Alginate-Coated Chitosan Membrane for Guided Tissue Regeneration

Ta Wei Chen,[2] Shwu Jen Chang,[1] Gregory Cheng-Chie Niu,[1] Yun Ting Hsu,[1] Shyh Ming Kuo[1]

[1]*Department of Biomedical Engineering, I-SHOU University, Kaohsiung County, Taiwan*
[2]*Division of Dentistry, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan*

Received 9 May 2005; accepted 3 June 2006
DOI 10.1002/app.24945
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Chitosan membranes were first prepared by a thermally induced phase separation method and then alginate was coated on one side of the membranes by a modified dialysis apparatus to prepare alginate/chitosan membranes (A/C membranes). Electron spectroscopy for chemical analysis (ESCA), scanning electron microscope, and contact angle measurements were conducted to evaluate the surface characteristics. The mechanical strength, degradation behavior, and cell adhesion test were performed to evaluate the feasibility of using A/C membrane in guided tissue regeneration applications. The results revealed that alginates could effectively be coated onto the chitosan membrane. As observed in ESCA results, the *N*-atomic emission peak was decreased from originally 6.2% on the untreated chitosan surface to 2% on the alginate-treated surface. The contact angle decreased on the alginate-modified side substantially, compared with the untreated side (from 88.4° to 34.2°). The A/C membrane had a higher water content of 71.8% in comparison to the chitosan membrane of 61.8%. Consequently, A/C membrane became stiffer and had a higher Young's modulus and strength. After a 30-day *in vitro* shaking test, the weight of membranes was degraded to about 75% from the original. The 3T3 fibroblast cells showed less adhesion to alginate-modified side as compared to the untreated chitosan-side in cell adhesion test. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 4528–4534, 2006

Key words: polysaccharides; biomaterials; barrier; adhesion; membrane

INTRODUCTION

Guided tissue regeneration (GTR) is a technique utilizing membranes to serve as a physical barrier or an occlusive membrane, to separate and create a secluded space around the defects. This permits the lost periodontal tissues to regenerate by reducing the competitive and fast growth of other connective tissues.[1–3] Barrier membranes are able to prevent infection, stabilize wound site, and limit the epithelial cells migration or down-growth into the bony area. Moreover, they are stiff enough to sustain a protected space during the tissue-healing period. Among the materials used in GTR applications, nonresorbable membranes (such as e-PTFE membrane) and resorbable membranes (collagen membrane) have been successfully applied and are commercially available. However, they have inherited disadvantages such as the need for a second surgical procedure to remove it (for e-PTFE) and a localized chronic inflammatory response and fast degradation behavior (for collagen).[4,5] When polyglycolic acid-based membranes were used in GTR techniques, gingival recession, exposure of the device, and surrounding soft tissue inflammation were presented in clinical findings.[6,7] In this study, we utilized another biopolymer, chitosan, to replace these expensive materials as GTR barrier membranes. In addition, alginate was coated onto one side of the prepared chitosan membrane to prepare alginate/chitosan (A/C) barrier membrane. We expected the A/C membrane to have a dual-purpose: antiadhesive effect on the alginate side and osteoinductive effect on the chitosan side. Furthermore, in absence of commonly used cross-linking agents, such as glutaraldehyde to strengthen the membrane material with potential toxic, it would be beneficial to the biocompatibility of these A/C membranes.

Chitosan [poly(1,4),-β-D-glucopyranosamine], a natural occurring polysaccharide, could normally be obtained from crab or shrimp shells, by alkaline deacetylation of chitin. Highly deacetylated chitosan (e.g., > 85%) exhibits low degradation rate in aqueous media and may last several months. This slow degradation leads to a great potential in the development of inexpensive and versatile drug encapsulating systems.[8,9] Besides, its excellent gel-forming ability to be shaped into various forms by simple thermal-induced separation method strongly enhances its potential applications in the biomedical field. Chitosan may

Correspondence to: S. M. Kuo (smkuo@isu.edu.tw).
Contract grant sponsor: Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan; contract grant number: VGHKS 93-11.

