JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 9 (1998) 503–508

Interaction between self-assembled protein vesicles and microporous apatite surface

M. SHIRKHANZADEH
Department of Materials and Metallurgical Engineering, Queen's University, Kingston, Ontario, Canada, K7L 3N6

Self-assembled structures such as vesicles have generated immense interest in recent decades due to their potential in mimicking biological membranes and in acting as drug-delivery systems. Despite the importance of the interaction between these organized assemblies and the surface of biomaterials, little is known about the mechanism involved. In this study, the interaction between giant proline-rich mussel adhesive protein (MAP) vesicles and the microporous apatite surface was investigated by scanning electron microscopy (SEM). We have found that MAP vesicles incubated on the apatite surface similar to osteoclasts, induce site-specific resorption of the apatite surface. However, in contrast to the osteoclastic resorption, the vesicle-induced resorption process appears to be accompanied by an organic matrix-mediated remineralization process. This results in the formation of a variety of complex three-dimensional site-specific ''remodelled zones'' on the apatite surface of micrometre scale. The mechanism of the formation of ''remodelled zones'' is discussed in terms of surface phenomena, such as adsorption and deformation of vesicles, site-specific release of resorptive agents, organic matrix-directed remineralization, and Ca-induced fusion, collapse and reshaping of the vesicles on the apatite surface.

1. Introduction

Amphiphilic molecules, such as surfactants, synthetic polymers and proteins, are known to self-assemble into a variety of aggregate shapes in aqueous solutions, including spherical micelles, vesicles and planar bilayers [1]. Much of the growing interest in self-assembling structures arises from the close association of this process with living systems [2–5]. Vesicles in particular, have generated immense interest due to their potential in mimicking biological membranes and in acting as drug-delivery systems for therapeutic applications such as cancer chemotherapy [6]. In this context, the study of the interaction between self-assembled vesicles and natural and synthetic tissues is of significant importance. The understanding of adhesion, reshaping and rupture of vesicles, which may lead to the release of therapeutic agents, is especially crucial for developing drug-containing vesicles that can effectively interact with specific target tissues.

Synthetic hydroxyapatite (HA) and particularly the non-stoichiometric forms of this compound are chemically similar to the natural hard tissues. Yamada *et al.* [7] have demonstrated that microporous apatite layers formed on glass ceramics exhibit bone-like properties and can be used as bone substitutes for assaying the resorptive behaviour of osteoclasts in cell cultures. Unlike sintered apatite and calcite surfaces previously used as artificial substrates [8], the resorption lacunae formed on microporous apatite layers were shown to exhibit a track-like appearance similar to those observed on natural calcified tissue [9, 10] suggesting that osteoclasts were actively resorbing and migrating.

We have previously reported an electrochemical process for fabricating similar microporous apatite layers on conductive substrates [11]. It was also reported that transparent apatite layers can be electrodeposited on glass substrates pre-coated with an electrically conductive film of indium tin oxide (ITO) [12]. Such transparent microporous apatite layers may be useful as artificial substrates for observing the resorptive patterns of bone cells, using conventional light transmission microscopes. During the course of study, it has been observed that when self-assembled protein aggregates derived from the marine mussel *Mytilus edulis* are incubated on these substrates, they induce site-specific remodelling of the apatite surface, which lead to the formation of highly complex mineral structures of micrometre scale. In view of the significance of the interaction between self-assembled aggregates and bone-like apatite this phenomenon has been examined in more detail.

2. Experimental procedure

Microporous apatite was electrodeposited on glass slides (2×3 cm) pre-coated with a thin film of electrically conductive ITO. The electrolyte used for the

0957–4530

electrodeposition of apatite layer was made by mixing 1 l 0.042 M $Ca(NO_3)_2$ and 1 l 0.025 M $NH_4H_2PO_4$ solutions. The solutions were prepared with reagent grade chemicals and deionized water. The pH of the electrolyte measured at 25 °C was 4.4. The electrodeposition of apatite was carried out for 30 min at 65 ± 1 °C in a conventional electrolytic cell fitted with a saturated calomel electrode (SCE) acting as a reference electrode, and two graphite rods acting as counter electrodes. The ITO-coated glass slide was used as the cathode of the cell. A Hokuto Denko (HD) HAB-151 potentiostat–galvanostat operating in potentiostatic mode was employed to maintain the cathode potential at $E_c = -1400$ mV (vs. SCE).

