Evaluation of Chitosan-g-PEG Copolymer for Cell Anti-Adhesion Application

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Received 26 Dec 2006; Accepted 7 Mar 2007

Abstract

When using chitosan in biomaterial applications, a frequently encountered problem is that chitosan is difficult to dissolve in water of neutral pH range. This impeded its potential in biomedical field. In the present study, chitosan was modified by grafting a highly hydrophilic poly(ethylene glycol)-aldehyde onto the chitosan backbone to prepare water-soluble chitosan derivatives (defined as chitosan-g-PEG copolymers). The characteristic peaks of the copolymer at 2900 cm$^{-1}$ and 1110 cm$^{-1}$ showed that PEG was successfully grafted onto the chitosan main chain. Also, the obtained graft ratios increased with feed ratios, and a similarity between targeted and obtained graft ratio was found by $^1$H NMR examination. Chitosan derivatives in powder form prepared by a freeze-dry process were readily water-soluble. The contact angles of the membranes of a PEG-grafted chitosan copolymer decreased significantly, as compared to those of a pure chitosan membrane, from 85.7° to about 30°. Significantly, the surface of the membranes prepared from the copolymers showed a lower cell adhesion behavior in the cell flow-chamber test.

Keywords: Chitosan, PEG, Copolymer, Anti-adhesion

Introduction

Chitosan [poly(1,4)-,$\beta$-D-glucopyranosamine] is a natural polysaccharide widely present among marine and terrestrial invertebrates [1-2]. Chitosan can normally be obtained from crab or shrimp shells by alkaline deacetylation of chitin. Highly deacetylated chitosan (i.e., $>$85%) exhibits low degradation rate in aqueous media and may last several months, and thus offers great potential in the development of inexpensive and versatile drug encapsulating systems [3-4]. In medical applications, chitosan has been observed to accelerate wound healing and blood clotting, and chitosan-based implants also evoked a minimal foreign body reaction in tissue response, revealing the good biocompatibility of chitosan [5-6]. Two of chitosan’s promising features in biomaterial application are its excellent gel-forming properties and ability to be processed into different structures by simple thermal-induced separation method from chitosan-acetic acid solutions. This pH-dependent solubility provides chitosan a versatile processing mechanism for different applications. However, the poor solubility of chitosan in neutral aqueous solutions, due to its rigid crystalline structure, indeed limits its effective utilization in some biomedical applications. In order to overcome this shortcoming and increase the potential of chitosan materials, it is often necessary to convert chitosan to water-soluble derivatives for ease of application [7-8]. For example, in general, intestinal obstruction is a clinical complication that frequently occurs after abdominal surgery and is commonly due to postoperative intraperitoneal adhesions, which are responsible for about 70% of intestinal obstructions. Currently, the remedy often applied today is to insert physical barriers to prevent adhesion from occurring. The barriers separate the intestines from surrounding tissues to prevent them from adhesion [9-11]. However, if those barriers are in the form of membranes, they may change location after surgery in the abdominal cavity. Therefore, it is necessary to suture onto the intestinal wall to prevent the membrane from migration. Also, the need to carry out a second operation just for the removal of enclosed barrier materials might limit their application. If the barriers are in the form of powders, it might facilitate the adsorption of fibroblast and eventually result in adhesion. If in the liquid form, they might flow inside of the body and not be effective in the target places. On the other hand, if in the form of permeating, swelling and softening of a gel, they could be adsorbed readily to the surfaces of tissues in situ and provide a barrier between such tissues. Accordingly, using the gel form to separate the various tissues may prove to be a more advantageous choice. Recently, there has been much interest in the development of materials as anti-adhesive agents, such as oligosaccharides to ensure the proper segregation among the tissues and organs. It appears that there is good potential for soluble yet viscose oligosaccharides or derivatives with improved viscosity to act as anti-adhesive ingredients or materials to inhibit binding of
of these copolymers were examined. The hydrophilicity and flow-chamber adhesion test. The cell anti-adhesion ability of these copolymers in membrane (chitosan-method described by Sugimoto onto chitosan (mol. wt. 300,000 and 70,000), according to the characteristics of PEG can be attributed to the flexibility of the polymer backbone and the volume exclusion effect of this polymer in solution. In general, surfaces modified with PEG were found to be less thrombogenic and more resistant to fibrous tissue on-growth, due to the flexibility of the backbone and hydrophilicity of the polymer [16-18]. Some approaches have been conducted to modify chitosan with PEG through a complexation-interpenetration method to yield water-soluble or, preferably, water-affinitive copolymers. This modified chitosan could improve the blood compatibility, and resisted plasma protein adsorption and cell adhesion of chitosan [20-21].

