Preparation of Carbon Nanotube-alginate Nanocomposite Gel for Tissue Engineering

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Received August 29, 2006/Accepted September 22, 2006

A novel scaffold material based on an alginate hydrogel which contained carbon nanotubes (CNTs) was prepared, and its mechanical property and biocompatibility evaluated. Soluble CNTs were prepared with acid treatment and dispersed in sodium alginate solution as a cross-linker. After which, the mechanical property (elastic deformation), saline sorption, histological reaction, and cell viability of the resultant nanocomposite gel (CNT-Alg gel) were evaluated. The CNT-Alg gel showed faster gelling and higher mechanical strength than the conventional alginate gel. Saline sorption amount of freeze-dried CNT-Alg gel was equal to that of the alginate gel. In terms of histological evaluation and cell viability assay, CNT-Alg gel exhibited a mild inflammatory response and non-cytotoxicity. These results thus suggested that CNT-Alg gel could be useful as a scaffold material in tissue engineering with the sidewalls of CNTs acting as active sites for chemical functionalization.

Key words : Carbon nanotube, Alginate, Scaffold material

INTRODUCTION

Since their discovery in 1991, carbon nanotubes (CNTs) have captured the public imagination and been catapulted into research limelight due to their diverse range of beneficial features: remarkable mechanical properties, unique electric properties, as well as high thermal and chemical stability. However, due to their insolubility in organic or aqueous solution, CNTs have very limited usefulness in chemical and biochemical applications. On this note, soluble CNTs in aqueous systems are of immense interest to many researchers since it implies the beginning of many chemical and biomedical designs to create functional CNTs.

To date, some attempts have been made to dissolve or disperse CNTs in aqueous or organic solution by chemical or physical modification of CNTs. For example, Hamon et al. found that CNTs could be dissolved and dispersed in an aqueous solution when they were subjected to surface chemical modification by ultrasonication in strong acids such as H$_2$SO$_4$ and HNO$_3$. This acid treatment produces carboxylic acid groups on CNTs. The carboxylic acid groups on CNTs then allow the formation of ester, ionic, or amide linkages with various functionalities. Hydrolyzed CNTs, i.e., CNTs with carboxylic group, are more hydrophilic than the original CNTs, and can be combined with hydrogels such as alginate. It is expected that the nanocomposite of hydrolyzed CNTs and hydrogel will be useful for biomedical applications, such as carrier for drug delivery system or scaffold for tissue engineering. This is because some drugs can be encapsulated into CNTs and that CNTs have high affinity towards proteins and DNA.

Alginate is a bioresorbable seaweed-derived polysaccharide composed of β-D-mannuronic acid and α-L-guluronic acid. Although calcium alginate gel sheets have been applied as surgical dressings and scaffolds for regenerating dermis, nerve, and bone, it has been reported that calcium alginate gel exhibited calcium ion cytotoxic effect on fibroblasts via its extract medium. In a bid to circumvent the cytotoxicity effect, Suzuki et al. produced an alginate dressing gel cross-linked with collagen and showed that the gel could reduce the cytotoxicity to fibroblasts as well as foreign body reaction. However, the alginate gel remained weak and a further improvement in the mechanical properties of the gel...
is still needed. One approach to improve the mechanical properties of alginate gel is to forge a higher degree of cross-linking between the chains using cross-linking chemicals. In this connection, the aim of the present study was to improve the mechanical properties of alginate gel by using chemically functionalized carbon nanotubes (CNTs) as a cross-linking agent.

Indeed, we expected the mechanical properties of alginate gel to be improved by means of incorporating functionalized CNTs as a cross-linking agent. The resultant alginate gel would then have potential applications as a scaffolding material or carrier of drug molecules and cytokines for tissue engineering. In this paper, we described the preparation of an alginate-CNT nanocomposite gel (CNT-Alg gel) and examined its gelling characteristics, microstructure, histopathological changes, and cell viability.

### MATERIALS AND METHODS

**Preparation of water-soluble CNTs**

CNTs (400 mg, single-walled type, main range diameter: <2 nm, length: 5-15 μm; L-SWNT, Shenzhen Nanotech Port, Shenzhen, China) were poured into a mixture of 300 ml of sulfuric acid and 100 ml of nitric acid, and the mixture was sonicated in a bath-type sonicator for seven hours at 40°C. After this treatment, the product was vacuum-filtrated using a PTFE membrane with a pore size of 0.2 μm. Resultant solid was then washed with deionized water and dried in a vacuum oven for 24 hours at 80°C. It has been previously shown that after this treatment, carboxylic groups were left on the CNTs. Fig. 1 shows a photo of CNTs in water (left: untreated CNTs, right: treated CNTs). Untreated CNTs were insoluble in water. It was evident that an optically transparent black-colored dispersion/solution of CNTs was obtained by this treatment.