Purified mussel adhesive protein (MAP) dissolved in 5 vol% acetic acid solution (commercially known as Cell-Tak) was obtained (Becton Dickinson Labware) and stored at 4 °C. This protein is a formulation of polyphenolic proteins extracted from the marine mussel *Mytilus edulis* [13]. This family of related proteins is the key component of the adhesive secreted by the mussel to anchor itself to solid structures in its natural environment. The concentration of protein in the stock solution was 1.95 mg ml^{-1}. Micellization of MAP was initiated by neutralizing the acidic stock solution with a 0.1 M sodium bicarbonate ($NaHCO_3$, pH = 8.1) solution in a dilution tube. The final concentration of the protein was 26 μg ml^{-1}. Some 300 μl aliquots of this solution were placed on a small area (1 cm^2) of the apatite-coated glass slides defined by a plastic frame that was fixed to the slides. After incubation for 1 h at 37 °C, the apatite-coated glass slides were rinsed of any residual protein using distilled water. Scanning Electron Microscopy (SEM) was employed to examine the surface morphological changes induced by the self-assembled aggregates adsorbed on the apatite surface.

3. Results and discussion

3.1. Characterization of the apatite substrate prior to interaction with MAP assemblies

Fig. 1 shows the SEM micrograph of the microporous apatite layer electrodeposited on the ITO-coated glass slide. The apatite layer (~10 μm thick) consists of an interlocking network of plate-like crystals in the range 2–3 μm. The Fourier transform infrared (FTIR) spectrum of the apatite layer reported earlier [14] has shown, in addition to bands associated with HA (1100–1032, 962, 632, 3570 cm^{-1}), bands attributed to octacalcium phosphate (OCP) at 525 and 865–900, 1200 and 1280 cm^{-1}. The appearance of both OCP and HA bands may signify the presence of OCP–apatite interlayering similar to bone apatite.

3.2. Interaction of MAP assemblies with the apatite surface

The polyphenolic adhesive protein of the marine mussel *Mytilus edulis* is a protein containing large amounts of hydroxyproline (13%, 130 residues per 1000) and 3,4-dihydroxyphenylamin (dopa 11%, 110 residues per 1000) [13]. Waite and Tanzer [15] have reported that prior to polymerization the polyphenolic protein consists of a rather large polypeptide chain (relative molecular weight $M_r = 130\,000$) in which seven amino acids, lysine, hydroxyproline, alanine, serine, threonine, tyrosine, and dopa account for about 80% of all residues. It would be expected, therefore, that similar to other amphiphilic proline-rich proteins, such as human salivary proteins [16], MAP may be highly asymmetric and may exhibit both hydrophobic and hydrophilic domains, and thus may form various micelle-like assemblies in aqueous solutions above the critical micelle concentration (CMC). While the study of aggregation of amphiphilic molecules in solution is in general well established [1], the study of the interaction between these assemblies and solid surfaces is relatively new [17–19]. In particular, the study of the interaction between self-assembled structures and reactive materials such as apatite is non-existent.

