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Cell/surface interactions and adhesion on bioactive glass 45S5

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Abstract This paper examines the effects of surface texture (smooth versus rough) on cell/surface interactions on the bioactive glass, 45S5. The cell surface interactions associated with cell spreading are studied using cell culture experiments. Subsequent energy dispersive x-ray spectroscopy is also used to reveal the distributions of calcium, phosphorous, sodium and oxygen on the surfaces of the bioactive glasses. The implications of the results are then discussed for the applications of textured bioactive glasses in medicine.

1 Introduction

Many devices implanted into the body have a limited lifetime due to loss of adhesion between the implant material and the surrounding tissue. This is particularly important in load-bearing implants, such as the metal implants that are inserted into a bone. A loss of adhesion, in these cases, will lead to relative movement between the implanted material and the surrounding tissue, causing much pain to the patient. Implants often separate from the tissue because the material properties of the implant are incompatible with those of the surrounding tissue. There is, therefore, a great need for new materials for use in implants and coatings on implants, which are able to integrate more effectively with the surrounding tissue.

One way to assure that an implant will integrate completely with the surrounding tissue is to use a material that will completely dissolve in the body, serving as a scaffold for bone. In this situation, as the material of the implant is resorbed by the body, it will eventually be replaced with natural tissue. One example of a completely resorbable material is $Ca_3(PO_4)_2$, which has ions that rapidly dissolve when exposed to a biological environment [1]. The material will be remodeled by the activity of osteoclasts, a type of bone cell. The calcium and phosphorus ions will be incorporated into the newly forming bone tissue. Eventually, when the entire material has been resorbed, osteoids will have replaced the implant [1]. This process will ultimately leave natural tissue at the implant site. However, there is a disadvantage to this procedure. While the implant material is being replaced by bone, a process that could take months, there will be a long period of mechanical instability at the implant site. During this period, the patient would need to remain immobilized in order to not disturb the osteoblast activity.

Partly, for this reason, completely resorbable materials are still not a practical solution in load bearing applications. However, materials that are only reactive on the surface could be used in the body as implant materials. These materials, also called bioactive materials, will not significantly change their mechanical properties, while still reacting and bonding chemically with the surrounding tissues. Clinically, the use of such materials would be a more practical solution since the joint can be functional much sooner after implantation.

Bioactivity, in this study, is defined as the ability of a material to strengthen the bond between the implant material and the surrounding bone, thus leading to more bone formation at the implant site [1]. Several bioactive materials have been developed [1–8]. These materials all contain bonding

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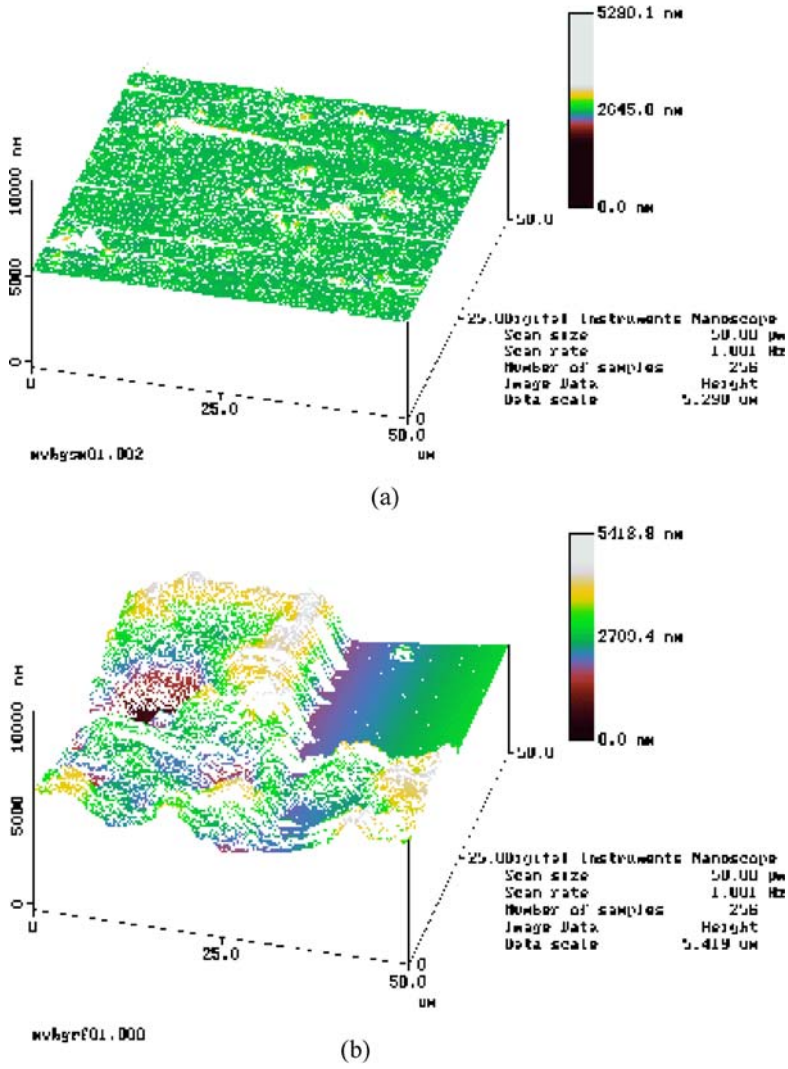

Fig. 1 Tapping mode AFM images of surface topography: (a) Smooth surface and (b) Rough surface

sites on the surface for the various proteins of soft tissues and cell membranes [1]. Also, these bioactive materials have the ability to release ions from the surface into the biological fluid. The presence of the various ions near the surface promotes hydroxyapatite (HA) nucleation ($Ca_5(PO_4)_3OH$) on the surface of these materials [1]. The formation of the HA is significant, since it is one of the major components of bone. This hydroxyapatite layer is the first step in the process that leads to the formation of structured bone tissue.

In 1971, a particular composition of glass was discovered to have the ability to bond to bone [2]. This glass is known as Bioactive Glass 45S5. It has a composition of 45 wt% SiO_2, 6 wt% P_2O_5, 24.5 wt% CaO, and 24.5 wt% Na_2O. This "optimized" composition has been shown to promote bone growth, since exposure to a biological environment causes an apatite layer to form on the surface. After exposure to the biological environment, a complex series of reactions occur on the surface of the bioactive glass. During the entire healing process, there will be changes in the pH at the surface that will lead to the release of ions including calcium, phosphorous, and sodium [1]. Initially, there is a loss of sodium ions, Na^+, from the surface. This occurs via ion exchange with hydrogen, H^+ or H_3O^+, within minutes of exposure to bodily fluids [4]. The depletion of sodium from the surface causes a breakdown of the silica network near the surface, as the Si─O─Si bonds are broken [5]. As a result, silanol, or $Si(OH)_4$ will go into solution [4]. The silanol will then repolymerize into a silica-rich surface layer (SiO_2) [4].