**Preparation of CNT-Alg gel**

Two percent of sodium alginate (Wako Chemical, Osaka, Japan) and CNTs (0.05 or 0.1 g) were first dissolved in 30 ml of deionized water, and then ethylenediamine-di(hydroxysuccinimide) (EDSI) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (WSC, Wako Chemical) were added to the solution. EDSI was synthesized by reaction of ethylenediamine and hydroxysuccinimide in ethyl acetate according to a previously described method. The solution was poured into a plastic mold (50×30×15 mm) and allowed to set for 24 hours at 25°C.

Table 1 shows the concentration of each component, whereby four different types of CNT-Alg gel were prepared. EDSI and WSC concentrations were employed as shown in Table 1 because preliminary experiment revealed that these concentrations enabled gelation within a mere few hours, hence making it easier to prepare the test samples. Note that alginate gels without CNTs were also prepared.

**Elastic deformation of CNT-Alg gel**

Elastic deformation of the prepared CNT-Alg gels was monitored over a 24-hour period. A stainless steel probe (10-mm diameter and 5-mm thickness) with a digital dial gauge (ID-U1025, Mitutoyo, Kawasaki, Japan) was placed on the surface of the gel with an 80-g load (weight of the probe) in the mold for 10 seconds, and compressive deformation was re-
corded at six, 12, and 24 hours after being poured into the mold (n=3). Compressive deformation (%) was then calculated using the following equation:

\[
\text{Compressive deformation (\%)} = (L_0/L_1) \times 100
\]

where \(L_0\) and \(L_1\) are the thickness of gel before and after loading, respectively. All measurements were carried out in an incubator at 37°C.

**SEM observation**

At 24 hours after setting, CNT-Alg gel was washed with deionized water for 24 hours to remove unreacted reagents, freeze-dried, and then subjected to SEM observation. Microstructure of the freeze-dried CNT-Alg gel was observed using a SEM (JEOL T-330, Tokyo, Japan) after ion-coating with platinum.

**Saline sorption amount of freeze-dried CNT-Alg gel**

Blocks of freeze-dried gel (10×10×10 mm) were cut to prepare the specimens for saline sorption measurement. Each specimen (n=3) was placed on a polyurethane sponge (5×5×5 cm) saturated with saline at 37°C for 24 hours, and the amount of saline sorption (weight gain per initial weight) was measured.

**Cell viability assay**

Two prepared gels, CNT-Alg-4 and Alg-2 (with and without CNTs respectively, but with the same EDSI and WSC concentrations), were subjected to cell viability assay. Pulverized freeze-dried gel (0.5 mg) was placed in each well of a 24-well multilayered plate and sterilized by irradiation with ultraviolet light for 48 hours. MG-63 osteoblast-like cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 1% non-essential amino acids. Then, 1 ml of cell suspension (1×10^6/ml) was added to each well and incubated for five days at 37°C in a humidified 5% CO₂ atmosphere. After the five-day incubation, the medium was removed and cells were washed with Dulbecco’s phosphate-buffered saline. Into each well again was added 1 ml of medium containing 0.2 ml of CellTiter 96 Aqueous MTS (Promega Corp., Madison, WI), and the plate was incubated for two hours at 37°C in a humidified 5% CO₂ atmosphere. Optical density was measured with a SpectraMax Plus spectrophotometer (Molecular Device Corp., Sunnyvale, CA) at 490 nm, with 650 nm used as the reference wavelength. Cell viability of CNTs (0.5 mg in well), which was expressed as a percentage compared to untreated CNTs, acid-treated CNTs, and alginate gel without CNTs (n=5), which were also assayed.

**Implantation and histological evaluation**

Animal experiments were performed in accordance with the ethical guidelines for animal experiments of Fukuoka Dental College. CNT-Alg gel was implanted into the back of six-week-old male Sprague-Dawley rats weighing approximately 200 g. Surgery was performed under general anesthesia induced by an injection of 0.4 ml/kg pentobarbital sodium (Nembutal, Dainippon Pharmaceutical Co. Ltd., Osaka, Japan). An incision was made in the back, and a 40-mg sample (10×10×10 mm) was implanted subcutaneously in the rat (total 18 rats). After implantation, the soft tissues were closed in separate layers by suturing with intracutaneously resorbable Vicryl 3-0 (Ethicon Inc., Somerville, NJ).

At different observation times (seven and 15 days), the animals were sacrificed by injection with an overdose of Nembutal. After the animals were sacrificed, the subcutaneous tissues containing the implanted samples were immediately excised. Following fixation in 10% neutral buffered aqueous formaldehyde solution (pH 7.4), the specimens were prepared for histological evaluation. The tissue blocks were dehydrated through a graded series of ethanol solutions and then embedded in paraffin. Embedded samples were sectioned at 3 mm thickness with a microtome (Dx 78-11, Erma Optical Works Ltd., Tokyo, Japan), and sectioned samples were stained with Mayer’s hematoxylin-eosin solution (Merck Ltd., Tokyo, Japan). Tissue reactions were observed under a Nikon Eclipse 5i light microscope (Nikon, Tokyo, Japan).

