45S5 Bioactive glass surface charge variations and the formation of a surface calcium phosphate layer in a solution containing fibronectin

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Abstract: This study investigated the effect of fibronectin adsorption on surface charge variations and calcium phosphate (Ca-P) layer formation kinetics on the surface of 45S5 bioactive glass (BG). We hypothesize that the adsorption of fibronectin on BG changes the surface charge and alters the kinetics of Ca-P layer formation on the glass surface. The charge at a material's surface modulates surface chemistry, protein adsorption, and interactions with bone cells. The zeta potential of BG in a solution containing human plasma fibronectin (TE-FN) was measured as a function of time by particle electrophoresis, and Ca-P layer formation was characterized using SEM, EDXA, and FTIR. Si, Ca, and P solution concentrations also were determined. It was found that the adsorption of fibronectin reduced the initial electronegativity of the BG surface and delayed the formation of both the amorphous and the crystalline Ca-P layers. The delayed formation of these surface layers may be attributed to the competitive binding of Ca^{2+} ions by the fibronectin molecule. In addition, the formation of an amorphous Ca-P layer correlated with the reversal from a negatively to a positively charged surface, independent of the presence of fibronectin. The addition of a single protein (in this case fibronectin) can significantly alter material surface parameters, such as charge, and subsequently affect the formation of a surface Ca-P layer. Furthermore, the formation of an amorphous Ca-P layer is an important event in the reactions leading to bioactive behavior, and proteins such as FN are actively involved in the transformation of the surface into a Ca-P layer. © 2000 Wiley & Sons, Inc. J Biomed Mater Res 54: 454–461, 2001

Key words: bioactivity; surface charge; fibronectin; protein adsorption; calcium phosphate layer

INTRODUCTION

By forming a surface calcium phosphate layer *in vivo*, bioactive materials, such as 45S5 bioactive glass (BG), are able to enhance bone formation and to establish a contiguous chemical bond to surrounding bone tissue.[1] Such an interface is particularly advantageous in orthopedic applications as it will promote long-term implant stability and integration with surrounding bone. Currently, the mechanism governing bioactive behavior is not fully understood. This study is part of an ongoing investigation into the mechanism of bone bioactivity, specifically focusing on the interaction between proteins and the charged, bioactive surface.

Variations in BG surface charge are likely to alter the kinetics of Ca-P layer formation, protein adsorption, and cellular response to bioactive surfaces. Various authors have emphasized the importance of surface charge in the formation of the Ca-P layer and in surface interactions with the biologic environment.[2–7] These suggestions are related to the fact that endogenous bone potentials have been implicated in normal bone synthesis and remodeling.[8] Furthermore, short-range electrostatic interactions can determine the site of protein adsorption and the orientation and conformation of the adsorbed protein, thereby modulating cellular response to the biomaterial.

Upon implantation, the BG surface immediately is exposed to numerous extracellular proteins; thus the role of protein adsorption on the overall bioactive response must be addressed. In this study, we examine the effect of protein and, in particular, of fibronectin adsorption on BG surface charge variations and Ca-P layer formation kinetics. Fibronectin (FN) is an abundant extracellular matrix glycoprotein found in many

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tissues. It is actively involved in cell adhesion, spreading, wound healing, cytoskeletal reorganization, and bone tissue formation.[9–13] By focusing on a single protein, the inherent simplicity of such a system allows us to begin unraveling the complex interfacial reactions occurring between the biomaterial surface and the biologic environment. Previous works from our laboratory suggest that FN also plays an important role in cellular response to bioactive surfaces: El-Ghannam et al. found that compared to hydroxyapatite, the reacted BG surface selectively adsorbed more FN[14]; and Garcia et al. reported on the adhesion strength of osteoblast-like cells on FN-coated surfaces and measured an enhanced FN-mediated cell attachment on the reacted BG surface compared to both unreacted BG and hydroxyapatite controls.[15]

Previously, we examined BG surface charge variations in an electrolyte solution and found that temporal BG surface charge variations directly corresponded to the formation of a Ca-P surface layer *in vitro*.[16] In the current study, we continue our investigation into the mechanisms of bioactivity by focusing on the effect of fibronectin adsorption on surface charge variations and the formation of a Ca-P surface layer. This work is based on the hypothesis that fibronectin adsorption on the BG surface will alter its surface charge and change the kinetics of Ca-P layer formation on the glass surface.

