Transactions of the Materials Research Society of Japan 25 [4] 919-922 (2000)

Cell Adhesion and Separation Materials with a Molecular Recognition Function: Cell Attachment and Growth on a PVA-Chitosan Hydrogel

Tomoe Koyano, Masanobu Nagura* and Norihiko Minoura**
Japan Chemical Innovation Institute, Higashi, Tsukuba, Ibaraki 305-8565, Japan
Fax: +81-298-54-4680, e-mail: koyano@home.nimc.go.jp
*Faculty of Textile Science and Technology, Shinshu University, Tokita, Ueda, Nagano 386-8567, Japan
Fax: +81-268-25-9417, e-mail: mnagura@giptc.shinshu-u.ac.jp
**National Institute of Materials and Chemical Research, Higashi, Tsukuba, Ibaraki 305-8565, Japan
Fax: +81-298-54-4680, e-mail: minoura@home.nimc.go.jp

We prepared poly(vinyl alcohol) (PVA) hydrogels that included the polysaccharide chitosan, a material with an important role in cell recognition. Hydrogels in which chitosan (86 mol% deacetylation) comprised 40 wt% of the initial mixture showed cell attachment and growth that exceeded those seen on collagen, a widely used mammalian cell-culture substrate. This superior cell attachment and growth may be the result of cells recognizing not only the chemical structure of the chitosan molecules but also the two-dimensional distribution pattern of those molecules on the surface of the hydrogel.
Key words: tissue engineering, biomaterial, hydrogel, chitosan, oligomer, fibroblast cell, molecular recognition

1. INTRODUCTION

Tissue engineering is one of the most promising technologies for developing biomedical materials and artificial organs [1, 2]. Tissue engineering requires substrates that promote superior cell attachment and growth, because mammalian cells are anchorage-dependent. Effective substrates include polymeric materials such as collagen [3] and polystyrene with a galactose moiety [4].

Recently, we found that chitosan-containing PVA hydrogels prepared according to the method we developed led to marked cell attachment and growth [5]. We designed these hydrogels to be substrates for tissue engineering with the rationale that chitosan likely will play a role in cell recognition, whereas PVA, which maintains the hydrogel's high elasticity, modulates the chitosan content in changing the blending ratio. The final goal of our research is a cell adhesion and separation material (hydrogel) with a molecular recognition function. The formation of a three-dimensional cell sheet (tissue) on the hydrogel is shown schematically in Fig. 1. Small molecules such as dissolved oxygen and other nutrient substances for the attached cells easily permeate hydrogels. In addition, hydrogels can achieve highly selective cell attachment through ligand binding. Furthermore, after appropriate incubation, mild external stimuli such as cooling can be used to smoothly detach a formed cell sheet from the hydrogel.

Here we show the effects of the deacetylation (50, 86, or 100 mol%) of chitosan and the chitosan content of the initial mixture (2, 5, 10, 15, 20, 30, or 40 wt%) on the cell attachment and growth behavior of the composite hydrogels. In addition, by adding oligomers of either chitin or chitosan to the cell culture medium, we evaluated the function of chitosan as a ligand for cell attachment to the hydrogels.

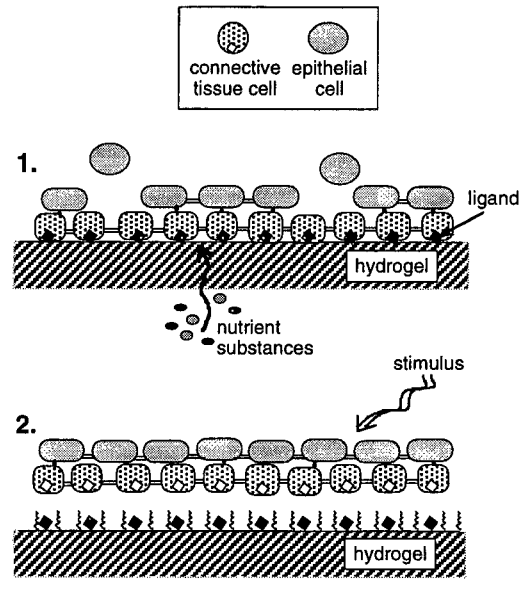

Figure 1 Formation of a three-dimensional tissue on a hydrogel substrate. 1. The hydrogel contains ligands, which could play a role in recognition; connective tissue cells with the appropriate receptor selectively attach to the ligands. These cells then proliferate. Subsequently, epithelial cells (on the basis of self-tissue formation) attach to the connective tissue cells and proliferate. 2. The cell sheet (tissue) is detached from the hydrogel by a mild stimulus.

In the case of skin tissue, the connective tissue cells correspond to dermis, and the epithelial cells are epidermis.