After the silica-rich layer is formed, the Ca^+ and PO_4^{3-} ions migrate to the surface and form clusters of $CaO - PO_4^{3-}$ on top of the silica layer [4]. Eventually, an entire amorphous Ca-P layer is formed [4]. Finally, the amorphous Ca-P layer is recrystallized when it reacts with the OH^- and CO_3^{2-} from the body fluid. This hydroxyl-carbonate apatite layer on the surface serves as the template for bone growth [5]. Hydroxyapatite crystals are nucleated on the surface at these sites, within the oriented collagen matrix [1]. The initial hydroxyapatite

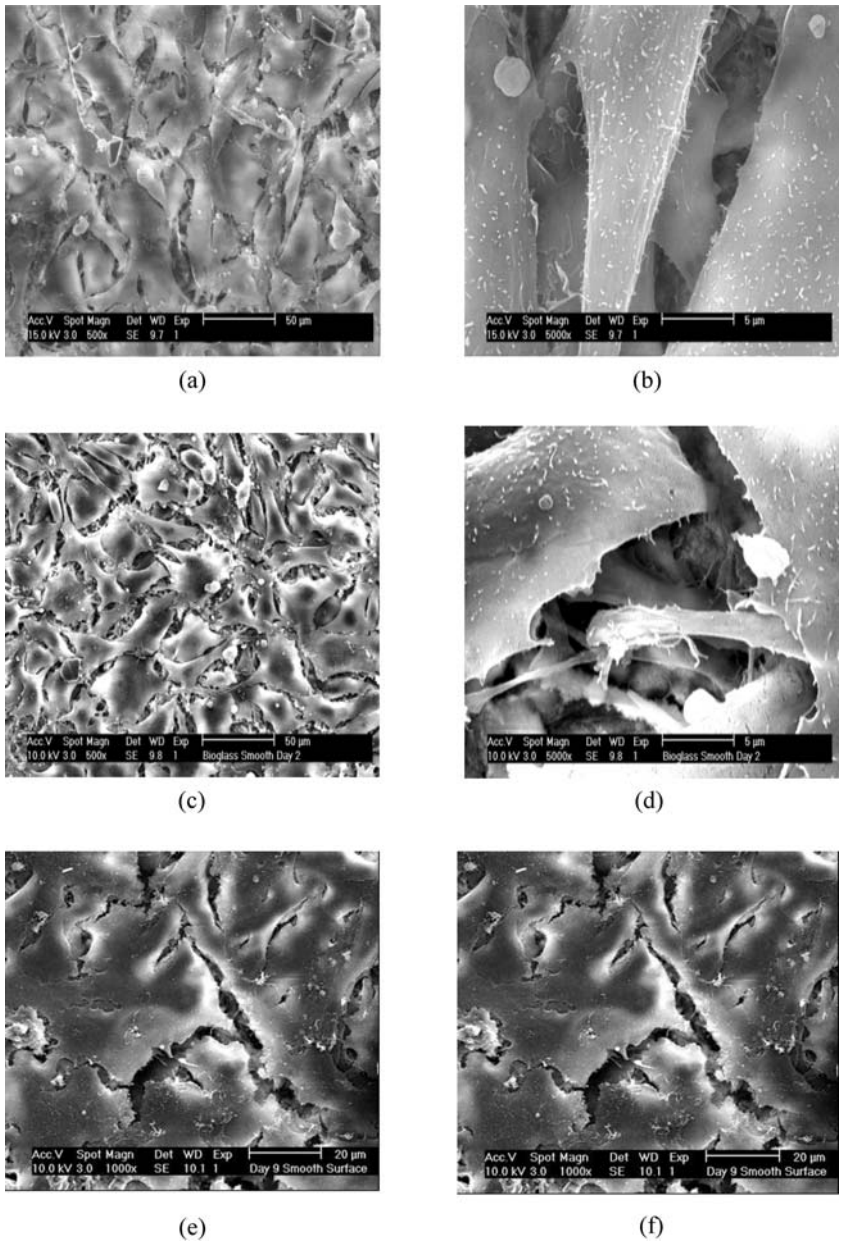

Fig. 2 Spreading and proliferation of MC3T3 cells on smooth 45S5 after: (a, b) 1 day; (c, d) 2 days; (e) 5 days, and (f) 9 days

crystallization leads to the growth of mineralized bone within a few weeks [1].

The reactions at the surface of the bioactive glass when immersed in a biological environment are time dependent. These will only last until the material has bonded with the bone. Upon immersion, the bioactive glass forms surface layers of SiO_2 and calcium and phosphate. These layers will form a protective barrier, preventing the rest of the glass from reacting with the biological fluid, and thus degrading [3]. The rate of formation of the calcium-phosphate layer must match the rate of biomineralization. If the material forms a layer at a rate that is too slow, the material will not be bioactive. If the layer is formed too quickly, the material will resorb into the body [3]. For bioactive glass 45S5, however, the rate of formation is such that the material will be bioactive with a bonding layer of 200 μm [3]. As this layer is mineralized over the period of several weeks, the interfacial bond strength will increase, while the elastic modulus of the layer also increases [3].

Thus far, most of the clinical applications for bioactive glass 45S5 as an implant are in non-load bearing and low-load bearing situations applications [4]. It has not found many applications in high load-bearing applications, despite the fact that it is a relatively hard material, with a microhardness of 8–10 GPa. Also, bioactive glass 45S5 is more than able to withstand the compressive stresses that are typical of bone