In this study, we prepared a series of copolymers (chitosan-g-PEG, Table I) by grafting PEG (mol. wt. 2000) onto chitosan (mol. wt. 300,000 and 70,000), according to the method described by Sugimoto et al. [12]. The basic properties of these copolymers were examined. The hydrophilicity and cell anti-adhesion ability of these copolymers in membrane form were evaluated, by contact angle measurements and cell flow-chamber adhesion test.

### Materials and Methods

Chitosans with molecular weights of 300,000 and 70,000, respectively, and deacetylation degree 85% were purchased from TCI, Tokyo, Japan. MeO-PEG (methoxy poly ethylene glycol) with mol. wt. of 2000 was purchased from Sigma (St. Louis, MO). Acetic anhydride, anhydrous dimethyl sulfoxide (DMSO), chloroform, acetic acid, diethylether, methanol, and NaCNBH₃ were purchased from Merck-Schuchardt (Germany). All chemicals used in this study were of reagent grade and all organic solvents were HPLC grade.

### Synthesis of chitosan-g-PEG copolymers

MeO-PEG was utilized as the PEG source, by blocking one of the two –OH ends to avoid the crosslinking reaction by the bifunctional OHC-PEG-CHO, in this study. MPEG was oxidized to MPEG-CHO with DMSO/acetic anhydride. The coupling reaction of MPEG-CHO to chitosan was conducted as follows: First, chitosan was dissolved in a mixture of acetic acid and methanol. Aqueous MPEG-CHO was added into the chitosan solution and stirred for 30 minutes at room temperature. After that, the solution was adjusted to pH 6.5 and stirred for one hour at room temperature. Then, aqueous NaCNBH₃ was added dropwise into the solution for 20 minutes to reduce the Schiff’s base. After the mixture was stirred for 18 hours at room temperature, the reaction solution was dialyzed, with 0.05M NaOH and distilled water alternatively, until the pH of dialyzed water reached 7.5. The final dialyzed solution was centrifuged under 37,000 g for 20 minutes. The supernatant was lyophilized and then washed with acetone several times to remove the un-reacted MPEG-CHO. After drying in vacuum, the final powder was obtained as final product, chitosan-g-PEG copolymer.

### Characterization of chitosan-g-PEG copolymers

Infrared (IR) spectra of chitosan-g-PEG copolymers were taken with KBr pellets on Perkin-Elmer System 2000 (U.S.A). Samples for FTIR analyses were scanned from 400 to 4000 cm⁻¹ at a resolution of 4 cm⁻¹. The degree of substitution value of PEG on chitosan was estimated by NMR analysis. Samples (30 mg) were dissolved in 0.7 ml D₂O containing one drop of 20 wt % DC/DC/D.O. ¹H-NMR spectra were recorded on a Varian Unity-INOVA 500 MHz NMR spectrometer, and the chemical shifts were referenced from DDS.