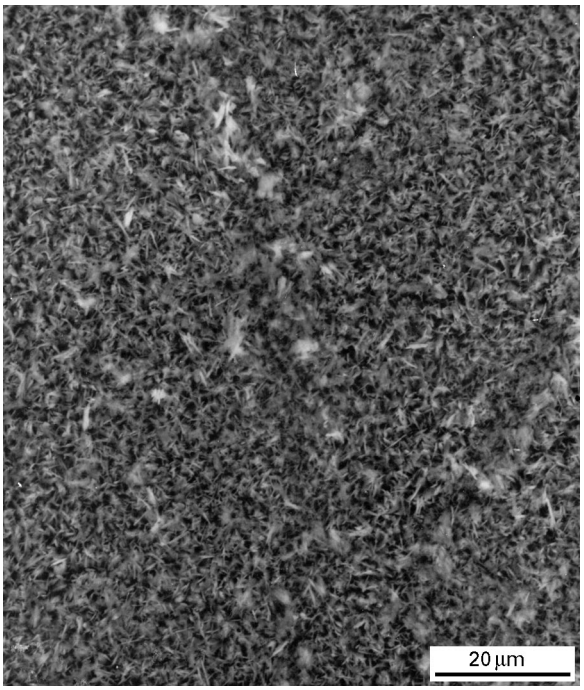

Figure 1 SEM photomicrograph of the apatite layer electrochemically deposited on the ITO-coated glass slide. The apatite layer shows plate-like crystals uniformly distributed on the glass slide.

In the present work, SEM observation of microporous apatite substrates that have interacted with MAP provided evidence for the formation of highly energetic self-assembled aggregates, which resulted in site-specific remodelling of the surface of the apatite. These observations have revealed a variety of "remodelled zones" including circular depressions and highly ordered structures, of micrometre scale, apparently at various stages of their development (Figs 2–7). Perhaps the most noticeable feature of the "remodelled zones" is that their microstructure is very similar to the microstructure of biological skeleton and synthetic organic–inorganic apatite materials prepared in the presence of polypeptides [20] (see, for example, Fig. 3). Such materials are often characterized by a fine microstructure in which organic molecules are intimately

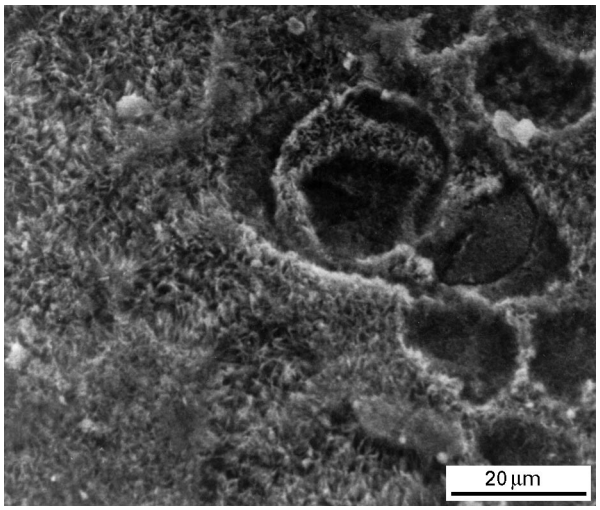

Figure 2 Surface features appear as overlapping rings may indicate involvement of giant spherical vesicles in the resorption and remineralization process. Note the lateral expansion of the remodelled zone beyond the original site of one vesicle (centre right), which may have occurred as a result of the leakage of the vesicle's contents.

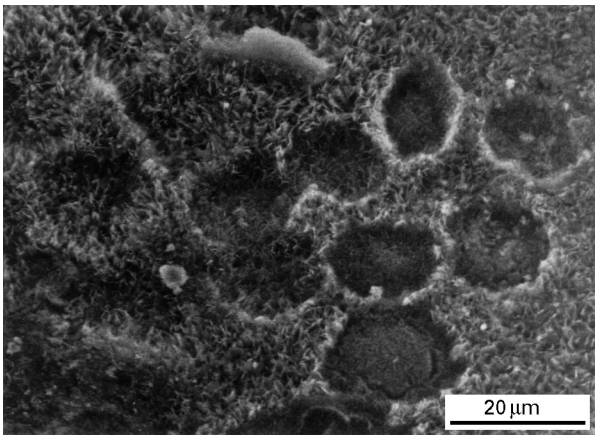

Figure 4 Extensive dissolution and excavation of the apatite surface made by fusion of three giant vesicles (centre). Note also the remodelled zone at the lower edge of the micrograph characterized by newly formed fine crystals distinguishable from the original apatite crystals. Lateral expansion of the remodelled zone due to vesicle leakage is also apparent (lower edge). Shallow bowl-shaped domains (centre left) characterized by the appearance of relatively large and irregular crystals may signify site-specific resorption at early stages during the remodelling process.