prove to be useful as an aid to hemostasis in patients with coagulopathies. Nonwoven fabrics made of chitosan were recently developed for use of wound dressings.[10] Many researchers also found that chitosan has osteoinduction and osteoconduction potentials when used as a bone scaffold material.[11,12] Alginate is a copolymer composed of (1–4)-linked β-D-mannuronic acid (M units) and α-L-guluronic acid (G units) monomers. Alginates are also naturally derived polysaccharides; they have been extensively used as an ideal matrix material to prepare synthetic extracellular matrices or for immobilization of enzymes and microencapsulation of tissues and cells.[13–17] Because of the highly hydrated anionic surface characteristics, alginate matrix could resist cell adhesion and spread.[18] It could be chemically modified by simple reaction; such as modified by carbodiimide chemistry, alginate becomes a promising material for biomedical applications in recent years. However, the alginate matrices prepared by crosslinking alginate molecules with Ca^{+2} ions were less stable and may easily lose their mechanical strength in physiological environment, due to an outward flux of the crosslinking ions into the surrounding medium. This seemed to be a main drawback of the alginate material. According to the other research literatures,[19–21] Ca^{+2} and Cu^{+2} ions caused gelation of the alginate molecules into negatively charged beads, which interacted with positively charged chitosan to form microcapsules. It appears that alginate and chitosan are compatible to each other even when they are opposite charged to each other.[22–25]

In this study, we take advantage of the specific properties of chitosan (with positively charged and osteoinduction potential) and alginate (with negatively charged and resisting cell adhesion potential) to prepare A/C membranes. To retain the good biocompatibility of these two unique materials on the same device, we avoided using any crosslinking agents in the process of preparing A/C membrane. Electron spectroscopy for chemical analysis (ESCA) and SEM observation were conducted to examine the coating efficiency of alginate onto the chitosan membrane. Also, some basic properties of the A/C membrane were tested, such as water content measurements, contact angle measurements, mechanical strength, and degradation tests, to survey the feasibility of the A/C membranes in GTR applications. Cell adhesion test was carried out under a flow chamber system to assure the antiadhesion characteristics of alginate.

EXPERIMENTAL

Materials

Chitosan was purchased from TCI (Tokyo, Japan), with the molecular weight of 300,000, deacetylation degree 83%. Alginate was purchased from Sigma (St. Louis, MO), with the molecular weight of 150,000. Acetic acid was purchased from Merck-Schuchardt (Germany). All chemicals used in this study were of reagent grade.

Preparation of an A/C membrane

Chitosan was dissolved in acetic acid (0.1*N*) to prepare a 2% (w/v) chitosan solution. The chitosan solution was filtered and degassed overnight and then 19 mL of chitosan solution was poured into a Petridish and placed this dish in a drying air oven at 40°C overnight. This oven-dried chitosan membrane (about 1 mm in thickness) was then washed under running distilled water for 1 h. This washed membrane was transferred to a modified dialysis apparatus for coating alginate (Fig. 1.) The modified dialysis apparatus was described elsewhere.[26] Briefly, the apparatus was composed of two chambers and, in between, separated by a chitosan membrane. One chamber was filled with 0.05 wt % alginate for coating and the other chamber was filled with distilled water to maintain the chitosan membrane in hydrated state. This apparatus was put on the stirrer for continuous stirring. Let us be reminded that as chitosan is a cationic polysaccharide consisting of glucosamine residues and

Figure 1 The modified dialysis apparatus used to prepare the A/C membrane.