2. EXPERIMENTAL

PVA powder, supplied by Kuraray Co., Ltd., Japan, has a degree of polymerization of 1700 and a saponification degree of 99.85%. Katakura Chikkarin Co., Ltd., Japan, supplied chitosan powders with deacetylation degrees of 99.5 mol% and 86.0 mol% (as estimated from a elementary analysis). We obtained chitosan powder with 48.1 mol% deacetylation (as estimated from a elementary analysis) from Ajinomoto Co. Ltd. Japan. All powders were used as supplied and without further purification.

PVA-chitosan hydrogels were prepared as described previously [5]. Using the chitosan powder whose deacetylation was 48.1 mol%, we put the chitosan content of the initial mixture at 40 wt%, thereby creating the hydrogel D.D.=50. We similarly used the 86.0 and 99.5 mol% powders to create D.D.=86 and D.D.=100, respectively. We then used the powder with 86.0 mol% deacetylation to set the chitosan content of the initial mixture at 2, 5, 10, 15, 20, 30, or 40 wt%; these reagents were labeled as Chitosan-X (X = wt% chitosan).

We used cultured L-929 cells, which are derived from a mouse connective tissue fibroblast, to complete the experiments on cell attachment and growth. We used the air-surface side of the hydrogels for the cell culture test. The test procedure has been described [6]. In addition, we performed cell culture tests to which we added oligomers of chitin or chitosan. Briefly, cell suspensions (6 mL) containing 6.0×10^5 cells were mixed with Eagle's minimum essential medium (1 mL) containing the oligomer (0.6 mg). Chitin oligomers containing 1 and 2 repeating units of N-acetylglucosamine and chitosan oligomers with 1 to 6 repeating units of glucosamine were purchased from Seikagaku Kogyo Co. Ltd., Japan, and chitin oligomers with 3 to 6 repeating units of N-acetylglucosamine were supplied by Yaizu Suisan Kagaku Kogyo Co. Ltd., Japan. All of the oligomers were purified to > 99%.

3. RESULTS AND DISCUSSION

3.1 Effect of the deacetylation of chitosan

We evaluated the attachment and growth of cultured fibroblast cells on the D.D.=50, D.D.=86, and D.D.=100 hydrogel samples and on collagen film, which served as a reference (Fig. 2). The hydrogels of D.D.=86 and D.D.=100 exhibited an affinity for L-929 cells. Further, the attached cells not only remained viable but also proliferated on the surface of these hydrogels, as indicated by the positive slope of the cell growth curves. In contrast, hardly any L-929 cells attached to the D.D.=50 hydrogel, and those few cells that attached did not proliferate.

We calculated the relative cell attachment as the ratio of the number of cells attached to the hydrogel after incubation for 20 h to that attached to the control film (Plastic Sheet for Tissue Culture, Wako Pure Chemical Co. Ltd., Japan) after a 20-h incubation. For the D.D.=50 hydrogel, the relative cell attachment was nearly zero. However, the values for D.D.=86 (149%) and D.D.=100 (161%) exceeded that of collagen (115%), a widely used mammalian cell-culture substrate.

We calculated the relative rate of cell growth by dividing the slope of the cell growth curve of each hydrogel by the slope of the curve for growth on the control film. For D.D.=50, the relative rate of cell growth was nearly zero. In comparison, the relative rate of cell growth was 2.0 for D.D.=86 and 1.5 for D.D.=100. In particular, the value for the D.D.=86 hydrogel (2.0) exceeded that of collagen (1.8).

Neither the relative cell attachment nor the relative rate of cell growth was linearly proportional to the degree to which the chitosan was deacetylated. The hydrogel prepared from the chitosan that had 86.0 mol% deacetylation is an acceptable biomaterial that promotes high cell attachment and growth.

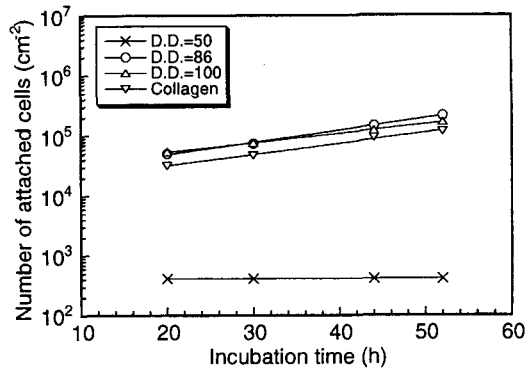

Figure 2 Typical cell growth curves of L-929 cells on collagen film and hydrogels containing chitosan with various degrees of deacetylation.