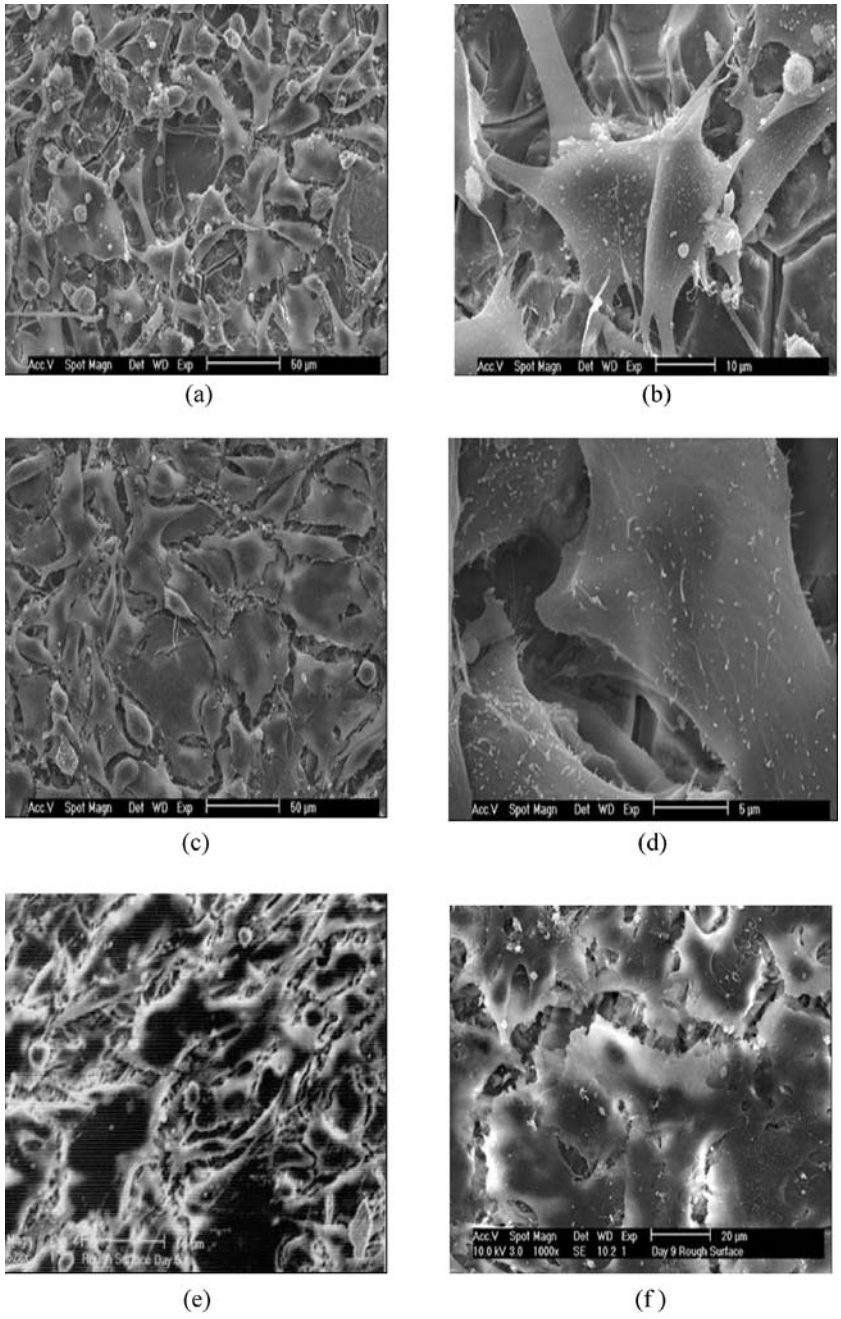

Fig. 3 Spreading and proliferation of MC3T3 cells on 45S5 after: (a, b) 1 day; (c, d) 2 days; (e) 5 days, and (f) 9 days

in the human body. Typically, compact bone has a tensile strength of 135–160 MPa, while bioactive glass 45S5 has a much greater compressive strength of 500 MPa [6]. However, bioactive glass does have several poor mechanical properties, which limit its use in high load bearing applications. These include: poor fatigue crack growth resistance, which could lead to early failure under periodic loading. Bioactive glass 45S5 is also a brittle material, with the strain to failure being only ∼0.7%. The material also has a relatively low tensile flexural strength of around 60 MPa [7].

Another problem that could occur with bioactive glass in load bearing applications is the phenomenon of stress shielding. This occurs when the Young's modulus of the implant material is much greater than that of the surrounding bone. Under such conditions, the implant supports most of the applied loads, and the surrounding bone would atrophy because the bone remodels to adjust to the reduced stress conditions. The stress shielding phenomenon occurs in bioactive glass 45S5, since it has a much higher modulus of elasticity than bone (Bioactive glass 45S5 has a Young's modulus of 78 GPa [8], while compact bone only has a Young's Modulus of 17–20 GPa [9]).

Beyond the surface chemistry and mechanical property issues, prior work on cell/surface interactions and cell/tissue

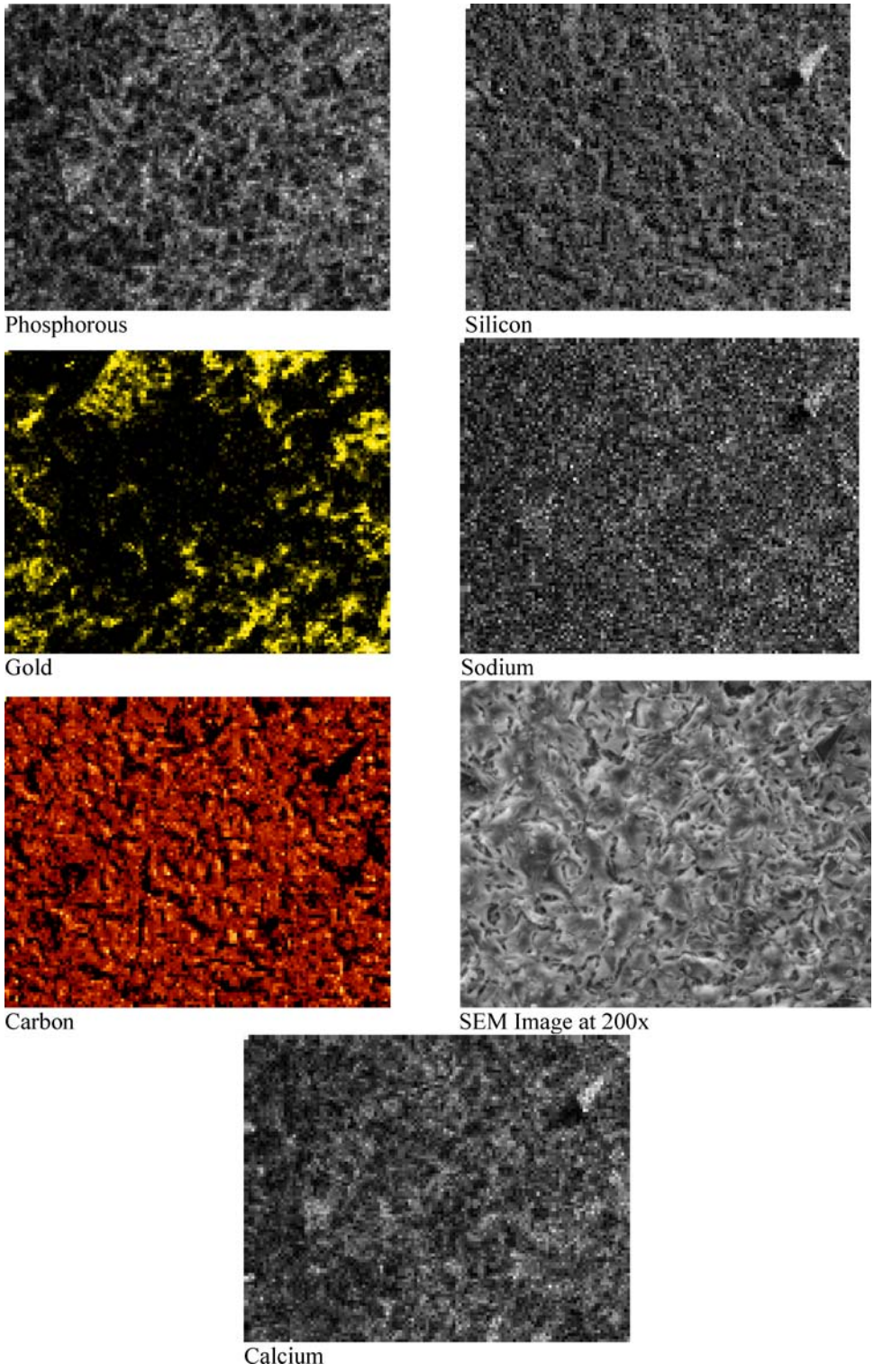

Fig. 4 EDS map of MC3T3 cells on smooth 45S5 surfaces after 1 day culture

interactions has shown that the adhesion of implants is significantly affected by the surface texture of the implants. For example, rough surface textures, produced by surface blasting techniques, have been shown to significantly improve cell adhesion to biomedical surfaces [10, 11]. However, these also restrict cell spreading, and may lead to scar formation due to random cell orientations that act as a template for scar tissue formation.