MATERIALS AND METHODS

Sample and solution preparations

45S5 Bioactive glass particles (BG, <10 μm, weight %: 45.2% SiO_2, 24.2% Na_2O, 24.6% CaO, 6.0% P_2O_5) were used in this study (Orthovita, Malvern, Pennsylvania). A qualitative examination of the as-received BG particles by scanning electron microscopy (SEM) confirmed that the largest dimension of the majority of the particles was less than 10 μm. X-ray diffraction (Rigaku–Geigerflex diffractometer) analysis revealed that the BG structure was amorphous.

Human plasma fibronectin (FN, 1 mg, GIBCO BRL, Grand Island, New York) was reconstituted in 1 mL of sterile tissue-culture-grade H_2O and diluted with a sterile electrolyte solution. The electrolyte solution (TE) was prepared by adding plasma-equivalent electrolytes to 0.05*M* of Trizma® buffer (Sigma, St. Louis, Missouri). The protein solution (TE-FN) used in the immersion experiments had a FN concentration of 1 μg/mL and a pH of 7.4 at 37°C. TE-FN has an ionic strength of 0.17*M* and an electrolyte concentration of 152.0 m*M* of Na^+, 135.0 m*M* of Cl^-, 5.0 m*M* of K^+, 2.5 m*M* of Ca^{2+}, 27.0 m*M* of HCO_3^-, 1.5 m*M* of Mg^{2+}, 0.4 m*M* of SO_4^{2-}, and 1.0 m*M* of HPO_4^{-2}.

BG particles were immersed in TE-FN at 37°C at the weight-to-volume ratio of 1.0 mg/mL. Samples were incubated at 37°C, 5% CO_2, for 15 min, 30 min, 1, 3, 5, and 12 h, and 1, 3, 6, and 7 days. Unreacted BG and TE-FN without BG particles served as controls.

Zeta potential measurements

The zeta potential of bioactive glass particles in TE-FN was measured by particle electrophoresis using the Pen Kem System 3000 Electrokinetic Analyzer (Pen Kem Inc., Bedford Hills, New York). The value of zeta potential represents the effective surface charge of a biomaterial in contact with solution. The theory and operation of the electrokinetic analyzer, as well as particle size effects, have been described in detail elsewhere.[16–19] Before and after the measurements, the instrument was calibrated using standardized latex particles suspended in a 0.01*M* solution of KCl.

Surface analysis (FTIR, SEM, EDXA)

At each time point, the particles were separated from the solution by centrifugation at 3400 rpm and left to dry overnight in an oven at 37°C. The sample surfaces were analyzed using Fourier transform infrared spectroscopy (FTIR, Nicolet 5DXC FTIR spectrometer, Madison, Wisconsin) in the diffuse reflectance mode, and 300 scans were collected per sample. Surface morphology and composition of the glass particles before and after immersion were examined using scanning electron microscopy (SEM, JEOL JSM 6300, operated at 2 keV) and energy dispersive X-ray analysis (EDXA, JEOL JSM 6400, operated at 10 keV). The samples were coated with carbon in order to minimize surface charging effects.

Solution chemical analysis (pH, Si, Ca, and P concentrations)

The pH of sample solutions was measured at 37°C (Brinkmann 632 digital pH meter) before centrifugation. After separation of BG particles from the solution, calcium and silicon concentrations in the post-immersion solutions were determined using flame atomic absorption spectroscopy (Perkin–Elmer Model 5100, Norwich, Connecticut). The system was calibrated using Ca and Si standards prepared in a range of concentrations. Phosphorous concentrations were measured by an UV visible light spectrophotometer (Biochrom, LKB 4053 Ultrospec K) using a colorimetric assay.[20]

Statistical analysis

Data in the graphs are presented in the form of mean ± standard deviation (mean ± SD), with *n* equal to the number of samples analyzed per immersion treatment. An unpaired *t* test was used to determine significant differences between two means. In the case of multiple comparisons, one-way

and two-way analyses of variance (ANOVA) were performed, and the Tukey–Kramer test was used to compare the means. Significance was attained at $p < 0.05$.

RESULTS

Surface zeta potential

Kinetic zeta potential variations of BG immersed in TE-FN are shown in Figure 1 ($n = 9$). In the same graph, the dashed line connecting circles represents BG zeta potential as a function of time in TE without protein.[16] Statistically significant differences in surface zeta potential between BG immersed in the two solutions are marked with asterisks (*). The charge of the BG surface in the presence of FN was negative within 15 min of immersion (−3.3 mV), and it remained statistically constant during the first 5 h in solution. After 24 h, the surface continued to maintain a negative zeta potential of −2.95 mV. However, as immersion continued, the surface became increasingly positive, and a sign reversal from "−" to "+" was measured at day 3. The long-term BG surface was positively charged, with a zeta potential of +6.4 mV after 7 days.