3.2 Effect of varying the chitosan content

We evaluated cell attachment and growth on the hydrogels containing PVA and various concentrations of chitosan (86.0 mol% deacetylation). The chitosan content clearly affected the interaction between the hydrogels and the cells. The relative cell attachment after incubation for 30 h increased markedly with increasing chitosan content above 15 wt% (Fig. 3, top). The relative cell attachment of the Chitosan-40 (380%) far exceeded that of collagen (256%).

In the relative rate of cell growth (Fig. 3, bottom), there appears to be a switch. In the hydrogels with more than 15 wt% chitosan, the relative rates of cell growth were independent of chitosan content and exceeded the relative rate on the collagen film (0.68). These results suggest that the affinity between the cell and hydrogel increases with increasing chitosan content (especially at concentrations exceeding 15 wt%).

Electron spectroscopic analysis revealed that the chitosan component was concentrated on the surface of

the hydrogels; in the hydrogels containing 10 to 40 wt% chitosan, the surface concentration of chitosan component in each hydrogel was about the same [7]. Furthermore, results from electron-probe microanalyzer tests showed that the two-dimensional distribution of the chitosan component on the surface of the Chitosan-15 sample was heterogeneous compared to that on the surface of the Chitosan-40 sample. In particular, the chitosan component on the surface of the Chitosan-15 sample was localized in islands [7].

These results suggest that the deacetylation of the chitosan molecules and the chitosan content affect the cell attachment and growth behavior on the hydrogels and that a homogeneous distribution of the chitosan components on the surface of the hydrogels favors these processes. Unclear as yet is why the hydrogel containing 40 wt% chitosan with 86.0 mol% deacetylation is superior to other hydrogels and collagen film for cell attachment and growth. A biospecific interaction between the cell and chitosan molecule should be considered.

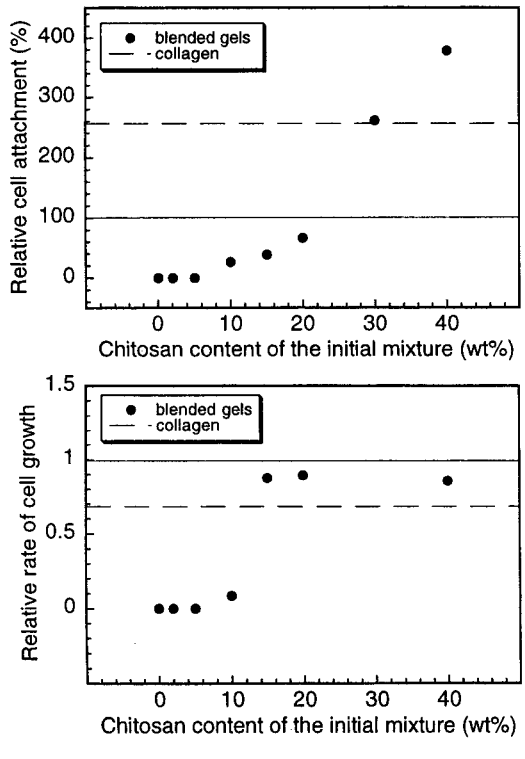

Figure 3 Top, cell attachment after incubation for 30 h relative to that on the control film. Bottom, rate of cell growth relative to that on the control film.

3.3 Effect adding chitin and chitosan oligomers

The cell culture experiments were carried out on hydrogels of 40 wt% chitosan with 86.0 mol% deacetylation by using cell suspensions containing chitin oligomers $(GlcNAc)_n$ and chitosan oligomers $(GlcN)_n$, (n = 1 - 6). If a biospecific interaction between L-929 cell and chitosan molecule exists, the cells would conjugate with the oligomers before incubation and therefore would be unable to attach to the hydrogel surface.

When chitin oligomers were added, the number of attached cells increased with increasing incubation time independent of the number of N-acetylglucosamine units of the chitin oligomers (Fig. 4). Clearly the addition of each chitin oligomer promoted the remarkable cell attachment to the hydrogel because the cell growth curves in the presence of the chitin oligomers were higher than those in the absence of the chitin oligomers. This promotion effect was unexpected.

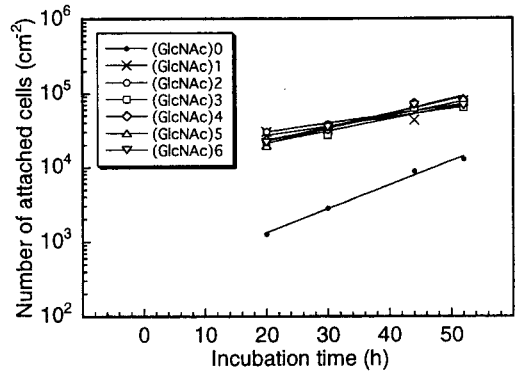

Figure 4 Typical cell growth curves of L-929 cells on a hydrogel (Chitosan-40) in the absence $(GlcNAc)_0$ and presence of various chitin oligomers $(GlcNAc)_{1\text{-}6}$.