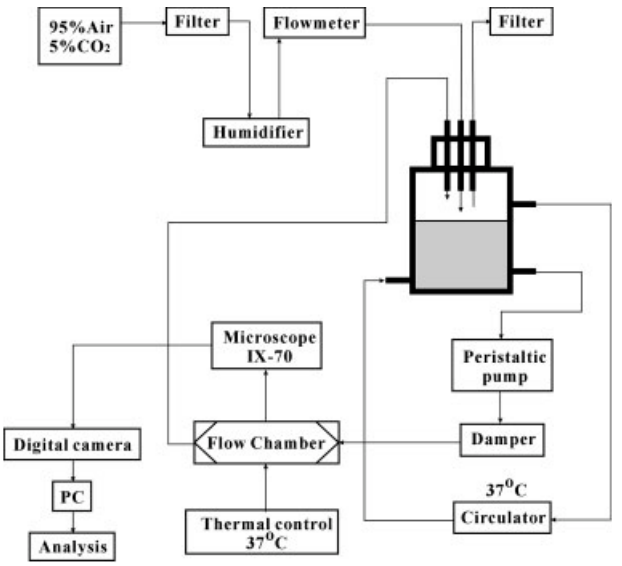

Figure 2 The apparatus of flow chamber used for cell adhesion test.

alginate is known for its anionic behavior, a tight electrostatic interaction was expected between both polymers. In general, chitosan and alginate could undergo C-A interpolyelectrolyte reaction even without the introduction any crosslinking agent.[27,28] For that, we chose not to use any crosslinking reagents to prepare or strengthen the A/C membranes.

Characterizations of the A/C membrane

Electron spectroscopy for chemical analysis

Electron spectroscopy for chemical analysis (ESCA) was used to obtain the elemental compositions of the A/C membrane. The A/C membrane was dried and analyzed by using a Physical Electronics (Quantum 2000, U.S.A.) ESCA spectrometer. The *N*-atomic percentage of A/C membrane (two sides) was estimated and assured of the coating of alginate.

SEM observation

The surface microstructures of the A/C membrane were examined by scanning electron microscope (SEM). Before SEM observation, the A/C membrane was dried and, sputter-coated with gold. Then it was examined under a scanning electron microscope (JEOL, JSM-5300, Japan).

Water content measurement

For comparison, the water content (WC) of the prepared A/C membrane and the chitosan membrane was determined by swelling the membrane in pH 7.4 of phosphate-buffered saline (PBS) at the temperature. The wet weight of the A/C membrane was determined by blotting the membrane with filter paper to remove adsorbed water on the surface. The WC was calculated as:

$$\mathrm{WC} = (W_w - W_d)/W_w$$

where W_w and W_d are the weights of the wet and dry membrane, respectively. The experiment was conducted three times, and a mean and standard deviation was calculated.

Mechanical properties measurement

The mechanical tensile properties of the A/C membranes were determined in hydrated condition. The samples, $1 \times 6 \times 0.1$ cm^3, were hydrated in 0.1*M* phosphate buffer, pH 7.4, before being subjected to mechanical testing. The tensile strength measurements of prepared membranes were charted up to the point where they were broken. The mechanical parameters of these membranes were calculated and recorded automatically using a MTS Systems (Eden Prairie, USA) at a crosshead speed of 10 mm/min.

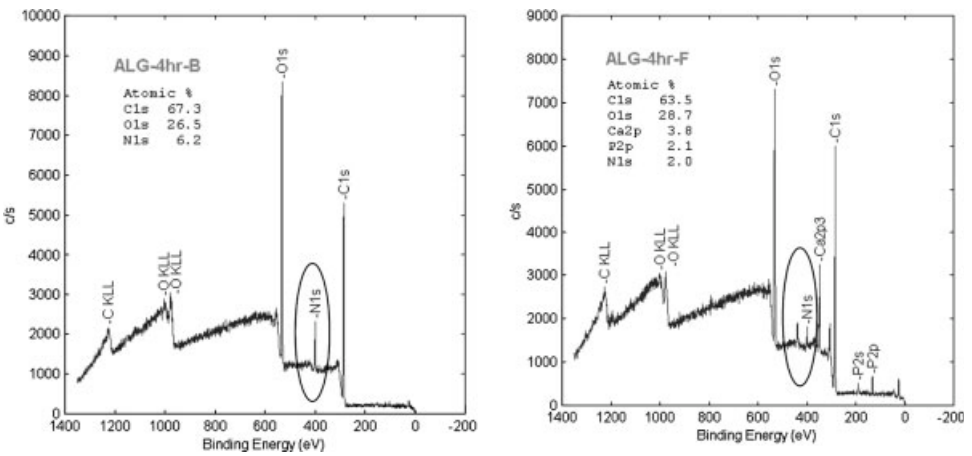

Figure 3 ESCA analyses of A/C membrane: (a) chitosan side; (b) alginate side.