**Statistical analysis**

Statistical analysis was performed using one-way ANOVA for elastic deformation, saline sorption, and cell viability, followed by Scheffe’s multiple comparison test at 5% level of significance.

**RESULTS**

**Elastic deformation of CNT-Alg gel**

The change in elastic deformation of CNT-Alg gel as a function of time is shown in Table 2. Gel formation was affected by the concentrations of EDSI and WSC. Gels with lower EDSI and WSC concentrations (CNT-Alg-1 and Alg-1 of Table 1) were too weak for elastic deformation evaluations before six hours. Higher concentrations of EDSI and WSC accelerated the gel formation of composites and improved the resistance against elastic deformation. At all measuring periods, CNT-Alg gels showed significantly lower deformation (p<0.05) than the alginate gels of Alg-1 and Alg-2 (controls without CNTs). Further, increase in CNT content improved the resistance against deformation of CNT-Alg gels, as shown by the deformation results of CNT-Alg-4 versus CNT-Alg-2 and CNT-Alg-3.
Microstructure of CNT-Alg gel
Freeze-dried CNT-Alg gel exhibited a black-colored sponge-like appearance (Fig. 2). In SEM images of the cross-section of CNT-Alg-4 (Fig. 3), interconnected pores with 100-200 μm diameter were observed. There were no apparent differences in structure among the four types of CNT-Alg gel and the alginate gels without CNTs.

Saline sorption of the gels
Higher concentrations of CNTs and EDSI tended to result in lower amount of saline absorption, although there were no significant differences in the amount of saline absorption for different CNT-Alg gels and alginate gels without CNTs (Fig. 4) (p > 0.05).

Cell viability
Fig. 5 shows the cell viability results of CNTs, CNT-Alg-4, and Alg-2. Control refers to cell viability on tissue culture treated polystyrene well. There were no significant differences among control, CNTs, CNT-Alg-4, and Alg-2 (p > 0.05).

Histopathological findings
Representative histopathological images of rat subcutaneous tissues after the implantation of CNT-Alg-4 and Alg-2 gels are shown in Fig. 6. Five days after implantation, there was acute inflammatory reaction to the gel, and no evidence of any degradation or breakdown of the implanted CNT-Alg-4 gel (arrow in Fig. 6A). Likewise, Alg-2 gel showed a similar observation after five days' implantation. After 15 days' implantation, both the CNT-Alg-4 and Alg-2 gels showed the formation of granulation tissue surrounding the gel particles as well as slight cellular infiltration (arrow in Fig. 6B and Fig. 6D).

<table>
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<tr>
<th>Sample Code</th>
<th>Time (hr)*</th>
<th>CNT-Alg-1</th>
<th>CNT-Alg-2</th>
<th>CNT-Alg-3</th>
<th>CNT-Alg-4</th>
<th>Alg-1</th>
<th>Alg-2</th>
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<td></td>
<td>37.7% (0.9)</td>
<td>31.9% (0.4)</td>
<td>23.3% (0.9)</td>
<td>–</td>
<td>49.7% (0.6)</td>
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<td>49.7% (0.6)</td>
<td>29.4% (1.5)</td>
<td>22.9% (0.8)</td>
<td>15.7% (0.6)</td>
<td>54.6% (1.3)</td>
<td>43.3% (0.4)</td>
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<tr>
<td></td>
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<td>10.5% (0.6)</td>
<td>33.2% (0.7)</td>
<td>31.2% (0.7)</td>
</tr>
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</table>

*: Number of setting hours after gel was prepared

Table 2 Changes in elastic deformation (%) of CNT-Alg and alginate gels. Results are shown as mean ± SD. There were significant differences in elastic deformation among the types of gels within each reaction time (p < 0.05) and among the reaction times within each gel group (p < 0.05)

Fig. 2 Freeze-dried CNT-Alg gel with absorbed saline.

Fig. 3 SEM microphotographs of the cross-section of freeze-dried CNT-Alg-4 gel (A) and alginate gel (B: Alg-2, control).
In the present study, a new type of nanocomposite gel was prepared from water-soluble carbon nanotubes and alginate. Alginate gels have the capability for controlled release of growth factors and cytokines. Previously, a hydrogel which consisted of alginate and heparin which was used as an artificial extracellular matrix was prepared, and the release behavior of heparin reported accordingly\(^1\)\(^-\)\(^16\). Although this alginate-heparin hydrogel could bind basic fibroblast growth factor (bFGF), the release rate of bFGF in PBS was very fast. This disadvantage of initial burst in bFGF

**Fig. 4** Saline sorption of CNT-Alg gels and alginate gels after 24 hours. There were no significant differences in saline sorption among all of gels.