Surface analysis

Figure 2 compares FTIR spectra of unreacted BG with surfaces immersed in TE-FN for 1, 3, and 7 days. An amorphous Ca-P layer was detected after 3 days of immersion. A crystalline Ca-P layer, as indicated by the divided P-O bending vibration band between 500 and 600 cm^{-1}, formed after 7 days in TE-FN. A C-O stretching vibration band also appeared between 890 and 800 cm^{-1} after 7 days, indicating the formation of carbonated calcium phosphate.[21–24]

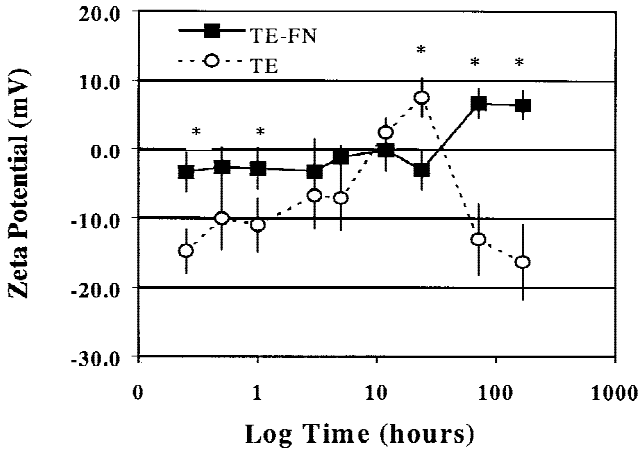

Figure 1. 45S5 bioactive glass (BG) zeta potential variations as a function of immersion time in solutions with fibronectin (TE-FN) and without fibronectin (TE). Immersion ratio = 1 mg/mL. Statistically significant differences between zeta potentials of surfaces formed in the two solutions are marked with asterisks (*), with $p < 0.05$. The mean zeta potential of each data point is calculated based on the average electrophoretic mobility values obtained from nine sets of electrophoretic mobility histograms ($n = 9$).

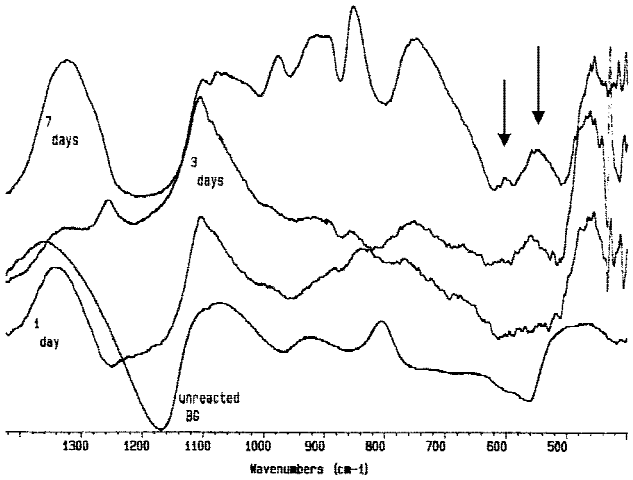

Figure 2. Fourier transform infrared (FTIR) spectra of unreacted 45S5 bioactive glass (BG) particles and of BG after immersion in a solution containing fibronectin (TE-FN) for 1, 3, and 7 days. Note the appearance of the P-O bending vibration band between 600 and 500 cm^{-1} on day 3, indicating the formation of a predominantly amorphous calcium phosphate (Ca-P) layer. Incipient crystallization of surface calcium phosphate was detectable at this time with other surface analysis techniques. At 7 days, the P-O bending band became divided, indicating the presence of a crystalline Ca-P layer. When compared to bioactive surfaces formed after immersion in a solution without proteins,[16] the addition of fibronectin delayed the formation of the Ca-P surface layer.

SEM micrographs of BG particles immersed in TE-FN revealed that BG particles were almost completely covered by a film not seen on particles immersed in TE without FN.[16] This film was found on both 1- and 3-day samples, and a representative image of the 1-day BG surface is shown in Figure 3(a). It is likely that this surface covering film is due to the adsorption of FN. Calcium phosphate nodules and their aggregates were found on all surfaces examined. As seen in Figure 3(b), after 1 day, calcium phosphate nodules continued to form even beneath the film covering the particle surface. The uncovered region of the BG particle shown in the SEM image was made up largely of calcium, phosphorus, and silicon, and it is similar in structure and appearance with areas of the surface underneath the film.