For various biomaterials other than bioactive glass, it has been shown *in vivo* that there is an increased attachment of

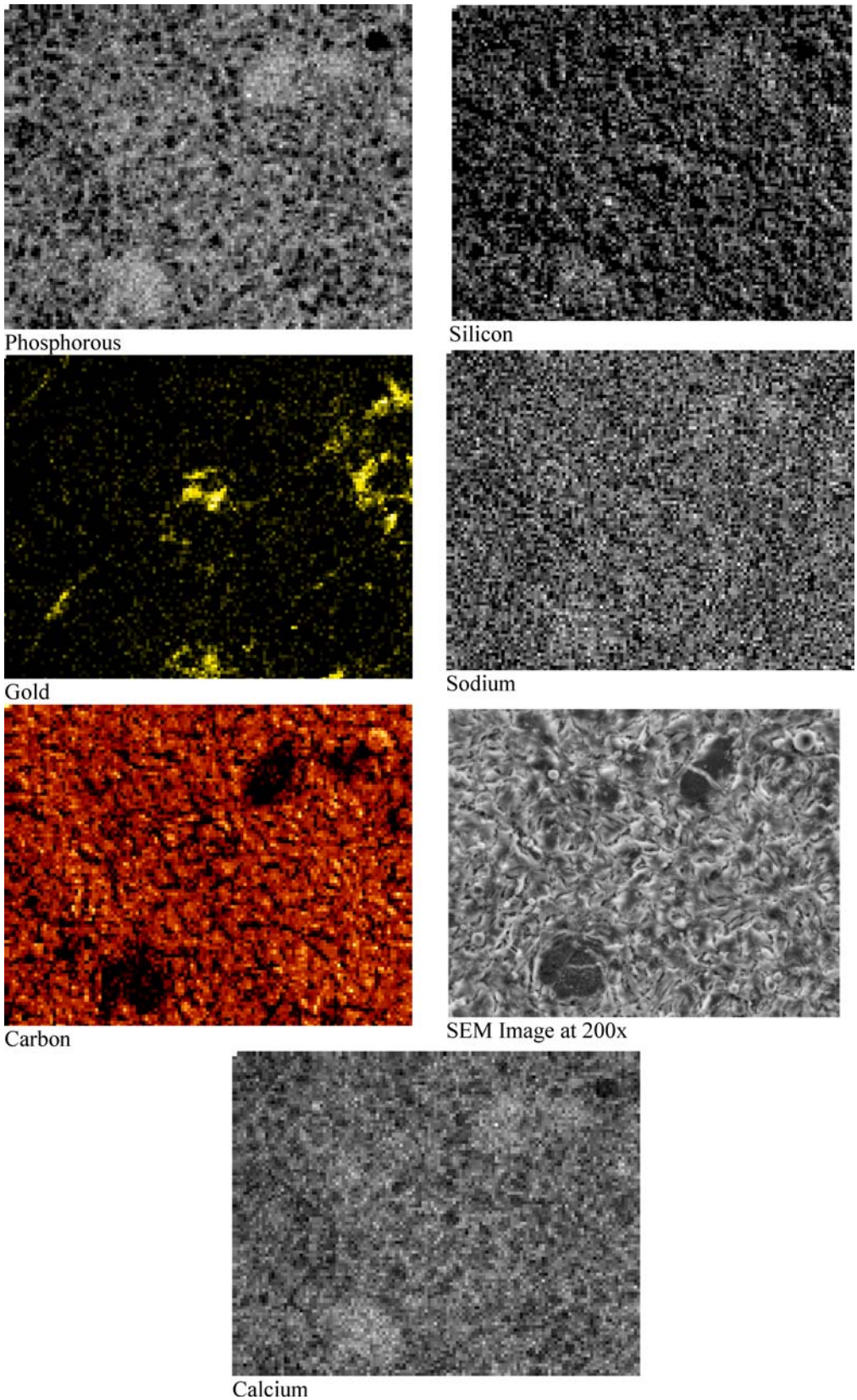

Fig. 5 EDS map pf MC3T3 cells on smooth 45S5 surfaces after 2 days culture

tissue to implants with surface features as opposed to smooth surfaces. There are several possible reasons why there is increased attachment to surfaces with features. On a smooth surface, the cells will spread fairly easily, since there is no physical barrier to impede their motion. Thus, soft tissue cells, which spread at a faster rate than bone cells, forming tissue as early as the blood clot formation stage [10], would have encapsulated the substrate by the time the osteoblasts had the opportunity to attach.

However, on textured surfaces, i.e. cells with features, cells adhere to the surfaces at a slower rate, as the cells must navigate around the surface features. This lower rate of adhesion allows the osteoblasts time to attach since bone formation does not occur until a later point in the healing process, during the proliferative phase [12]. Furthermore, texturing the surface works to increase integration, since there is an increase in clot adhesion and wound contracture, because cells and proteins adhere better to textured or rough surfaces [12]. Another explanation for the increased adhesion to surfaces with features is that there might be mechanical attachment and ingrowth into the pores or grooves of the surface. Also, with the addition of surface features, there is an increase in the surface area that is exposed to the cells, thus it might be possible for more cells to attach.

Although the potential benefits of surface texturing have been studied carefully in several biomedical materials, these authors are unaware of prior studies of the effects of surface texture on cell/surface interactions to bioactive glasses such as 45S5. There is, therefore, a need for studies of the cell/surface interactions to bioactive glasses. In this study, we compare the cell/surface interactions of rat osteoblasts (MC-3T3) cells to smooth and rough surfaces of bioactive glass 45S5. The paper is divided into 5 sections. In Section 2, the bioactive 45S5 materials are described along surface topography and microchemistry of the smooth and textured surfaces. This is followed by Section 3 in which the experimental techniques are described. The results and discussion are presented in Section 4, before summarizing the salient conclusions in Section 5.

2 Materials

2.1 Materials processing and surface topography

The 2 mm diameter disk samples that were used in this study were obtained from U.S. Biomaterials Corp. Alchua, FL. The bioactive glass 45S5 consists of 45 wt% SiO_2, 6 wt% P_2O_5, 24.5 wt% CaO, and 24.5 wt% Na_2O. The 45S5 is a melt-derived solid that was derived from fine-grained particulate solids that were then heated to temperatures above the melting point [3]. As the mixture is subsequently cooled, the SiO_2 forms a network, and the oxides (P_2O_5, CaO and Na_2O) function as network modifiers [3]. The disks provided by US Biomaterials had one polished smooth side, while the opposite side of the sample contained was a rough surface. The rough surface was formed by a sandblasting technique, in which air at high velocity carrying small particles (i.e. sand) passed across the surface of the disks to form the region of roughened surface.

The differences in the surface morphologies were characterized with a Dimension 3100 atomic force microscope (Veeco, Santa Barbara, CA) that was operated in the tapping mode. Atomic force microscopy (AFM) images of the smooth and rough surfaces are presented in Figs. 1(a) and (b). The smooth surfaces had a root-mean-squared (r.m.s.) roughness of $\sim$71 nm compared to r.m.s. roughness values of $\sim$794 nm in the case of the smooth samples.

3 Experimental procedures

Cells were cultured on smooth and rough surfaces, in order to observe the effects of surface morphology on cell spreading and proliferation on each of these surfaces. In all cases, 1 cm square Bioglass® samples (US Biomaterials Corp. Alchua, FL) were used. MC-3T3 cells obtained from The University of Pittsburgh were used in the experiments. These are mouse calvarian cells, or, undifferentiated, precursor cells to mouse osteoblasts. As a cell differentiates, it will become more specialized for a specific function within the system, while at the same time losing the ability to perform other functions [13]. These changes in the cells are usually irreversible [13]. Since the cells have yet to differentiate, this line of cells is often used in experiments, since the experimenter is able to observe which markers react with the substrate and how the cells differentiate.