#### Preparation of Chitosan-g-PEG membrane

Chitosan-g-PEG copolymer was dissolved in distilled water to prepare a 2% (w/v) chitosan-g-PEG copolymer solution. The solution was filtered and degassed overnight, and then 5 ml of solution was poured into a petri-dish. This dish was placed in an air-circulating oven at room temperature 7 days to prepare chitosan-g-PEG membrane. In order to preserve the non-toxic characteristics of these two raw materials, we did not use any cross-linking reagents to process or strengthen the chitosan-g-PEG copolymer membranes.

### Contact angle measurements

Contact angle measurement was often conducted to survey the changes of hydrophilicity of tested materials. The contact angles of chitosan-g-PEG copolymer membranes were measured by using a contact angle system (Dataphysics Instruments, GmbH, Germany). A distilled water droplet (20 μl) was released from a syringe and dropped on the prepared membrane, the contact angles were immediately measured using the technique developed by Hamilton [22].

#### Cell adhesion test

Typically, methods used to analyze cell adhesion behaviors on tested material fall into three categories: centrifugation, micromanipulation and hydrodynamic shear. The hydrodynamic shear assay utilizes fluid flow between two parallel plates, and that may generate a wide range of defined forces on cells that are adhered on the material. This assay provides a direct observation of the detachment of adherent cells from the substrate. In addition, the percentage of cells that remain attached after exposure to a given shear stress also

<table>
<thead>
<tr>
<th>Substitution targeted</th>
<th>Low-MW chitosan (70 kDa)</th>
<th>High-MW chitosan (300 kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>LC</td>
<td>HC</td>
</tr>
<tr>
<td>25%</td>
<td>L-25</td>
<td>H-25</td>
</tr>
<tr>
<td>50%</td>
<td>L-50</td>
<td>H-50</td>
</tr>
<tr>
<td>75%</td>
<td>L-75</td>
<td>H-75</td>
</tr>
<tr>
<td>100%</td>
<td>L-100</td>
<td>H-100</td>
</tr>
</tbody>
</table>

L: 70 kDa chitosan, H: 300 kDa chitosan, C: chitosan
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Figure 1. FT-IR spectrums of chitosan derivatives (a) low-MW chitosan (70 kDa), (b)L-50, (c) high-MW chitosan (300 kDa) and (d)H-50 provides information on the adhesive force of cells.

In the present study, the cell adhesion of 3T3 fibroblast cells to chitosan-g-PEG copolymer membrane was measured by using a flow chamber system according to the procedure described by Chang et al [23]. Briefly, the prepared chitosan-g-PEG membrane was cut into 6 cm × 1 cm in size and placed inside the chamber to equilibrate in the culture medium. About 1 ml, 1×10^5 cell/ml of the 3T3 fibroblast cell suspension was injected into the flow chamber and incubated for 2 hours so that the cells could descend and adhere onto the test substrate. 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 was counted under an optical microscope, after various flow rates of culture medium flushing. The fraction of the adhered cells on the substrate was represented by the ratio of adherent cells at the designated flow stress to that at zero flow rate.

Results and Discussion

Preparation of chitosan-g-PEG copolymer

Reductive amination of chitosan is a very attractive synthetic method because it could offer the versatility of introducing a series of substituents. We utilized different mole ratios of MPEG-aldehyde to the amine groups of chitosan, in order to vary the structural patterns and the degree of hydrophilicity along the chitosan chain. IR spectrum of MPEG-aldehyde, compared to the spectrum of initial MPEG, exhibited a new absorption band at 2220 cm^{-1}, which was attributed to the aldehyde (not shown). MPEG-aldehyde exhibited 1H-NMR signal of aldehyde proton at 7.3 ppm, indicating the formation of an aldehyde group (not shown). According to chemical analysis data, we could demonstrate that the hydroxyl groups were successfully oxidized into aldehyde groups. After modification, MPEG-aldehyde was coupled to the amino groups of chitosan, forming a graft copolymer, i.e., chitosan-g-MPEG. In comparison with chitosan, 1H-NMR and FTIR spectra of chitosan-g-MPEG copolymers are illustrated in Figure 1 and 2, respectively. According to FTIR analysis, the coupling of chitosan and MPEG was verified by the decrement of the NH₂ scissoring peak of the primary amine at 1660 cm⁻¹ and the increment of the ether band of MPEG at 1050~1300 cm⁻¹. While performing 1H-NMR spectrums of chitosan-g-MPEG copolymers, we could get a remarkable peak of chitosan by further addition DCl in chitosan-PEG D₂O solution. Chitosan derivatives containing MPEG were characterized by a new signal at δ = 3.40 ppm, which was attributed to the oxyethylene group present in the copolymer. On the basis of 1H NMR calculation, the substitution results in chitosan derivatives are shown in Table II. Obviously, the obtained graft ratios increased with feed ratios, and a similarity between targeted and obtained graft ratio was found.
Table II. Chitosan derivatives with MPEG substituent