Figure 3 "Remodelled zones" made on the apatite surface by MAP self-assembled aggregates at various stages of their development. Less developed surface features (top right), characterized by an irregular pattern of broken and fused crystals, may signify site-specific resorption of the apatite surface at early stages. More developed zones (centre right) appear to consist of newly formed fine crystal that are clumped together presumably by MAP molecules. More elaborate structures, assuming polyhedral outlines, are also noted that may have formed as a result of flattening and lateral expansion of vesicles at a more advanced stage.

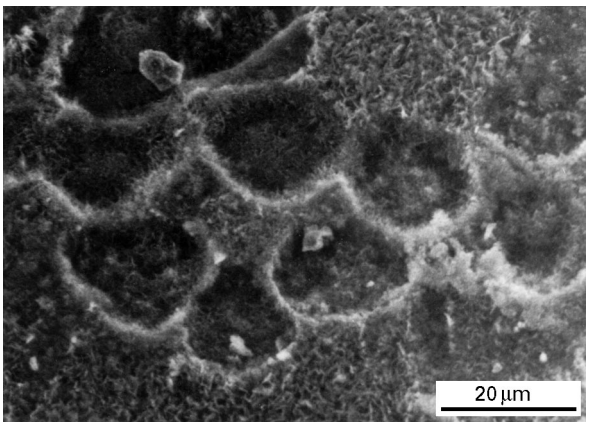

Figure 5 "Remodelled zones" made by giant vesicles in close contact with each other. Note the remodelled zone at the lower edge of the micrograph that is apparently made by a spherical vesicle squeezed and deformed by two other vesicles.

associated with the mineral phase. We propose that such ordered "remodelled zones" are formed through an "organic matrix-mediated" remodelling process by direct action of the self-organized MAP assemblies adsorbed on the surface of the apatite. It is interesting to note that the surface of the apatite layer outside the "remodelled zones" often remained undisturbed. Self-assembled MAP aggregates settled onto the apatite substrate appear to be solely responsible for the remodelling of the apatite surface.

It is possible that upon adsorption on the apatite surface, MAP assemblies may form a flat contact area with the apatite surface and function as both a source of agents for the resorption of apatite and templates for directing the remineralization process and defining the "remodelled zones". Indeed, the clear outlines of most remodelled zones indicate that organized MAP assemblies, similar to osteoclasts [21], may be able to make a tight seal at the periphery of their contact with the apatite surface, making a closed compartment. This apparently would allow the maintenance of a microenvironment favourable to a local concentration of organic resorbing agents required for the dissolution of apatite crystals.

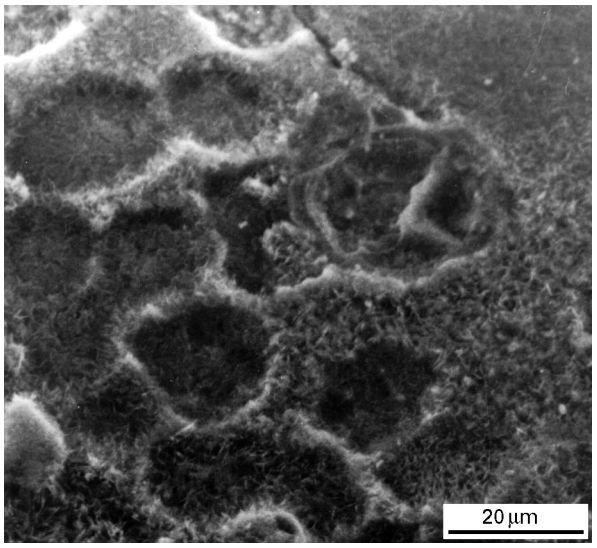

Figure 6 Expansion of the remodelled area caused by the excessive leakage and spreading of a bilayer subsequent to the rupture of one vesicle on the apatite surface (top right).