EDXA analysis revealed that the unreacted BG surface was comprised of Na, Si, Ca, and P, as expected. After exposure to TE-FN, the Na peak decreased and became barely detectable after 24 h of immersion. Its intensity, however, was relatively stronger than in TE at the same immersion duration.[16] Meanwhile, the Cl

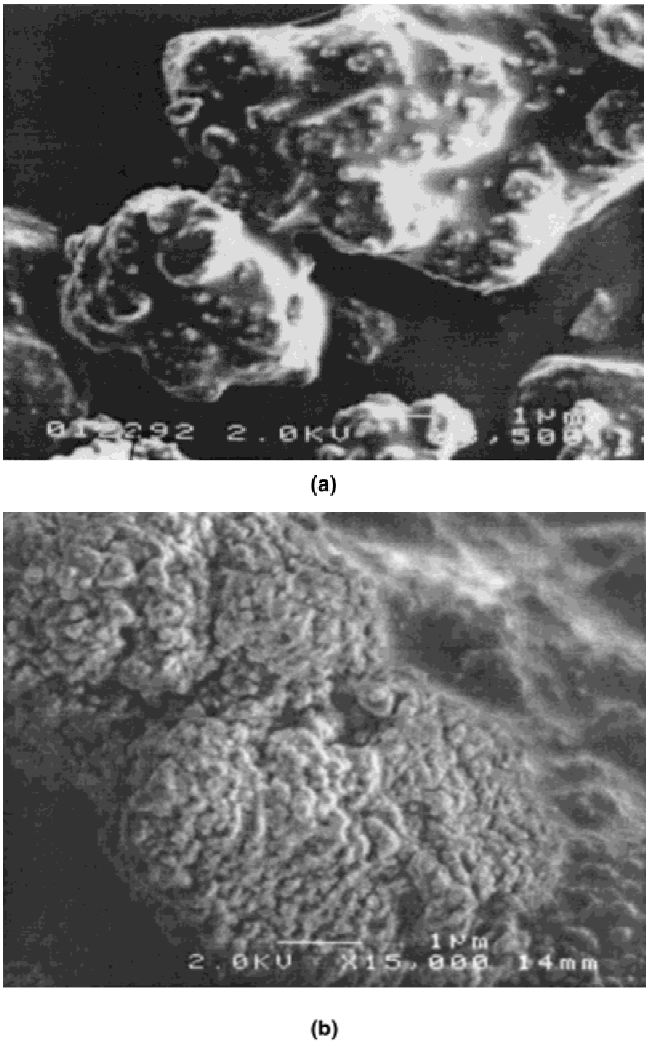

Figure 3. Scanning electron microscopy (SEM) images of 45S5 bioactive glass (BG) particles immersed in a solution containing fibronectin (TE-FN) for 1 day. (a) A film completely covered the BG particles after 1 day of immersion in a solution containing fibronectin (original magnification ×8,500). (b) Ca-P nodules were found on the entire particle surface, and underneath the surface film Ca-P aggregates could be observed. The film did not cover the area running from the top left to the bottom right corner of the image; X-ray analysis revealed that this area is made up of Ca-P nodules as well (original magnification ×15,000).