To prepare the samples for cell culture, they were cleaned in a bath of acetone for 15 min and then sterilized in dry heat for 1 h in a furnace that was heated to 110°C. The samples were then stored in pure ethanol, cell culturing overnight in medium consisting of Dulbecco's Minimum Essential Medium (DMEM). The solution also contained 10% Fetal Bovine Serum (FBS, Quality Biological) 1% Penicillin/Streptomycin (10^4 units/ml penicillin, 10^3 units/ml streptomycin, Quality Biological), and 1% Amphotericin B (250 μg/ml, Quality Biological). The surfaces needed to be conditioned since initially, there is a large release of Na^+ ions from the glass surface. Since these ions react with H^+, there is initially a rapid change in pH [14]. This can disrupt the cells. Also, the conditioning will aid in the effectiveness of the calcium phosphate and hydroxycarbonate appetite-rich layers, since they will already be present when the cells come into contact with the surface [13].

A working solution of 2.5% trypsin and .25% PBS was used to split and detach cells from the surface of the T-flasks

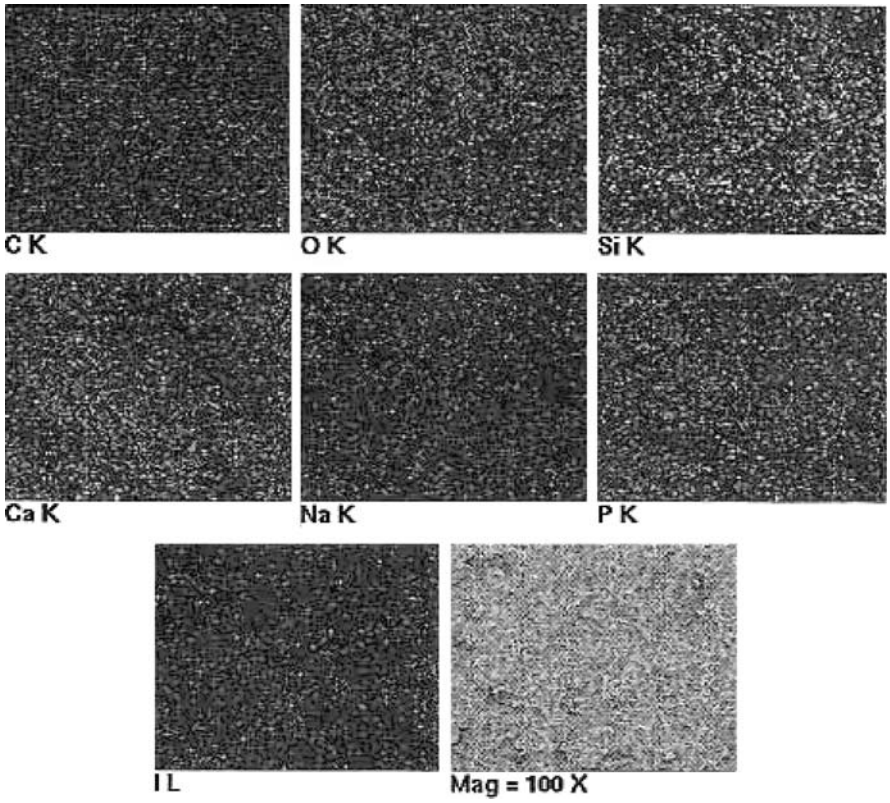

Fig. 6 EDS map of MC3T3 cells on 45S5 smooth surfaces after 5 days culture

they were cultured in. Once the cells were split, they could either be re-cultured or used for various experiments, such as seeding on the bioactive glass 45S5 surfaces. The resuspension of cells was split and seeded among the various surfaces for culture.

The cells were cultured on the surfaces for 1, 2, 5 and 9 days. They were placed in an incubator at 37°C during the culture period. After the culture period, the samples were removed from the incubator and fixed overnight using gluteraldehyde. The cells were then dehydrated through a step-wise dehydration procedure in ethanol. The samples were further dried using the method of critical point drying.

The samples were then sputter coated with gold so that they could be examined under the Scanning Electron Microscope (SEM). The sputter coating procedure coats the samples with a layer of metal atoms several nanometers thick. This is necessary to avoid a charge build up on the sample in the SEM. Under the SEM, the morphology of the cells on samples from days 1, 2, 5, and 9 were examined, along with evidence of cell spreading and proliferation.

4 Results and discussion

4.1 Cell culture on smooth surfaces

After one day of cell culture on smooth surfaces, the MC3T3 cells appeared to be fairly confluent, covering the entire surface of the sample (Figs. 2(a) and (b)). The cells appeared to be fairly large and spread out over the surface, thus indicating that they had attached and spread. Multiple layers of cells were observed in the higher magnification images (Fig. 2(b)). The rapid proliferation can be attributed to the fact that the surface was smooth and there were relatively small surface features impeding the spreading of the cells. The cells were not aligned in any particular direction, and they had pseudopodia directed in all orientations. The lack of alignment can be explained since there are no surface features—on the order of the size of the cell—for the cell to align with.

Similar results were observed in the 2 day culture samples (Figs. 2(c) and (d)). Once again, the cells appeared spread out and well attached to the surface. Several layers of cells were observed, and the cell orientations were random. The smooth Day 5 sample also showed that the cells were relatively spread out (Fig. 2(c)). On average, the cells were larger than those in the Day 1 and Day 2 samples, indicating that the cells continued to spread, even after the initial two days. Fewer pseudopodia were visible, as the cytoplasm had filled in the area in between the pseudopodia, covering the entire area of the surface. The cells appeared to spread until they impinged on the neighboring/adjacent cells.

After nine days of cell culture, the cells were even further spread out than on the 1, 2 and 5-day samples (Fig. 2(f)). There was less space visible between the cells than in the previous samples. Also, due to the continued spreading of