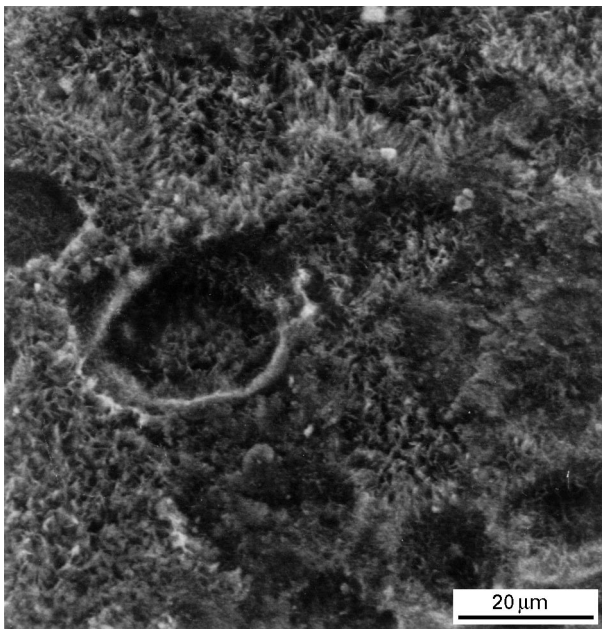

Figure 7 Expansion of the remodelled area apparently caused by the rupture of one vesicle on the apatite surface (centre left).

Considering that at the mechanistic level, the process of osteoclastic resorption is subjected to complex and regulated biological pathways and feedback mechanisms [22], the similarities observed in the present work are indeed remarkable. It may be possible that freely diffusing water-soluble acidic proteins in their monomer state released from the MAP aggregates act as the chelating or resorbing agents. It is known that one of the interesting characteristics of large aggregates such as vesicles is their ability to entrap within the assembly a portion of the aqueous phase and monomers present at the time of their formation in solution [1]. Upon adsorption on solid surfaces, these aggregates may undergo deformation and collapse or simply reorganize into new assemblies [18], which may result in the release of their contents.

It is also possible that acidic proline-rich proteins within the MAP assemblies may partially dissociate and provide the H^+ ions required for resorption of the apatite surface. Transfer of ions and soluble species through the membrane of assemblies, such as vesicles, and protein cages are well documented. For example, an important function of extra-cellular matrix vesicles (MVs) associated with the initial deposition of mineral crystals in calcified tissues is to provide enzymes that modulate the composition of the extra-vesicular tissue [23]. Studies by Eanes and Hailer [24], using lipsomes as MV models, have also indicated that the formation of calcium phosphate within these assemblies may be facilitated by diffusion of Ca^{++} ions through the liposome membrane. Furthermore, studies by Mann and coworkers [25] on the synthesis of inorganic nanophase materials in supramolecular protein cages has emphasized the significance of ion-transfer through hydrophilic and hydrophobic channels that penetrate the protein shell.

In the present work, apatite crystals can easily be solubilized under the action of H^+ ions or chelating agents [26] released by the isolated MAP assemblies. It would be expected that the rate of resorption would be determined, in part, by the size, composition, packing and orientation of the apatite crystals. The fine appearance of the newly formed mineral crystals in the "remodelled zones" (see, for example, Fig. 3) suggests that soluble proline-rich proteins not only act as resorbing agents but may also arrest growth and retard maturation of the newly formed crystals. During the course of the remodelling process, protein molecules may also become overgrown by the mineral and become trapped in the apatite crystals.

While the freely diffusing water-soluble acidic proteins appear to act as the resorbing agents and may inhibit crystal growth, the insoluble self-assembled MAP aggregates may also bind Ca and phosphates and act as templates for nucleation of apatite and for directing the remineralization process. The question arises as to whether by binding Ca and phosphate ions, MAP aggregates can be gradually reorganized into new ordered shapes as the remodelling process proceeds. Recent work by Firouzi *et al.* [27] has indeed indicated that self-assembled aggregates will often rearrange in the presence of inorganic species to form new and often unexpected morphologies. The structural rearrangements are particularly dependent on the nature of the inorganic species. For example, multidentate complexation of silicate oligomers to surfactant head groups in micellar aggregates has been shown to screen the intra-aggregate electrostatic head group repulsions among adjacent amphilphilic molecules and reduce the curvature of the aggregates. As a result, inorganic–organic aggregates are formed with structures that, in general, are entirely different from those of the precursor micelles [27].