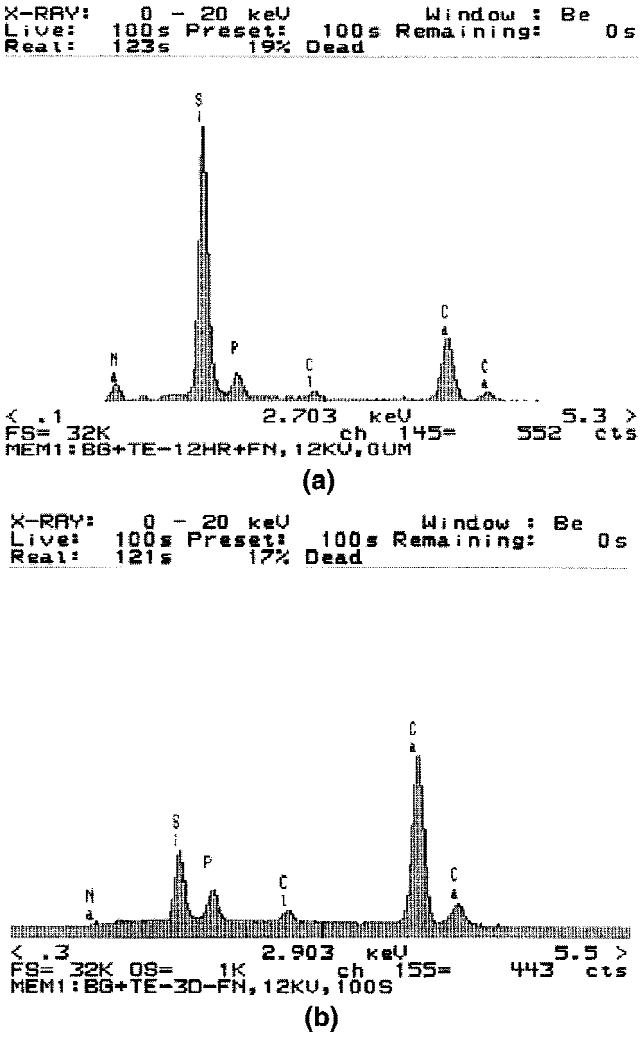

Figure 4. Energy dispersive X-ray analysis spectra of 45S5 bioactive glass (BG) immersed in a fibronectin-containing solution (TE-FN). (a) At 12 h the surface had a high content of Si, and Na was present on this surface at a relatively higher intensity than on BG surface formed in the absence of fibronectin. These results suggest that surface dissolution of Si and Na is delayed due to exposure to fibronectin. (b) At 3 days the relative intensity of the Si peak had decreased and the surface was rich in Ca. The amount of Na also had decreased as immersion continued.

peak, absent in the spectra for unreacted glass, was visible in the 12 h spectrum. Si was present on all surfaces analyzed, and its peak intensity began to diminish only after 3 days of immersion. Two typical EDXA spectra of BG immersed in TE-FN for (a) 12 h and (b) 3 days can be seen in Figure 4.

Solution chemical analysis

The pH of the sample solutions varied between 7.4 and 7.7 for the entire length of the experiment. Si dissolution occurred at the onset of immersion as Si concentration increased from 0.07 to 1.64 m*M* after 1 day. This concentration is below the solution saturation limit of Si. Subsequently, Si concentration fluctuated between 1.6 and 1.32 m*M* after 12 h in TE-FN (data not shown). However, a second release of Si was detected after 3 days, and steady state was reached after 7 days. Ca^{2+} concentration increased from 2.5 m*M* in the control solution to 3.6 m*M* after 15 min of immersion, and it remained at the same level for up to 5 h in TE-FN, as seen in Figure 5 (n = 9). After 12 h, an uptake of 1.1 m*M* of Ca^{2+} from solution was measured. A second release was found at day 3, and subsequently the Ca^{2+} concentration decreased from 3.12 to 1.36 m*M* at 7 days in TE-FN.

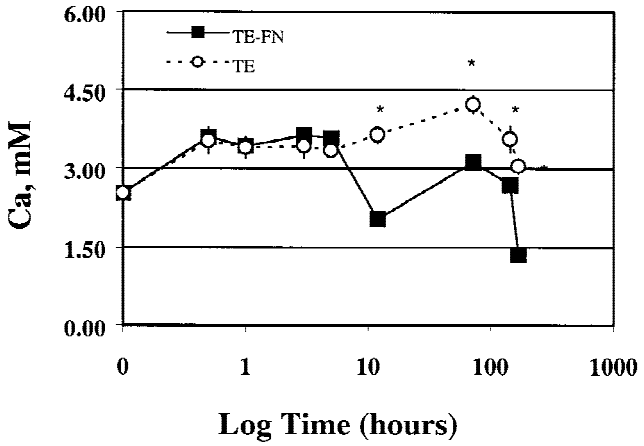

Figure 5. Solution calcium concentration as a function of time after 45S5 bioactive glass (BG) particles were immersed in solutions with fibronectin (TE-FN) or without fibronectin (TE). Immersion ratio = 1 mg/mL, with $n = 9$. While the presence of fibronectin did not alter the dissolution of calcium from the bulk glass, it did promote the precipitation of calcium from solution. Significant differences between two means are marked with asterisks (*), with $p < 0.05$.

An initial release of phosphorous (P) from the glass particles was not measured since P concentration remained at 1.0 m*M* for up to 5 h. This concentration value was not statistically different from the control solution. However, after 12 h in TE-FN, a significant uptake of phosphorous ions from solution was recorded as its concentration dropped to 0.32 m*M*. At day 7, the solution was almost completely depleted of phosphorus.

DISCUSSION

The goal of this study was to achieve a more comprehensive understanding of the interfacial reactions occurring at the junction between the bioactive surface and the physiologic environment. To this end we investigated the effect of fibronectin adsorption on BG surface charge and the formation of a Ca-P surface layer *in vitro*. We found that the addition of a single protein (in this case fibronectin) to the system decreased the initial electronegativity of the BG surface and delayed the formation of the Ca-P layer.