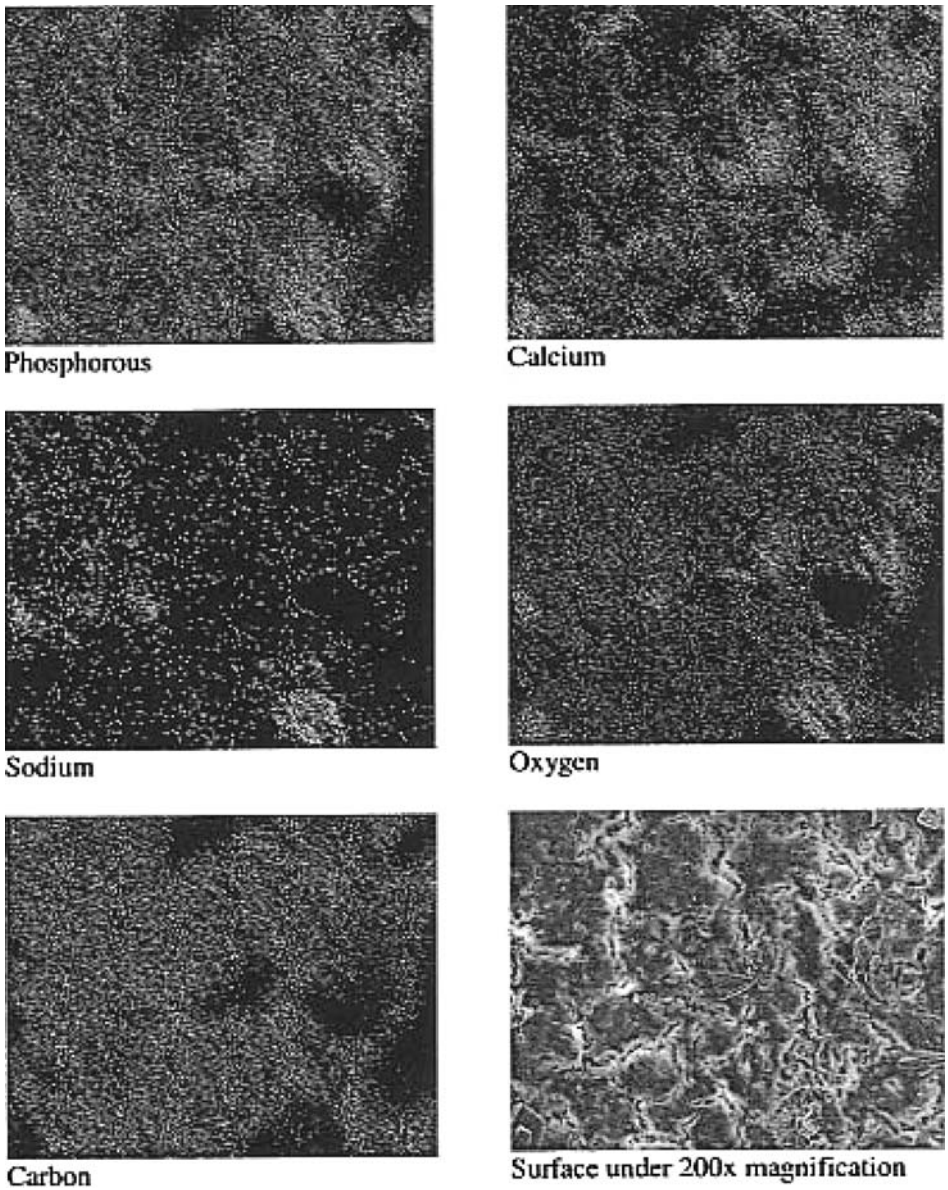

Fig. 7 EDS map of MC3T3 cells on 45S5 smooth surfaces after 5 days culture

the cells, the layers of cells underneath the top layer were not visible. Again, the cells spread until they reached the adjacent cells and the interfaces between the cells were randomly oriented.

4.2 Cell culture on rough surfaces

In the case of the rough surfaces, the cells were not confluent after 1 day of cell culture (Figs. 3(a) and (b)). The surface features inhibited spreading of the cells. Additionally, the cells appeared smaller in size and more rounded in shape. The cells had not spread out as much nor had they attached to the surface to the extent that the cells on the smooth surface had attached on Day 1.

After two days of cell culture, the cells appeared to be more spread out and better attached to the surface than the Rough Day 1 sample (Figs. 3(c) and (d)). However, there were still a few cells that appeared round and unattached. The process of spreading and attachment was not as far advanced as that in the smooth sample. When implanted into the body, this slower rate of attachment could have the effect that it allows for the attachment of the osteoblasts before the fibroblasts form a capsule around the surface. Overall, the cells were confluent, covering the entire surface of the sample. However, pseudopodia could still be seen, which indicated that the cells had not completed the spreading process. Multiple layers of cells were also observed. As with the Rough Day 1 sample, there was no evidence of contact guidance.

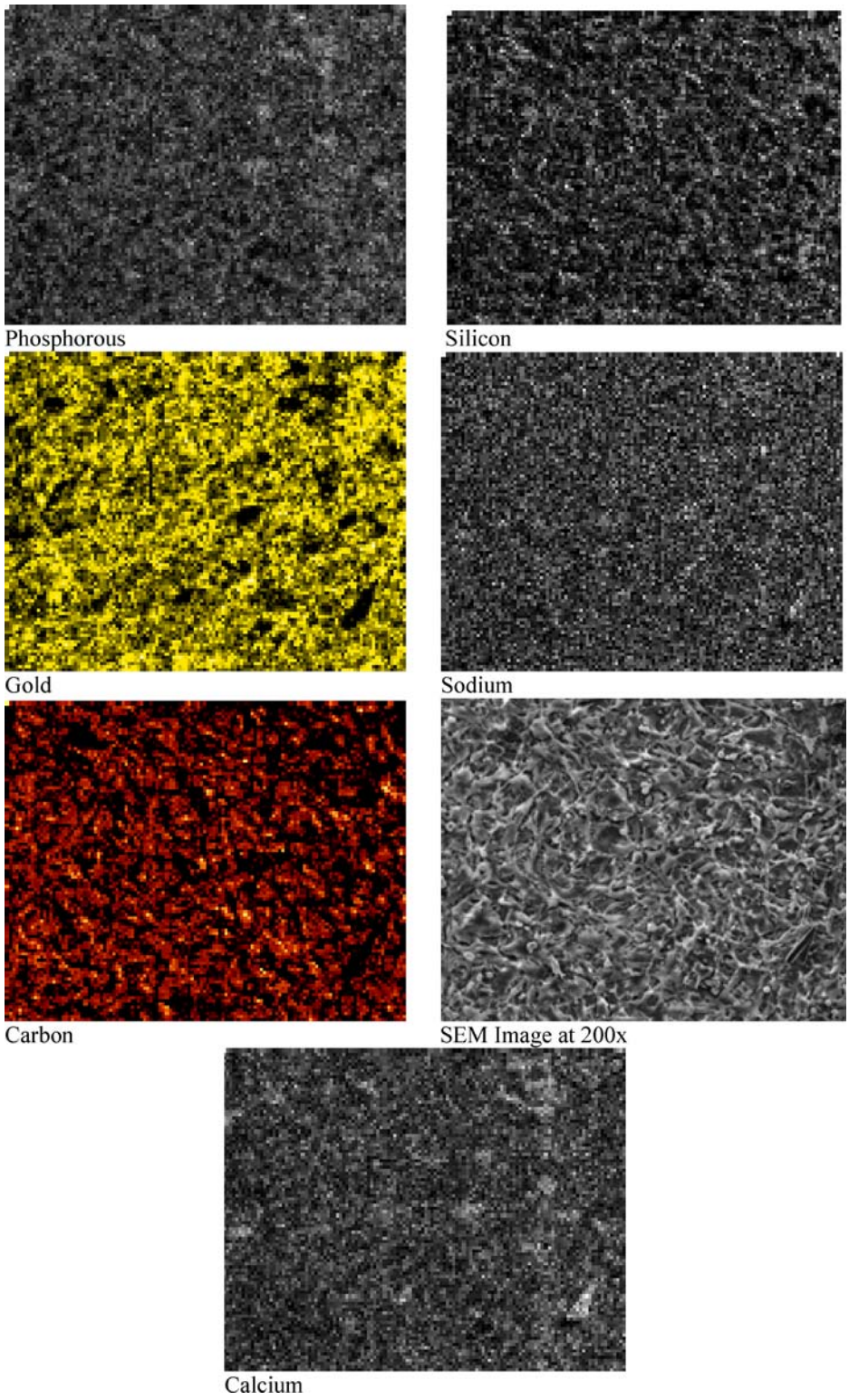

Fig. 8 EDS map of MC3T3 cells on 45S5 rough surfaces after 1 day culture

On the Rough Day 5 sample (Fig. 3(e)), the cells were well spread out and attached. This indicates that even after the initially slower rate of adhesion to the rough surface, eventually the cells do attach and form a bond with the surface. Similar to the observations made for Fig. 3(f), the cells appeared to be much larger and flatter than on the earlier days (Figs. 3(a)–(f)). Once again, the layers of cells underneath the top layer could not be observed. This was due to the large size of the

