. Med.* **5** (1994) 96.

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