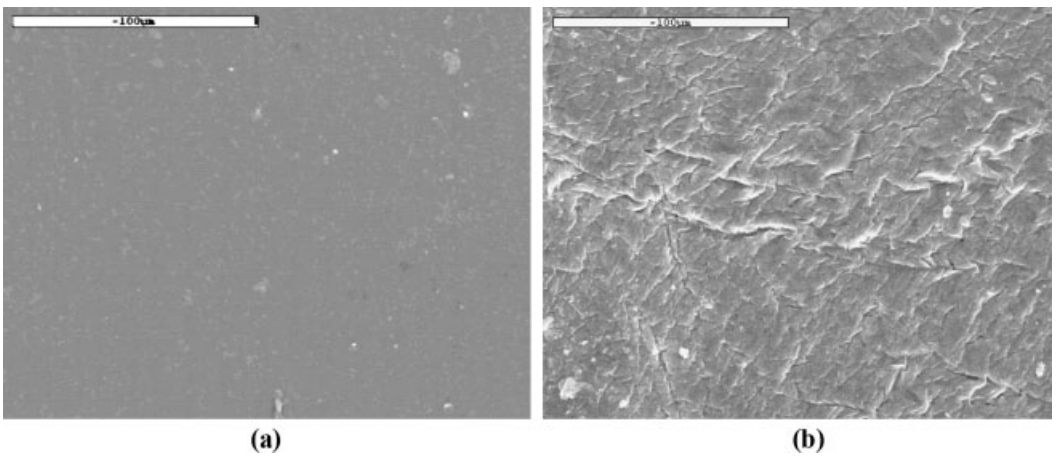

Figure 4 SEM observation of of A/C membrane: (a) chitosan side, (b) alginate side. Operating voltage: 15 kV, Magnification: ×300.

Contact angle measurement

Contact angle measurement was conducted to survey the changes of the hydrophilicity between the alginate and chitosan side. The contact angles of A/C membranes were measured by contact angle system (Dataphysics Instruments, GmbH, Germany). A distilled water droplet (20 μL) was released from a syringe and dropped on the prepared membrane. Contact angles were measured using the technique developed by Hamilton.[29]

Degradation test

The *in vitro* degradation test of the prepared A/C membrane was conducted by incubating the membrane in pH 7.4 of PBS (6 mL) on a shaker set at 40 rpm and 37°C. At predetermined time intervals, the membrane was taken out of the incubation medium, washed with distilled water, dried and the weight of this membrane was measured. Another fresh 6 mL PBS was added into the vial for continuum degradation test. The degradation profiles were expressed as the cumulative weight losses of the membrane.

Cell adhesion test

The cell adhesion of 3T3 fibroblast cells to A/C membrane was measured by using a method of flow chamber system (Fig. 2) according to the procedure described by Chang et al.[30] The prepared A/C membrane was cut into 6 cm ×1 cm size and placed inside the chamber for equilibrating in the culture medium. About 1 mL, 1×10^5 cell/mL of the 3T3 fibroblast cells suspension was injected into the flow chamber and incubated for 3 h so that the cells could spread and adhere onto the test substratum. The culture medium was then passed through the flow chamber at various flow rates to provide various shear stresses on the adhered cells. The number of remaining adherent cells, after culture medium flushing, was counted under an optical microscope. The fraction of the adhered cells on the substratum, alginate side and chitosan side, was represented by the ratio of adherent cells at the designated flow stress to that at zero flow rate.

RESULTS AND DISCUSSION

From the ESCA results on the surface of alginate (free from N atom) treated and untreated chitosan membrane sample, we could identify the reduction of *N*-atomic emission peak at 400 eV to 2.0% from 6.2% (for untreated surface) (Fig. 3).[31,32] It appears that there is a strong evidence of incorporation of alginate molecules onto the membrane surface due to the dialysis process.