Morphological changes induced by Ca^{++} ions are, in particular, remarkable. The morphological transition from spheres to vesicles in dilute solutions of polystyrene-poly(acrylic acid) diblock aggregates induced by Ca^{++} ions has been reported by Zhang and Eisenberg [28]. These morphological changes

have been attributed to strong Ca^{++} binding and bridging to the carboxylate groups of the poly(acrylic acid) (PAA) segments. Phosphatidylserine vesicles used as model membranes have also been reported to deform and change their shapes in the presence of Ca^{++} ions [29, 30]. These deformations often cause large elastic strains and stresses that result in increased fragility, leakage, lysis and fusion [31, 32].

We speculate that similar morphological changes may also occur as a result of slow interactions between dissolved Ca^{++} ions and the MAP aggregates adsorbed on the apatite surface. Thus the observation of various complex "remodelled zones" may be explained in terms of evolutionary changes in the aggregate structure induced by Ca^{++} ions and the subsequent occurrence of events such as deformation, fusion, collapse, rupture and spreading of a bilayer on the apatite surface. For example, we have observed regions on the surface of the apatite that appear as discrete or overlapping rings surrounding the well-developed "remodelled zones" (Fig. 2). Such features may indicate the involvement of self-assembled structures in the form of giant spherical vesicles in the remodelling process. It is possible that upon adsorption, spherical vesicles form a flat area of contact with the apatite surface and establish a sealing ring at the periphery of their contact. Thus, the borders of the "remodelled zones" may be set at early stages during the remodelling process. Calcium ions may contribute to the bonding strength and establishment of a firm contact with the apatite surface by forming Ca–MAP complexes that may strongly bind to the apatite surface through a phosphate link. Dissolution and remodelling of apatite within this border would be dependent on the successful establishment of a contact between the vesicles and the surface of the apatite. The dissolution process is also expected to be time-dependent and controlled by the rate of release of H^+ and/or freely diffusing acids. Thus, the appearance of less developed bowl-shaped domains [see, for example, Fig. 3 (top right) and Fig. 4 (centre left)] characterized by an irregular pattern of broken and fused crystals may signify the site-specific resorption of the apatite surface at early stages during the remodelling process. These features are indeed very similar to the irregular patterns of broken and fused crystals within the resorption lacunae caused by osteoclasts seeded on the bone-like apatite surface [7].

At a higher stage, remineralization apparently occurs within resorbed sites and this leads to site-specific remodelling of the apatite surface (Fig. 3) (centre right). The "remodelled zones" at this stage are characterized by the presence of much finer crystals that appear to be newly formed and clumped together presumably by MAP molecules. Thus, unlike osteoclasts, MAP vesicles may participate in a restructuring process that involves both resorption and remineralization. The clumping phenomenon may be associated with the formation of a Ca–protein complex that may link the newly formed apatite crystals together through a phosphate bond. This apparently results in considerable shrinkage in the "remodelled zones". More complex "remodelled zones" are also noted (Fig. 3), which often assume polyhedral outlines. Such surface structures may have developed at more advanced stages as a result of flattening and lateral expansion of vesicles in close contact with each other during the remodelling process. Thus, similar to the process of biomineralization in some biological systems these elaborate mineral structures may have formed through a remodelling process involving stressed membrane assemblies [33].