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>Substitution targeted</th>
<th>Substitution obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-25</td>
<td>25%</td>
<td>25.6%</td>
</tr>
<tr>
<td>L-50</td>
<td>50%</td>
<td>49.6%</td>
</tr>
<tr>
<td>L-75</td>
<td>75%</td>
<td>69.7%</td>
</tr>
<tr>
<td>L-100</td>
<td>100%</td>
<td>91.4%</td>
</tr>
<tr>
<td>H-25</td>
<td>25%</td>
<td>25.0%</td>
</tr>
<tr>
<td>H-50</td>
<td>50%</td>
<td>54.3%</td>
</tr>
<tr>
<td>H-75</td>
<td>75%</td>
<td>73.6%</td>
</tr>
<tr>
<td>H-100</td>
<td>100%</td>
<td>91.3%</td>
</tr>
</tbody>
</table>

L: 70 kDa chitosan; H: 300 kDa chitosan

Table III. Contact angles of the membranes of chitosan derivatives and pure chitosan

<table>
<thead>
<tr>
<th>Sample</th>
<th>Contact angle (°)</th>
<th>Sample</th>
<th>Contact angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-25</td>
<td>45.8±1.9</td>
<td>H-25</td>
<td>24.1±4.6</td>
</tr>
<tr>
<td>L-50</td>
<td>23.4±3.0</td>
<td>H-50</td>
<td>32.1±4.7</td>
</tr>
<tr>
<td>L-75</td>
<td>30.3±2.3</td>
<td>H-75</td>
<td>34.9±3.0</td>
</tr>
<tr>
<td>L-100</td>
<td>29.1±6.2</td>
<td>H-100</td>
<td>59.2±3.5</td>
</tr>
<tr>
<td>LC</td>
<td>85.7±2.6</td>
<td>HC</td>
<td>83.5±3.0</td>
</tr>
</tbody>
</table>

The values are means ± SD.

Prior to the following experimental tests, we first examined the hydrated status of these chitosan-g-PEG copolymer membranes and found that the membranes remained at their initial status and became hydrogel form after being immersed in distilled water about 4 hours, and completely dissolved 72 hours later.

**Contact angle measurement**

The hydrophilicity of chitosan membrane was increased by grafting hydrophilic PEG molecules on the chitosan (chitosan-g-PEG membranes), as indicated by the results obtained from contact angle measurement. As shown in Table III, Figure 3 and Figure 4, the contact angle decreased significantly on the chitosan-g-PEG membranes in comparison to the pure chitosan membrane (from 85.7° reduced to about 30°). These results, together with NMR and FTIR analysis, revealed that the PEG molecules were grafted onto the chitosan and thus effectively increased the membrane hydrophilicity.