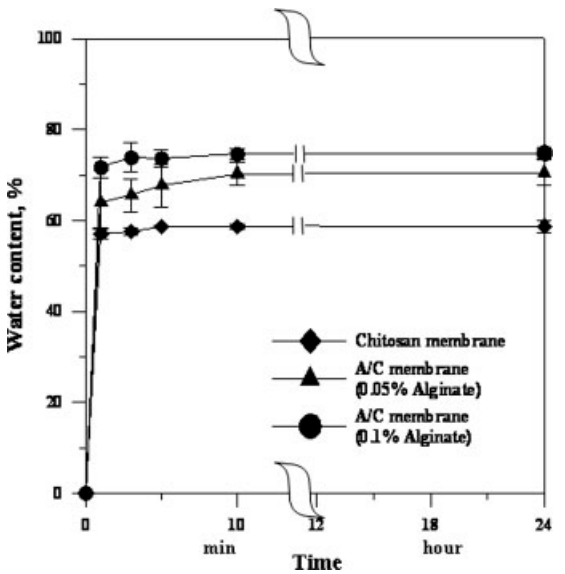

Figure 5 Water swelling profile of chitosan membrane and A/C membrane.

TABLE I
The Properties of A/C Membranes and Chitosan Membrane

	Young's modulus (MPa)	Ultimate strength (Pa)	Ultimate elongation (mm)	Contact angle at 25°C (°)	Equilibrium water content at 24 h (%)
Chitosan membrane	19.4	197.5	9	88.4°	58.6 ± 1.3
A/C membrane					
0.05% Alginate coated	21.1	207.5	9	34.2°	70.4 ± 2.8
0.1% Alginate coated	26.9	248.5	8	TLTM[a]	74.0 ± 1.1

[a] Too low to measure.

From SEM observations, the surface coated with alginate molecules exhibited a rougher, irregular surface morphology as compared with the untreated chitosan surface (Fig. 4). Combined with ESCA analyses and SEM observations results, we suggest that the alginate molecules might be coated or intertwined onto the chitosan membrane surface through a process by the modified dialysis apparatus. Also, this distinctively modified chitosan membranes surface exhibited much more hydrophilicity as indicated by the contact angle measurement: contact angle decreased from 88.4° (chitosan side) to 34.2° (alginate side). Furthermore, the modified membrane resulted higher equilibrium water content (65%) than that of plain chitosan membrane (Fig. 5).

Interestingly, A/C membranes became stiffer and stronger as indicated by higher Young's modulus and ultimate tensile strength. Higher Young's modulus and ultimate strength was obtained after coating with 0.05 and 0.1 wt % alginate (as shown in Table I). Instead of a simple coating phenomenon, there are indications that the interface between those two opposite charge polymers, chitosan and alginate, could be an intertwined blend by the complexation of alginate molecules onto the chitosan surface.[33] This intertwined phenomenon could be the cause for the surface roughness of the alginate-treated chitosan membrane (SEM photographs).

The prepared A/C membrane and plain chitosan membrane exhibited similar degradation profile in degradation test, and the A/C membrane degraded to 75% of the initial weight after 30-day shaking (Fig. 6). As a GTR barrier membrane, an appropriate degradation rate of membrane should be managed to fit the schedule requirements of remodeling of tissue regeneration. In clinical practice, barrier membranes are generally required to maintain their barrier functions for 4–6 weeks to assure successful restoration of bony tissues.[34] As can be seen in Figure 6, the A/C membrane degraded by about 25% of initial weight after 30-day shaking test. The SEM micrographs showed the surface morphologies of the A/C membrane (Fig. 7). After 30 days of shaking, the alginate was obviously seen on the chitosan surface, whereas, it decreased to a lesser content of alginate as compared with the unshaken one [Fig. 4(b)]. At the same time, the chitosan surface retained an intact and smooth surface morphology as before. Although, the resorption process could be facilitated by enzyme digestion in real applications, we suggested that the membranes prepared in this study could still meet the degradation requirement of bioresorbable membrane used for GTR from these observations. The bulk properties of prepared A/C membrane were summarized in Table I.