The results of the current study are best interpreted when compared to previous data obtained under identical experimental conditions but without fibronectin.[16] Table I summarizes the differences in both the zeta potential and the type of Ca-P layer formed on BG surface after reactions in electrolyte solutions with and without FN. From the earliest time points measured, at 15 min after immersion, the BG surface exhibited a zeta potential of −3.32 mV in TE-FN. This is close to a fourfold reduction in surface electronegativity compared to the situation without FN. In addition, in the solution with FN, BG surface zeta potential remained nearly constant within the first 24 h of immersion, while in the absence of FN a positively charged surface developed. The observed reduction in the initial negativity of the surface suggests that the adsorption of FN neutralized the intrinsically negative charge of the BG surface.

TABLE I
Comparison between 45S5 Bioactive Glass (BG) Surfaces Immersed in Electrolyte Solutions with (TE) and without Fibronectin (TE-FN)

Duration of Immersion	Surface Parameters	TE (no FN)	TE-FN (w/FN)
15 min	zeta potential (mV)	−14.72 ± 3.18	−3.32 ± 2.79
	Structure	*	*
1 day	zeta potential (mV)	7.47 ± 2.84	−2.95 ± 2.82
	Structure	Amorphous	*
3 days	zeta potential (mV)	−13.02 ± 5.05	6.67 ± 2.10
	Structure	Crystalline	Amorphous
7 days	zeta potential (mV)	−16.30 ± 5.47	6.44 ± 2.06
	Structure	Crystalline	Crystalline

*: surface structure not detectable by FTIR; zeta potential values are presented in mean ± standard deviation.

As immersion continued, the measured shifts in surface zeta potential reflected the formation of the Ca-P surface layer. In the absence of FN, we found that the formation of an amorphous Ca-P layer on BG after 1 day of immersion in TE corresponded to a sign reversal of the surface zeta potential from negative to positive.[16] Here a similar sign reversal, from a negative to a positive surface, was measured and found to correspond to the formation of an amorphous Ca-P layer. However, the appearance of this sign reversal was delayed; it was observed only after 3 days in TE-FN. The second sign reversal from a negative to a positive surface reported for BG immersed in TE was not found in TE-FN, not even after 7 days of immersion. Instead, the long-term BG surface was positively charged, with a zeta potential of 6.44 mV, while the charge of the surface formed after immersion in the solution without FN was −16.30 mV. The observed discrepancy in surface charge between the two crystalline Ca-P layers suggests that the adsorption of FN altered the surface reaction dynamics and resulted in a long-term BG surface that was distinct from the surface formed in the absence of FN.

FTIR analysis confirmed that the adsorption of FN delayed the formation of an amorphous Ca-P layer (3 days vs. 1 day) and a crystalline Ca-P layer (7 days vs. 3 days) on BG. However, although the amorphous Ca-P layer was not detected on BG by FTIR until day 3, SEM and EDXA analyses did reveal the presence of Ca-P deposits earlier on. These surface deposits contained high concentrations of Si and Ca but were low in P. Our results are similar to those of Radin et al,,[24]

who found that serum protein adsorption on BG disks did not limit the formation of the amorphous Ca-P layer; rather it inhibited the formation of the crystalline Ca-P layer. Here, in the presence of FN, the amorphous Ca-P layer continued to form; however, this layer became thick enough for FTIR detection only after 3 days of immersion.

Solution chemical analysis provided additional information on the effect of FN adsorption on BG surface charge and the formation of a Ca-P layer. We found that dissolution, ion exchange, and precipitation reactions continued to take place in the presence of FN.[25] However, the reaction rates were altered due to FN adsorption. Dissolution of the BG glass network remain unaffected in TE-FN since the same amount of Si was measured initially in the solutions with and without FN. Similarly, the initial release profiles of both calcium and phosphorus were unchanged in the presence of FN. Compared to the steady state plasma FN concentration of 150–300 μg/mL,[11] the concentration of 1 μg/mL of FN in TE-FN used here was significantly lower. In this case there would be a very small amount of FN adsorbed on the BG surface. Consequently, limited coverage of BG surface by FN was insufficient to prevent dissolution of ions from the bulk glass structure.