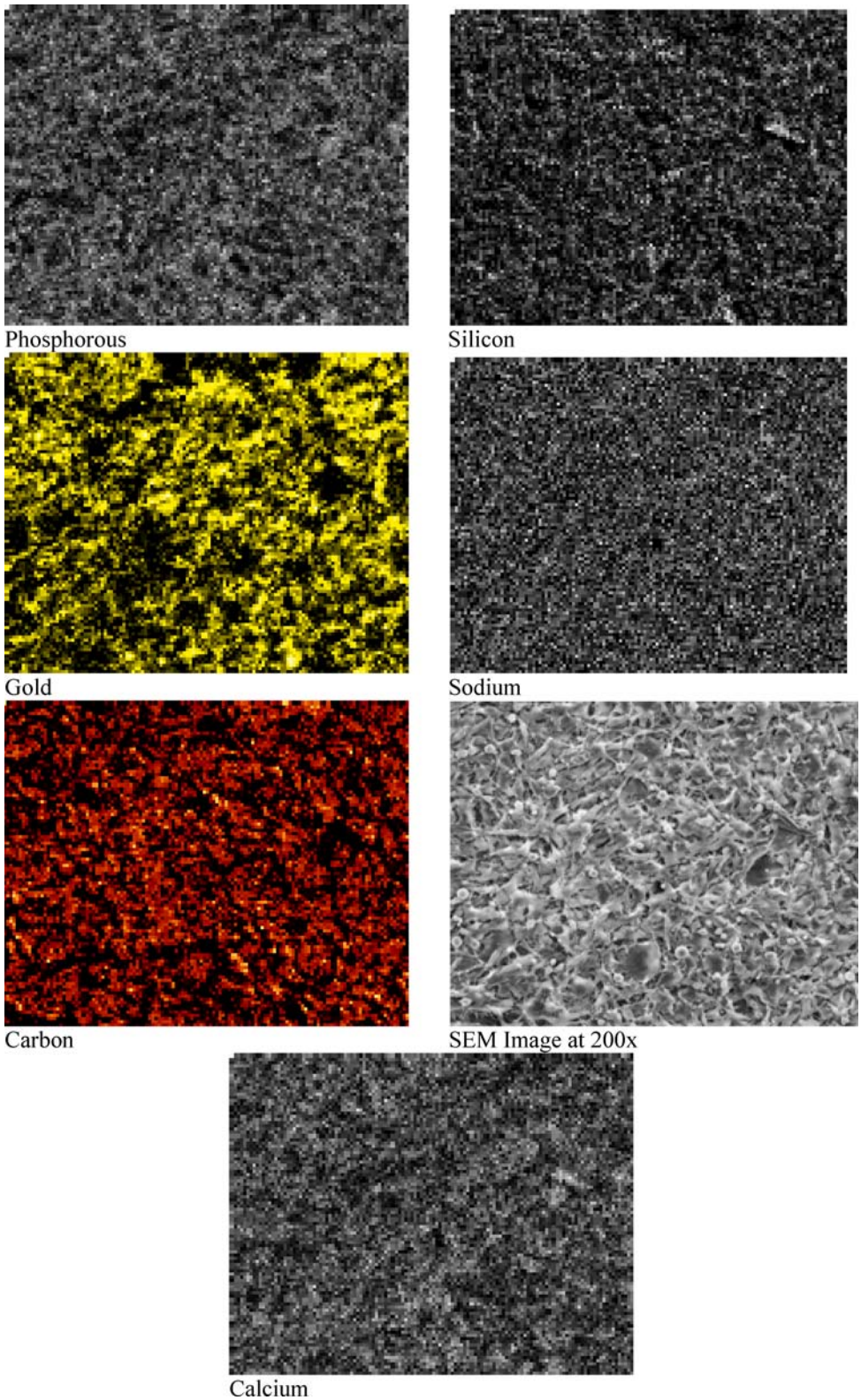

Fig. 9 EDS map of MC3T3 cells on 45S5 rough surfaces after 2 day culture

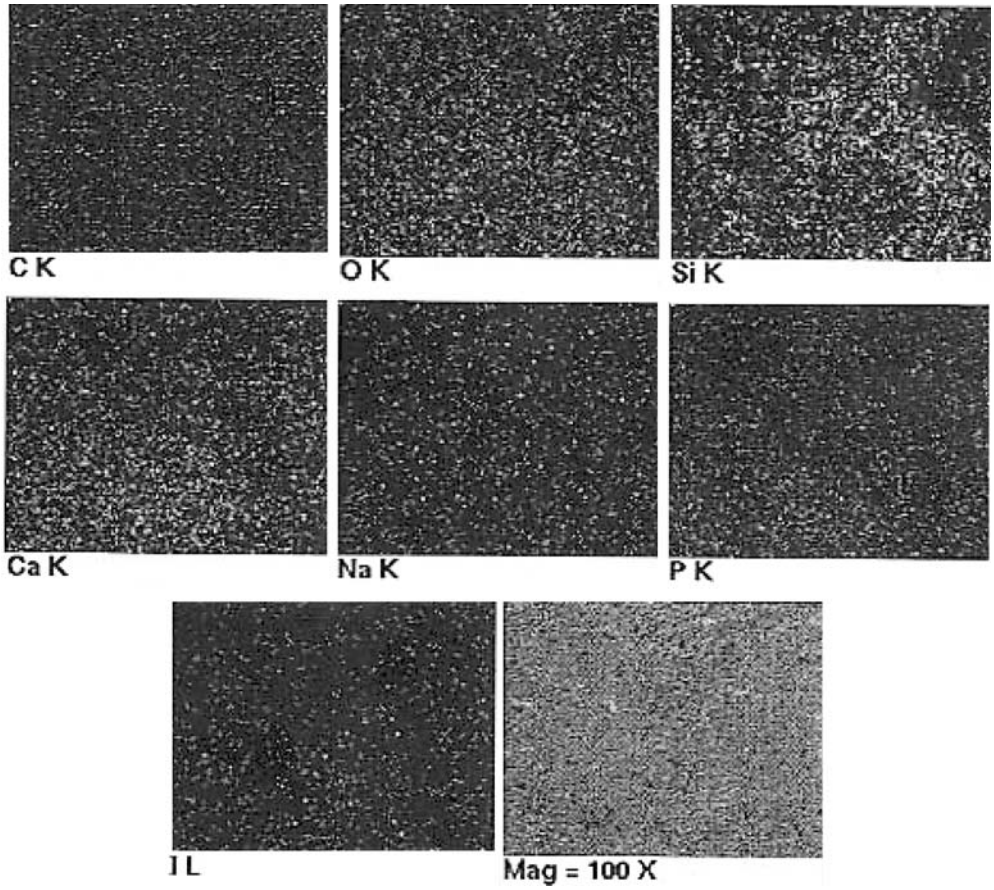

Fig. 10 EDS map of MC3T3 cells on 45S5 rough surfaces after 5 days culture

cells on the top. It appeared that the cells had continued to spread after the first several days, up to the point where they reached the neighboring cells. There were thus few pseudopodia observed on the surface since the cells had spread to their full area.