A cell adhesion assay was carried out to check 3T3 fibroblast cells adhesion onto the A/C membrane and chitosan membrane. 3T3 fibroblast cells showed a less adhering to the alginate-coated surface than that onto the chitosan surface. The fraction of 3T3 fibroblast cells adhering to the membrane surfaces, normalized to the initial number of cells was plotted as a function of shear stress in Figure 8. As shown, a difference in cell adhering ratios was observed between the chitosan surface and the alginate-coated surface. Probably because of the presence of negatively charged alginate and higher hydrophilic characteristics, the cells detached quickly at low shear stress of 0.82 dyn/cm^2 (cells retention ratio = 0.19), whereas the cells adhered a little strongly on the chitosan surface (retention ratio = 0.38). This flow chamber system provides a vehicle

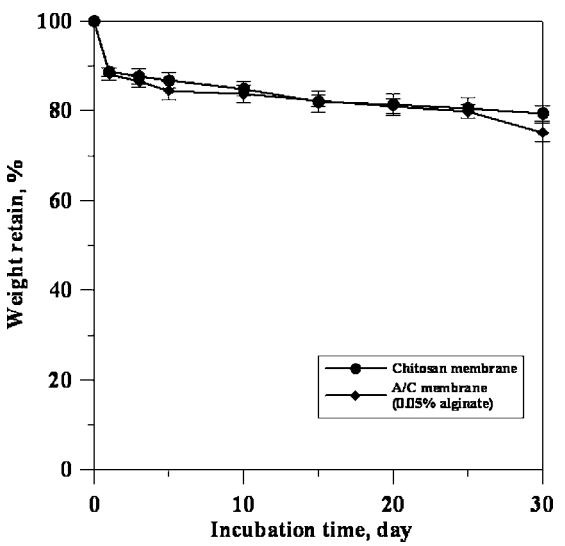

Figure 6 The degradation profile of chitosan membrane and A/C membrane.

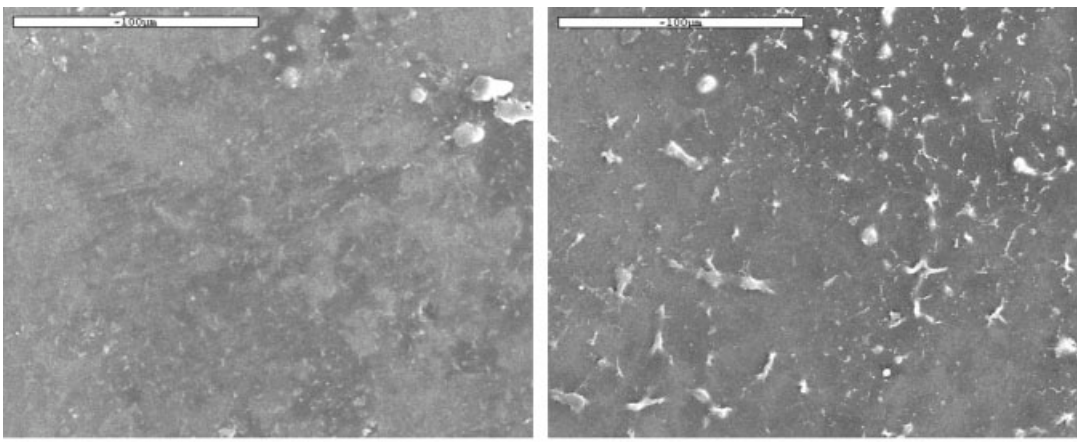

Figure 7 SEM observation of of A/C membrane after 30-day shaking: (a) chitosan side; (b) alginate side. Operating voltage: 15 kV, Magnification: ×300.

for determining the adhesion strength of cells on substratum; that is, the number of cells that remained attached decreased with the increasing shear stress. The results obtained from this flow chamber tests illustrated that the chitosan surface coated with alginate inhibited 3T3 cells attachment and provided a better antiadhesion effect than chitosan.