While FN adsorption did not affect dissolution reactions, it did alter the precipitation of both calcium and phosphorus ions from solution. After long-term immersion with TE-FN, we measured a statistically significant increase in Ca^{2+} precipitation in comparison to the solution without FN. After 7 days, the concentration of Ca^{2+} in TE-FN was half of that measured when BG was immersed in TE. In addition, greater Ca^{2+} presence on the surface was reflected in the increasingly positive surface zeta potential. We measured a positive zeta potential for the BG surface immersed in FN for more than 1 day. The structure of FN is known to bind divalent ions such as Ca^{2+} and Mg^{2+}, and the heparin binding domains of the FN structure are sensitive to changes in divalent ion concentration.[9,26] Therefore, increased uptake of Ca^{2+} from solution during the long-term immersions may be attributed to the competitive binding of Ca^{2+} ions by the FN molecule. These findings are consistent with those of Radin et al., where Rutherford backscattering spectrometry analysis of BG adsorbed with serum protein was enriched with Ca^{2+}. Furthermore, the Ca-rich layer formed grew thicker and the distribution of Ca^{2+} at the surface became increasingly homogenous. The authors concluded that serum protein adsorption enhanced the accumulation of calcium and phosphate ions on the BG surface. It is likely that the competitive binding of Ca^{2+} ions by serum proteins would lead to a depletion of Ca^{2+} ions available for the formation of the crystalline Ca-P surface layer. This is consistent with reports that the crystalline Ca-P layer did not form on BG surfaces in the presence of serum proteins. In this study, the formation of a crystalline Ca-P layer in the presence of FN was not completely inhibited since we used a relatively low concentration of fibronectin in TE-FN.

It has been postulated that a series of dissolution, ion exchange, and precipitation reactions occurring at the glass–solution interface contribute to the formation of the Ca-P surface layer. Adsorption of proteins onto BG concurrent to or before these reactions would alter the energy balance of interfacial reactions and thus affect the formation of the Ca-P layer. In this study, after FN adsorption, the over fourfold decrease in electronegativity of the BG surface would have a significant effect on the rates of interfacial reactions. Moreover, the associated reduction in electric field strength near the BG surface would translate into profound effects on protein and cellular function. Tanahashi et al.[27] studied calcium phosphate formation on self-assembled monolayers (SAMs) of alkanethiols with different surface functional groups and found that the negatively charged surface groups (PO_4H_2 and COOH) were potent Ca-P inducers while SAMs with nonionic ($CONH_2$,OH) and positively charged (NH_2) functional groups did not support rapid Ca-P formation. It was proposed that the Ca-P induction process likely began with the accumulation of Ca^{2+} on the material surface, accompanied by complexation of phosphate ions. Therefore a strongly negative surface would promote the formation of surface calcium phosphates. By the same token, the decrease in BG surface electronegativity observed here after FN adsorption would adversely affect the development of a Ca-P layer on the BG surface.

Interestingly, when comparing the amorphous Ca-P surfaces formed in the presence and absence of FN, their development was observed after a sign reversal from a negative to a positive zeta potential. Moreover, the resultant amorphous surfaces in both situations exhibited zeta potential values statistically identical in sign and magnitude. On the other hand, the crystalline Ca-P layers formed in solutions with and without fibronectin displayed zeta potentials that are distinctly different in magnitude and sign. Moreover, the fact that a crystalline surface formed in the presence of FN is surprising at first sight. It has been reported that by adding only 10% serum, the formation of the crystalline layer was completely inhibited on BG surfaces.[24,28] Because of the relatively low concentration of FN used in this study, it is possible that surface transformation from amorphous to crystalline was not sufficiently suppressed. Regardless, the effects of FN are extensive and have lead us to surmise that *in vivo,* the formation of an amorphous Ca-P layer on BG is the significant step, and that the subsequent formation of a crystalline Ca-P layer is not a solution-dependent

process per se but may be a cell-mediated process instead.

The results of this study suggest that the FN molecule is physically involved in the formation process of the amorphous Ca-P surface layer. Daculsi et al. studied the role of FN in biologic apatite crystal nucleation and found the FN molecule to be associated with numerous clusters of very small particles of Ca-P while the immersion with the control molecule albumin revealed no crystal precipitation.[26] Serum FN also has been shown to interact preferentially with bioactive surfaces. El-Ghannam et al. found that, compared to hydroxyapatite, the reacted BG surface selectively adsorbed more FN.[29] The affinity of FN for BG suggests that the protein plays an important role in bone bioactivity. The FN molecule is comprised of two nearly identical subunits held together at the carboxyl terminal end by a pair of interchain disulfide bonds. Divalent ions such as Ca^{2+} and Mg^{2+} are essential for subunit association, ligand binding, and receptor function.[30,31] Concentration of these cations influences both ligand affinity and receptor specificity.[31–33] Extracellular concentration of Ca^{2+} has been shown to affect fibronectin production by chicken granulosa cells.[34] It is possible that dissolution of Ca^{2+} ions into the extracellular fluid may alter the function of extracellular proteins such as FN.