It is interesting to note that while the "remodelled zones" are often seen to be associated with the action of isolated vesicles, occasionally they appear to have formed as a result of the fusion between two or more vesicles (Fig. 4, centre). Ca-induced fusion of vesicles is a well documented phenomenon. It is known that the initial contact between vesicles may result in the formation of flat double-bilayer diaphragms [29]. Diaphragm formation between MAP vesicles in the present work may depend on bridging adjacent vesicles by forming MAP–Ca–MAP complexes. Such interaction would lead to an increase in the area of contact between vesicles, deforming their spherical shape and stressing the bilayer membrane. Note, for example, the "remodelled zone" shown in Fig. 5 (lower edge), which appears to have formed by the action of deformed vesicles in close contact with each other. Bilayers normally resist stretching and do rupture at 3% increase in area [34]. The stress could be relieved by diaphragm rupture, which leads to fusion and mixing of the contents of contacting vesicles. This may result in the formation of much larger and more complex three-dimensional domains as shown in Fig. 4. It is interesting to note that changes in calcium concentration also affect the fusion of bone cells and this may regulate the size and resorptive capacity of osteoclasts [21]. It appears, therefore, that the observed effect of Ca^{++} ions in the system under investigation is related to the well known fact that this ion is a strong fusogenic agent, promoting the fusion of cells and vesicles [35].

Rupture of single vesicles settled on the surface of the apatite may also be initiated by Ca^{++} ions released from the substrate. Thus, upon adsorption and forming a flat area of contact with the apatite surface, vesicles may become increasingly deformed and stressed as a result of continued interaction with the apatite surface and eventually rupture after a short delay time. Rupture of vesicles may be followed by excessive leakage and lateral spreading of the bilayer. This will result in the expansion of the "remodelled zone" beyond the original site of contact of the vesicle with the apatite surface (Figs 6 and 7). The vesicle–bilayer transformation and lateral spreading of the bilayer on non-reactive supports are well documented [17–19]. Radler *et al.* [18] have proposed a model for the rupture of vesicles and spreading of bilayers on solid supports that involves four steps: approach, adhesion, rupture and lateral spreading of the membrane. Two fundamentally different mechanisms for spreading of bilayers on non-reactive substrates have been suggested depending upon whether the membrane slides on or sticks to the underlying solid: (i) the sliding of a single bilayer on a thin

lubricating water film, and (ii) the rolling of thin lobes of two juxtaposed bilayers in a "tank tread type" motion [18]. In the present work, a MAP bilayer spreading laterally would be expected to interact strongly with the reactive surface of the microporous apatite support. This would result in dissolution of the surface of the apatite and expansion of the remodelled zone beyond the original sites of contact of the vesicles. However, the extent of bilayer spreading may be severely restricted due to the formation of local sites of strong bilayer–apatite interaction that can effectively lead to local bilayer immobilization. Thus, the ultimate structure and shape of the "remodelled zones" may be determined by dynamic interaction between MAP vesicles and the surface of the apatite.

4. Conclusions

In this study we have shown that self-assembled vesicles derived from the marine mussel *Mytilus edulis* are capable of inducing site-specific remodelling of the apatite surface. This represents the first *in vitro* study demonstrating organized and dynamic remodelling of the apatite surface in the absence of bone cells. Further study in this area may lead to the development of specially engineered protein-based assemblies that can be used in combination with apatite materials for enhancing the process of remodelling and bone healing *in vivo*.

Acknowledgements

I thank Dr Lida Raptis and the members of her laboratory for providing the sample of mussel adhesive protein and for advice concerning its use. I also would like to thank Suzanne Sims for her assistance in preparing the apatite substrates and for conducting the adsorption tests. Funding for this work was provided by the Natural Sciences and Engineering Research Council of Canada (NSERC).