Addition of triglucosamine $(GlcN)_3$ inhibited cell attachment, whereas addition of other chitosan oligomers did not affect this measure (Fig. 5). This inhibition effect suggests that the L-929 cell may have a receptor for cell attachment, and this receptor likely recognizes the $(GlcN)_3$ structure.

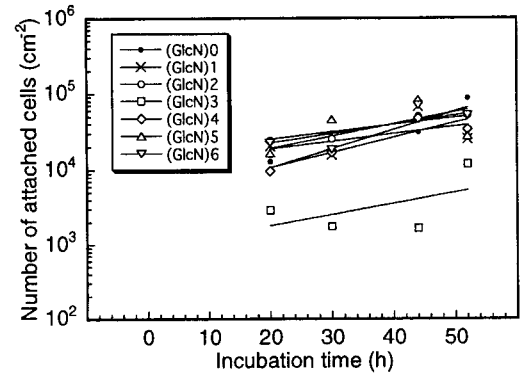

Figure 5 Typical cell growth curves of L-929 cells on a hydrogel (Chitosan-40) in the absence $(GlcN)_0$ and presence of various chitosan oligomers $(GlcN)_{1\text{-}6}$.

Compared to that of chitosan with 48.1 or 99.5 mol% deacetylation, chitosan molecules with 86.0 mol% probability. Because the cells recognize such a chemical structure unit, they can attach to the D.D.=86 hydrogel. This recognition accounts for the superior deacetylation have a chemical structure unit (Fig. 6) similar to $(GlcN)_3$ in the chemical structure in the high cell attachment and growth associated with the hydrogel containing chitosan with 86.0 mol% deacetylation.

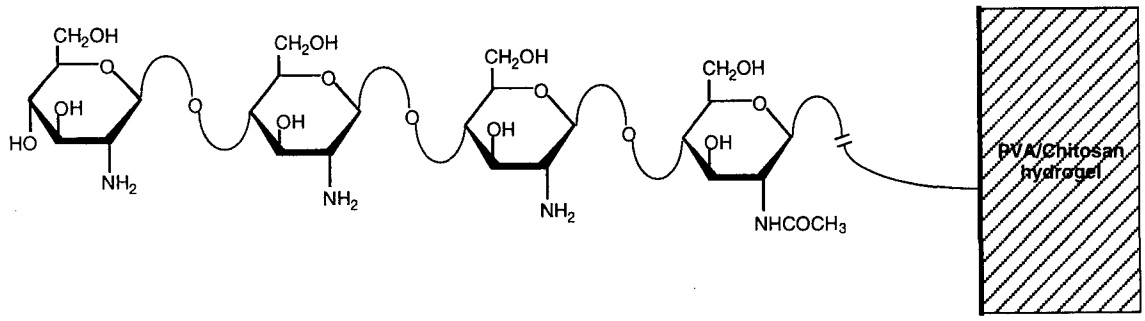

Figure 6 Schematic representation of a hydrogel surface composed of chitosan molecules whose unit of molecular structure is similar to $(GlcN)_3$.

ACKNOWLEDEMENT

The authors thank Kuraray Co. Ltd., Ajinomoto Co. Ltd., Katakura Chikkarin Co. Ltd., and Yaizu Suisan Kagaku Kogyo Co. Ltd. for supplying the samples.

REFERENCE

[1]R. Langer and J. P. Vacanti, *SCIENCE*, **260**, 920-926(1993).

[2]R. Langer and J. P. Vacanti, *SCIENTIFIC AMERICAN*, September, 100-103(1995).

[3]E. Bell, B. Ivarsson and C. Merrill, *Proc. Natl. Acad. Sci. USA.*, **76**, 1274-1278(1979).

[4]T. Akaike, M. Goto, H. Yura, A. Kobayashi, C. S. Cho, A. Maruyama and K. Kobayashi, "Advanced Biomaterials in Biomedical Engineering and Drug Delivery Systems", Springer Verlag, Berlin(1996) pp. 173-178.

[5]T. Koyano, N. Minoura, M. Nagura and K. Kobayashi, *J. Biomed. Mater. Res.*, **39**, 486-490(1998).

[6]N. Minoura, S. Aiba, Y. Fujiwara and N. Koshizaki, *J. Biomed. Mater. Res.*, **23**, 267-279(1989).

[7]T. Koyano, N. Koshizaki, H. Umehara, M. Nagura, and N. Minoura, *Polymer*, in press.

(Received December 16, 1999; Accepted September 30, 2000)