**Cell adhesion test**

Understanding the relationships between materials and cellular responses is important in design biomaterials, such as development of cell adhesion beneficial materials in cell culture application or contrarily, anti-adhesive materials for preventing postoperative intraperitoneal adhesions. One common approach to measure the adhesion strength between cells and substrate is using a parallel plate flow chamber system, which exposes adherent cells to a designed steady laminar flow and measure the cell detachment as a function of wall shear stress. This flow chamber system can provide
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Figure 4. Plots of contact angles of different chitosan membranes and targeted MPEG substitutions on chitosan

![Contact angle plots](image)

Figure 5. The attachment of 3T3 cells to different materials after 3-hour incubation

![Cell attachment images](image)

Figure 6. Retention of 3T3 cells on different materials as a function of shear stress

![Retention ratio graph](image)

real-time, in-situ image analysis for studying cells adhesion and deposition, and it further possesses functionality augmentation, such as adding different chemical or physical stimulants onto the adherent cells simultaneously and real-time observation of the behaviors of cells acquired.

In this study, we chose L-20 and H-50 membranes as the test samples to carry out flow chamber tests to evaluate the anti-adhesion of chitosan-g-PEG membranes. The adhesion of 3T3 fibroblast cells to the chitosan-g-PEG membranes surface with different hydrophilicity and pure chitosan membrane is shown in Figure 5. As expected from cell adherent behavior in general, the 2-hr incubated and aggregated 3T3 cells exhibited a greater adhesion on pure chitosan membrane than the ones on the H-50 and L-50 substrate. On the other hand, the poor adhesion for modified chitosan membranes was probably caused by the induction of grafted PEG chains to the chitosan backbone that led to a more hydrophilic skin and swollen surface. A reasonable picture could be visualized that the 3T3 cells could lose their footing more readily on those modified membrane surfaces. The fraction of adherent cells, normalized to the initial number of cells, was plotted as a function of shear stress (Fig. 6). As shown, remarkable differences in cell adhesion ratios were observed between chitosan-g-PEG membranes, pure chitosan membranes and glass. Most 3T3 cells were flushed out from the chitosan-g-PEG membranes surfaces by a low flow shear stress of 2.4 dyne/cm². The 3T3 cells adhered on pure chitosan membrane surface exhibited a stronger resistance on the medium flow and gradually flushed out on the increasing flow shear stress. However, 3T3 cells adhered well on the glass at the shear stress of 29 dyne/cm² (retention ratio was 60%). This indicated that even the conventional glass surface is high hydrophilic, because of its rigidity and unyieldingness at the interface, cells still held onto the surface well. One might also speculate that the copolymer membrane surface could provide a hydrophilic surface with deep-cushion characteristics.

As reported from other investigations [22, 24-26], cell attachment to material surface might be influenced by the physical and chemical properties of the material surface. Hydrophilicity is one of the principle parameters that affecting the adhesion of proteins or cells to a surface. It is accepted that the adhesion of proteins or cells to the surface where they have anchored would affect the cells’ migration, proliferation and differentiation. Consequently, researchers have devoted much effort to modifications of the surface hydrophilicity of materials by grafting hydrophilic functionalities onto the materials’ surfaces. PEG of preferable hydrophilicity and biocompatibility, is widely used as a reactant to react with the target material and is able to change the obtained material surface’s hydrophilicity. In this paper, we prepared chitosan-g-PEG copolymers with different levels of hydrophilicity as compared to pure chitosan. The decreased
contact angles and lower cell adhesion revealed that the membranes made by these copolymers exhibited distinct properties in a spectrum of hydrophilicities from those of neat chitosan to those of neat PEG. Furthermore, high solubility of these copolymers in water and neutral pH range also provides additional advantages such as offering soft and swelling interfaces in applications that involve a host of complex interaction between cells and surfaces of barrier materials.

### Conclusion

In this present study, we modified chitosan by grafting PEG through Schiff’s base reaction and obtained the highly hydrophilic copolymers, chitosan-g-PEG. These chitosan-g-PEG copolymers were more soluble in water and exhibited an anti-adhesion tendency toward cells as compared to ordinary chitosan material. Further experiments are ongoing to find out the optimal conditions for preparing anti-adhesion modified chitosan copolymers, based on the results obtained to date.

### References