References

1. J. N. ISRAELACHVILI, "Intermolecular and surface forces" (Academic Press, London, 1992).
2. *Idem*, in "Surfactants in solutions", Vol. 4, edited by K. L. Mittal and P. Bothorel (Plenum, New York, 1986) pp. 3–33.
3. G. CEVC and D. MARSH, "Phospholipid bilayers physical principles and models" (Wiley Interscience, New York, 1985).
4. D. D. LASIC, "Liposomes from physics to applications" (Elsevier, Armsterdam, 1993).
5. J. H. FENDLER, "Membrane mimetic chemistry" (Wiley, New York, 1982).
6. G. GREGORIADIS, *New England J. Med.* **295** (1976) 704.
7. S. YAMADA, T. NAKAMURA, T. KOKUBO, M. OKA and T. YAMAMURO, *J. Biomed. Mater. Res.* **28** (1994) 1357.
8. S. J. JONES, A. BOYDE and N. N. ALI, *Anat. Embryol.* **170** (1984) 247.
9. A. BOYDE, N. N. ALI and S. J. JONES, *Brit. Dent. J.* **156** (1984) 216.
10. A. BOYDE and S. J. JONES, *Scanning Microsc.* **1** (1987) 369.
11. M. SHIRKHANZADEH, *J. Mater. Sci. Lett.* **10** (1991) 1415.
12. *Idem*, US Patent 5 205 921 (1993).
13. J. H. WAITE, *J. Biol. Chem.* **258** (1983) 2911.
14. M. SHIRKHANZADEH, *J. Mater. Sci. Mater. Med.* (In press).
15. J. H. WAITE and M. L. TANZER, *Science* **212** (1981) 1038.
16. M. RYKKE, A. YOUNG, G. SMISTAD, G. ROLLA and J. KARLSEN, *Colloids Surfaces B: Biointerfaces* **6** (1996) 51.
17. G. PUU and I. GUSTAFSON, *Biochem. Biophys. Acta* **1327** (1997) 149.
18. J. RADLER, H. STREY and E. SACKMANN, *Langmuir* **11** (1995) 4539.
19. P. NOLLERT, H. KIEFER and F. JAHNIG, *Biophys. J.* **69** (1995) 1447.
20. S. I. STUPP and G. W. CIEGLER, *J. Biomed. Mater. Res.* **26** (1992) 169.
21. S. J. JONES, A. BOYDE, N. N. ALI and E. MACONNACHIE, *Scanning* **7** (1985) 5.
22. G. VAES, *Clinic. Orthop. Rel. Res.* **231** (1988) 239.
23. A. L. BOSKEY, B. D. BOYAN and Z. SCHWARTZ, *Calcif. Tissue. Int.* **60** (1997) 309.
24. E. D. EANES and A. W. HAILER, *ibid.* **37** (1985) 390.
25. F. C. MELDRUM, V. J. WADE, D. L. NIMMO, B. R. HEYWOOD and S. MANN, *Nature* **349** (1991) 684.
26. W. F. NEUMAN and M. W. NEUMAN, "The chemical dynamics of bone mineral" (University of Chicago Press, 1958).
27. A. FIROUZI, D. KUMAR, L. M. BULL, T. BESIER, P. SIEGER, Q. HUO, S. A. WALKER, J. A. ZASADZINSKI, C. GLINKA, J. NICOL, D. MARGOLESE, G. D. STUCKY and B. F. CHMELKA, *Science* **267** (1995) 1138.
28. L. ZHANG and A. EISENBERG, *Macromol.* **29** (1996) 8805.
29. R. P. RAND, B. KACHAR and T. S. REESE, *Biophys. J.* **47** (1985) 483.
30. S. W. HUI, S. NIR, T. P. STEWART, L. T. BONI and S. K. HUANG, *Biochim. Biophys. Acta* **941** (1988) 130.
31. A. E. SOWERS (ed.), "Cell fusion" (Plenum, New York, 1987).
32. J. M. WHITE, *Science* **258** (1992) 917.
33. S. MANN, D. D. ARCHIBALD, J. M. DIDYMUS, B. R. HEYWOOD, F. C. MELDRUM and V. J. WADE, *MRS Bulletin* **Oct**. (1992) 32.
34. E. EVANS and V. A. PARSEGIAN, *Annals NY Acad. Sci.* **416** (1983) 13.
35. D. E. LECKBAND, C. A. HELM and J. ISRAELACHVILI, *Biochemistry* **32** (1993) 1127.

Received 7 January
and accepted 26 January 1998