GTR techniques have been successfully applied in the treatment of periodontal lesions. However, the function of the currently available GTR membrane is limited to the creation of a space to separate the defects and surrounding tissues. It lacks of other desirable functions such as tissue integration or osteoinduction. Although we had prepared such chitosan membranes that improved bony cells adhering and growth, and also increased the proliferation of fibroblast cells,[3] the fibroblast cells would occupy the whole defect area due to their fast-growing and high proliferation characteristics. To delay the fibroblast cells adhering and growth, we developed a new barrier membrane (A/C membrane material) that possessed the basic functions for GTR application, but also resisted fibroblast cells adhering and delay the cells growth. This delayed growth of fibroblast cells could leave the defect space open until the slow-growing bony cells have enough time to occupy and proliferate into the defect area. There are several different concentrations of alginate solution (from 0.05 wt % up to 1 wt %) for preparing A/C membranes. However, with higher alginate concentrations, there was tendency of localized peeling-off of coating from the resulting membranes. We, therefore, chose A/C membrane from 0.05 and 0.1% formulas for this study. The preliminary results revealed that even low concentrations of alginate were able to effectively coat onto the chitosan membrane and fulfilled our expected results such as, different surface characteristics, morphologies, and antiadhesion characteristics. The applicability of these A/C membranes on animal model study of GTR application is under way in our laboratory.

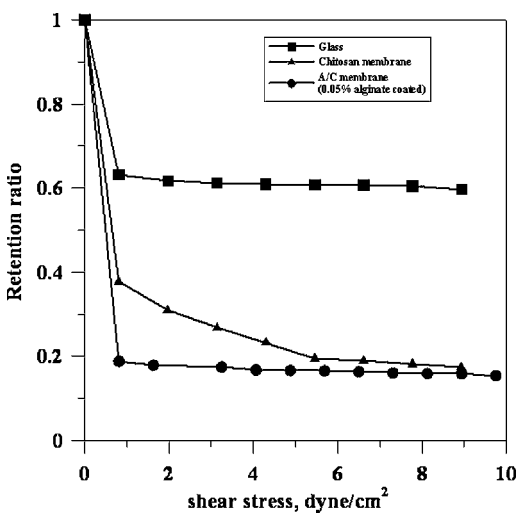

Figure 8 Retention of 3T3 fibroblast cells on glass, chitosan membrane, and A/C membrane as a function of shear stress.

CONCLUSIONS

In our previous studies,[3,26] we have reported the application of chitosan, which is in abundance, inexpensive, and possesses excellent biocompatible and biodegradable characteristics for preparation of chitosan membranes used in GTR techniques. The study results revealed that this chitosan membrane met the requirements of barrier membrane used in GTR. To expand the applications of chitosan membranes, we utilized here another biomaterial, alginate, which possesses negative charge, to coat onto one side of chitosan surface by ionic interactions to prepare an alginate-modified chitosan membrane for GTR application. The preliminary results indicated that the bulk

properties of the membrane were not extensively altered after coating one side with solution of alginate content from 0.05 to 0.1 wt %. However, A/C membrane exhibited different hydrophilicity and surface morphology. Furthermore, this alginate-coated surface exhibited a 50% improvement in the resistance to 3T3 cells adhering under low shear rate.

From the tissue engineering viewpoint, the guiding of growth of competing tissue has always been an issue difficult to resolve. Apparently, the alginate-modified chitosan membrane could be an ideal GTR material that presumably provides a means to regulate the growth of a soft tissue, such as gingival tissue, over a competing hard tissue, such as alveolar bone.