It has been shown that cells will bind to adsorbed FN but not to soluble FN. This observation led to the suggestion that FN underwent a conformational change upon binding to a surface.[35] The nature of the electric charge at a surface is an important parameter in determining the orientation of the adsorbed FN. FN has an acidic isoelectric point (pI) of 5.5–6.0; thus the entire molecule possesses a net negative charge within the pH range of the study (7.4–7.7). However, at the immediate interface between solid and solution, the electrostatic interaction between the protein and a charged surface likely is governed by the charge exhibited by functional binding domains located on the protein molecule. The structure of FN contains binding sites for collagen, heparin, fibrin, and gelatin as well as a cell binding domain consisting of a 12-kDa fragment with the arginine-glycine-aspartic acid (RGD) binding sequence.[36] The three high-affinity heparin-binding domains of the FN molecule have pIs between 8.2 and 9.0 and thus are positively charged at physiologic pH.[9] Electrostatically, the FN molecule most likely interacts with the negatively charged BG surface through these binding sites.

In addition to measuring temporal variations of BG zeta potential in TE-FN, we also pretreated the BG particles in TE alone for 24 h. This produced a BG surface with a positive zeta potential of 7.47 ± 2.84 mV. When this pretreated surface subsequently was immersed in TE-FN for 5 min, its surface zeta potential immediately became −3.52 ± 3.78 mV. This value is not statistically different from the zeta potential measured for the non-pretreated BG surface after exposure to TE-FN for only 15 min. These data suggest that the FN molecule interacts with the two surfaces differently. Above, we proposed that FN bound to the negatively charged BG surface through the heparin binding domains on its structure. However, when interacting with a positively charged surface, the collagen, gelatin, and fibrin binding domains of the FN molecule are likely candidates since they have pIs ranging between 4.9 and 5.8. Differences in the orientation and specific sites of FN binding to the bioactive surface would result in different conformations of the bound protein. Consequently, the availability of the RGD cell-binding region as well as the overall functionality of the protein may be affected.

Our examination of the parameter of surface charge in the presence and absence of protein may be further utilized for the design and development of future biomaterials. One of the goals of current biomaterial design is to modulate biologic responses of proteins and cells by fabricating surfaces with controlled properties upon which only the desired biologic behavior is expressed. The limiting factor in the successful implementation of this approach is the insufficient identification of the precise surface parameters required to modulate protein conformation upon adsorption and, subsequently, to direct cellular function. By improving our current understanding of bioactivity, we hope to define the surface parameters that are important in the dynamic interactions between a biomaterial and the physiologic environment. We have shown here that the charge at a protein-adsorbed surface is distinctively different from that of a surface without proteins, and examination of cell behavior on substrates with varied surface properties would yield valuable information on which material parameters are important in the modulation of cellular function.

CONCLUSIONS

In the current study, we continued our investigation into the mechanisms of bioactivity by focusing on the effect of fibronectin adsorption on time-dependent surface charge variations and the kinetics of Ca-P layer formation on the BG surface. Our results clearly demonstrate that the addition of a single protein (in this case fibronectin) can significantly alter material surface parameters, such as charge, and consequently can affect the formation of a Ca-P surface layer.

FN adsorption reduced the electronegativity of the BG surface and delayed the formation of both the amorphous and the crystalline Ca-P layers. The delayed formation of these surface layers may be attributed to the competitive binding of Ca^{2+} ions by serum

proteins, such as FN, which lead to a local depletion of Ca^{2+} ions available for the formation of the Ca-P surface layers. Increased presence of calcium ions at the surface resulted in a positively charged long-term BG surface. Moreover, there is initial evidence that the charge at bioactive surfaces may play an important role in modulating FN–substrate interactions.

The formation of an amorphous Ca-P layer correlated with time-dependent zeta potential variations. Specifically, the reversal from a negatively to a positively charged surface corresponded to the formation of an amorphous Ca-P layer, independent of fibronectin adsorption. Furthermore, the resultant amorphous surfaces formed in solutions with and without FN statistically were identical in charge.

Future work will focus on the response of bone cells to protein-modified BG surfaces, with the goal of achieving a more complete understanding of the interactions occurring at the interface between biomaterials and the physiologic environment.

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