4.3 Comparisons of cells on rough and smooth surfaces

The results from the studies of cell/surface interactions on rough and smooth surfaces show that, initially, there is a tendency for the cells to grow more slowly on the rough surface than on the smooth surface. Eventually, cells attach to the rough surface and begin to cover it entirely. The proliferation on the rough and smooth surfaces seems to even out by day 9. On both surfaces, even though the process of spreading continues after the initial days, the cells were completely spread out by day 9 (Figs. 2 and 3).

4.4 Chemical modifications between MC3T3 cells and bioactive glass

Bioactive glass is reactive in the biological environment. Hence, over time, the silicon will be depleted from the material, as it is absorbed by the biological fluids and cells. The other metal ions will also be depleted from the surface and absorbed by the cells. An EDS (Energy Dispersive X-ray Spectroscopy) analysis was used to characterize the relative concentration of each element, with the lighter areas indicating a higher concentration of the species. This method is only semi-quantitative, showing only relative concentrations.

Typical EDS maps that were obtained from the smooth and rough samples are presented in Figs. 4–11. In the areas of the image where cells were present, there were light spots in the carbon and maps. These were used to identify the regions of cell attachment, since cells contain large amounts of carbon and oxygen. In the areas where cells were not present, the EDS results for the Day 1 and 2 samples show that there was a greater concentration of the phosphorous, calcium, and sodium ions (Figs. 4, 5, 8, 9). The clearest example is the smooth Day 2 sample where there are two large areas without cells where the three elemental maps of the metals show lighter spots (Fig. 5). This change in the concentrations could be due to the fact that, when immersed in the biological environment, the surface releases many ions. Also, the presence of these ions is masked by the presence of the cells.

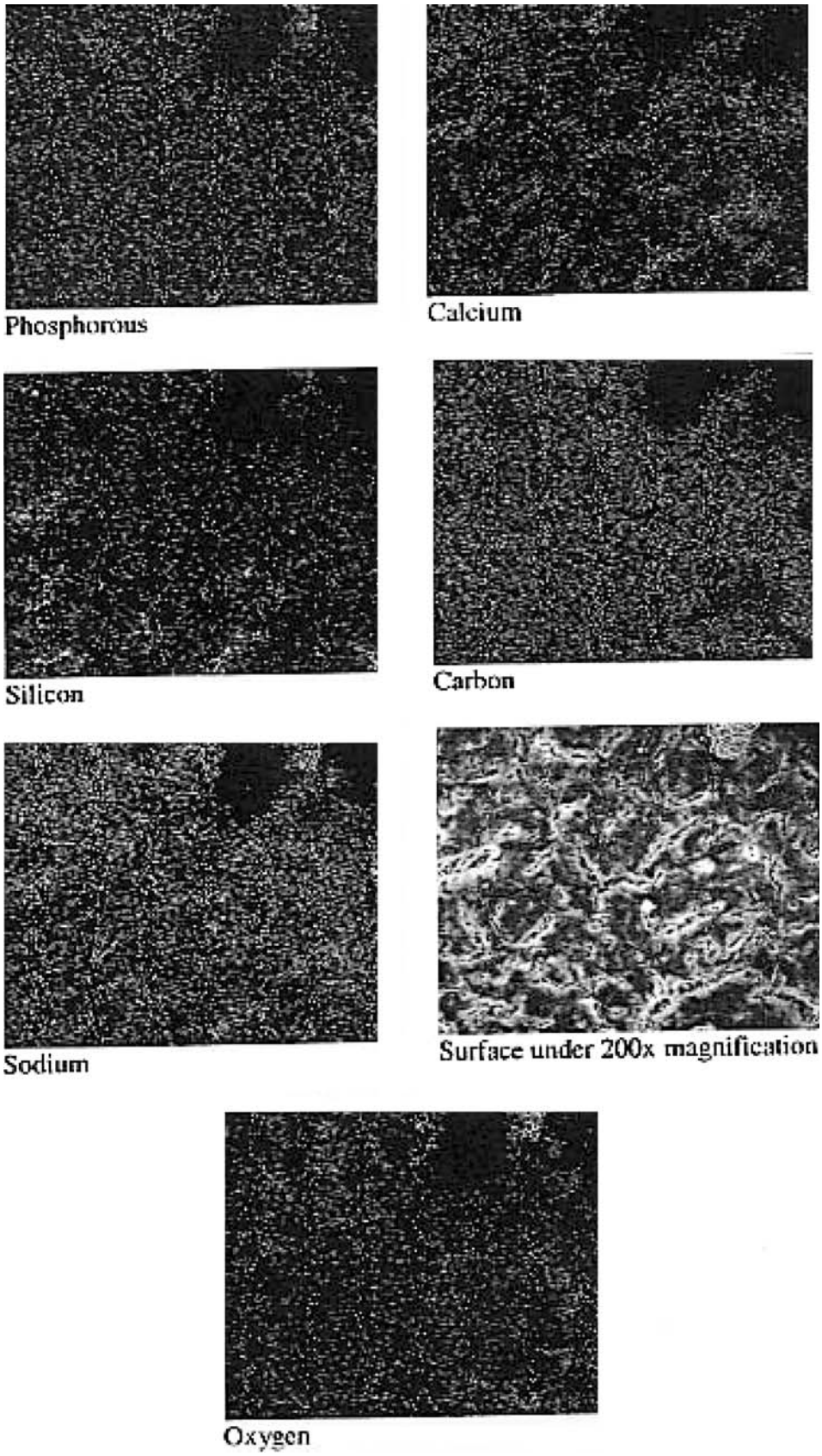

Fig. 11 EDS map of MC3T3 cells on 45S5 rough surfaces after 9 days culture

However, for the Day 5 and 9 samples, this trend was reversed (Figs. 6, 7, 10, 11). There are greater concentrations of the ions phosphorous, calcium and sodium in areas where the cells were present. This indicates that the cells have absorbed each of these species, making the relative concentration of the species greater within the cells. It thus appears that it takes several days before the cells absorb the ions.

The depletion of silicon was observed clearly in the EDS results (Figs. 4–11). For both the rough and the smooth surfaces, there is an appreciable amount of silicon on the Day 1 samples (Figs. 4 and 8). The Day 2 samples showed a significant decrease in the amount of silicon that was present (Figs. 5 and 9). It seems that the silicon was released from the surface, as the sample remained in the medium. It also appears that the cells did not absorb the silicon, since there was no correlation between the presence of cells, and the presence of silicon.

5 Summary and concluding remarks

This paper presents the results of an experimental study of cell/surface interactions between smooth/rough bioactive glass of 45S5 and MC3T3 cells. The salient conclusions arising from this study are summarized below.

1. The MC3T3 cells interact chemically with the bioactive glass surfaces. Following a release of P, Na, and Ca from the cells, during the first few days (days 1 and 2) of cell culture, the cells appear to absorb the same chemical species during the final stages (days 5 and 9) of cell spreading.
2. Since there was no appreciable amount of silicon in the regions containing the cells, the current work suggests that the silicon released from the surfaces of the bioactive glass 45S5 was not absorbed by the cells during cell spreading for up to nine days.
3. The extent of cell spreading is slower on rough 45S5 surfaces during the initial stages of cell adhesion (days 1–9). Also, the topographical features on the rough surfaces provide sites for cell (focal point) attachment. In contrast, the extent of cell spreading is greater on the smooth surfaces. However, cell spreading is completed within 9 days on both the smooth and rough surfaces of 45S5.

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