References

1. Blumenthal, N. M. J Periodontal 1988, 59, 830.
2. Milella, E.; Barra, G.; Ramires, P. A.; Leo, G.; Aversa, P.; Romito, A. J Biomed Mater Res 2001, 57, 248.
3. Chang, S. J.; Kuo, S. M.; Chen, T. W.; Kuan, T. C. J Biomed Mater Res A 2006, 76, 408.
4. Gottlow, J. J Periodontol 1993, 64, 1157.
5. Zellin, G.; Gritli-Linde, A.; Linde, A. Biomaterials 1995, 16, 601.
6. Simion, M.; Scarano, A.; Gionso, L.; Piattelli, A. Int J Oral Maxillofac Implants 1996, 11, 735.
7. Caffesse, R. G.; Nasjleti, C. E.; Morrison, E. C.; Sanchez, R. J Periodontol 1994, 65, 583.
8. Kiang, T.; Wen, J.; Lim, H. W.; Leong, K. W. Biomaterials 2004, 25, 5293.
9. Mi, F.-L.; Shyu, S.-S.; Wu, Y.-B.; Lee, S.-T.; Shyong, J.-Y.; Huang, R.-N. Biomaterials 2001, 22, 165.
10. Madihally, S. V.; Matthew, H. W. T. Biomaterials 1999, 20, 1133.
11. Kuo, S. M.; Chang, S. J.; Lin, L.-C.; Chen, C. J. J Appl Polym Sci 2003, 89, 3897.
12. Ito, M.; Hidaka, Y.; Nakajima, M.; Yagasaki, H.; Kafrawy, A. H. J Biomed Mater Res 1999, 45, 204.
13. Taqieddin, E.; Amiji, M. Biomaterials 2004, 25, 1937.
14. Ishikawa, K.; Ueyama, Y.; Mano, T.; Koyama, T.; Suzuki, K.; Matsumura, T. J Biomed Mater Res 1999, 47, 111.
15. Seo, S.-J.; Akaike, T.; Choi, Y.-J.; Shirakawa, M.; Kang, I.-K.; Cho, C.-S. Biomaterials 2005, 26, 3607.
16. Rowley, J. A.; Madlambayan, G.; Mooney, D. J. Biomaterials 1999, 20, 45.
17. Chang, S. J.; Lee, C. H.; Hsu, C. Y.; Wang, Y. J. J Biomed Mater Res 2002, 59, 118.
18. Rasmussen, K.; Østgaard, K. Water Res 2003, 37, 519.
19. Gotoh, T.; Matsushima, K.; Kikuchi, K.-I. Chemosphere 2004, 55, 135.
20. Ribeiro, A. J.; Silva, C.; Ferreia, D.; Veiga, F. Eur J Pharm Sci 2005, 23, 31.
21. Tamura, H.; Tsuruta, Y.; Tokura, S. Mater Sci Eng C 2002, 20, 143.
22. Simsek-Ege, F. A.; Bond, G. M.; Stringer, J. J Appl Polym Sci 2003, 88, 346.
23. Lee, K. Y.; Park, W. H.; Ha, W. S. J Appl Polym Sci 1997, 63, 425.
24. Gåserød, O.; Smidsrød, O.; Skjåk-Bræk, G. Biomaterials 1998, 19, 1815.
25. Becherán-Marón, L.; Peniche, C.; Argüelles-Monal, W. Int J Biol Macromol 2004, 34, 127.
26. Chen, T. W.; Kuo, S. M.; Chang, S. J.; Kuan, T. C. Biomed Eng Appl Basis Commun 2004, 16, 259.
27. Tamura, H.; Tsuruta, Y.; Tokura, S. Mater Sci Eng C 2002, 20, 143.
28. Yan, X. L.; Khor, E.; Lim, L. Y. J Biomed Mater Res B 2001, 58, 358.
29. Hamilton, W. C. J Colloid Interface Sci 1972, 40, 219.
30. Chang, S. J.; Kuo, S. M.; Lan, J. W.; Wang, Y. J. Artif Cells Blood Substit Immobil Biotechnol 1999, 27, 229.
31. Amiji, M. M. Colloids Surf B 1998, 10, 263.
32. Chiou, S. H.; Wu, W. T. Biomaterials 2004, 25, 197.
33. Agnihotri, S. A.; Mallikarjuna, N. N.; Aminabhavi, T. M. J Controlled Release 2004, 100, 5.
34. Park, Y. J.; Nam, K. H.; Ha, S. J.; Pai C. M.; Chung, C. P.; Lee, S. J. J Controlled Release 1997, 43, 151.