**Fig. 5** Cell viability of CNT-Alg gel (CNT-Alg-4), alginate gel (Alg-2), acid-treated nanotubes (CNTs), and untreated nanotubes (cont.). There were no significant differences in cell viability between each sample group.

**Fig. 6** Representative light microscopic views of a subcutaneous tissue with implantation of CNT-Alg gels after 5 days (A) and 15 days (B), and of alginate gel after 5 days (C) and 15 days (D).
release from the gel could be reduced by improving the binding and stabilizing of bFGF in the gel. To improve the stabilizing of bFGF in the hydrogel, an effective approach would be to incorporate functionalized single-wall CNTs into the hydrogel. The desired result would be CNT-Alg gel with controlled release of various growth factors, so that it can potentially deliver and modulate the local concentration of growth factors for prolonged periods of time.

Further, we expected the incorporation of soluble CNTs into alginate hydrogel to increase the cross-linking density between polysaccharide chains. Results of this study revealed that CNT-Alg gel indeed showed a faster gelling time and a superior resistance to compressive deformation. This finding suggested that CNTs effectively played the role of crosslinker in the alginate hydrogel. Nonetheless, mechanical properties of CNT-Alg gel in the present study were not adequate for measuring elastic recovery or shear strength. To increase the mechanical properties of CNT-Alg gel, more improvements in terms of cross-linking density or surface modification would be needed.

As-produced CNTs had a highly hydrophobic nature, and existed as bundles (aggregation of tubes) of 10-30 nm in diameter because of strong van der Waals interactions. This hydrophobic nature made the CNTs insoluble in water and organic solvents. By using a strong acid treatment in water under sonication, iron and nickel which are catalysts used for forming carbon nanotubes were removed, and the purity of CNTs thereby improved. This acid treatment also produced defect sites (carboxylic acid and quinone moieties) on the sidewalls of CNTs, thereby increasing the hydrophilic nature of CNTs and synthesizing soluble CNTs as a result. Alternatively, water-soluble CNTs can be obtained by functionalization via covalent bonding with polyethylene glycol, glycosamine, and DNA. In the present study, the carboxylic acid groups on the sidewalls of CNTs could be used for covalent bonding of primary amine molecules through amide bonds facilitated by the coupling reagent N-hydroxysuccinimide with ethyldimethylaminopropyl carbodiimide hydrochloride (WSC).

Freeze-dried CNT-Alg gel obtained in this study exhibited a sponge-like appearance. As shown in Fig. 3, SEM observation of the cross-section of CNT-Alg gel showed interconnected pores as with the alginate gel. In terms of saline sorption, there were no significant differences between the CNT-Alg gel and alginate gel. These findings thus suggested that the incorporation of CNTs into alginate gel improved the latter’s mechanical property without any change in the original microstructure.

If CNT-based materials were to be used as a novel scaffold material, it is mandatory to evaluate their toxicity and biocompatibility. It has been reported that CNTs inhibited HEK293 human embryo kidney cell growth by inducing apoptosis and decreasing cell adhesion ability. However, several studies have claimed otherwise whereby CNTs appeared non-toxic once internalized into mammalian cells and without any apparent adverse effect to cell viability. Furthermore, Wong et al. evaluated the application of the preparation CNTs as intracellular protein transporters, and found that the proliferation and viability of HL60 cells were unaffected by the internalized CNTs. Differences in CNT toxicity and biocompatibility findings and results could be due to the preparation method. Nimmagadda et al. investigated the physical and chemical effects of different SWNT (single-walled carbon nanotube) preparation methods on cell viability and metabolic activity. It was found that the cell viability and metabolic activity of 3T3 fibroblasts was dependent on SWNT preparation method and concentration. They suggested that the removal of production contaminants and the reduction of CNTs’ hydrophobicity played an important role in positively influencing cell-CNT interactions.

In the present study, we evaluated the in vitro toxicity using MG-63 osteoblast-like cells and in vivo tissue compatibility by implanting samples in the subcutaneous tissue in the back of rats. The incorporation of CNTs into alginate did not show any cytotoxicity, and CNT-Alg gel showed mild tissue response but no adverse effect. It was presumed that the acute mild tissue response was caused by virtue of the implantation surgery. To date, with the aim of functionalizing CNTs for biomedical applications, various kinds of chemical surface modification of CNTs have been successfully achieved such as esterification, non-covalent functionalization with DNA, and non-specific adsorption of proteins. Nonetheless, further studies on the chemical modification of CNTs will be needed to elucidate the release of some drugs to diminish the acute mild tissue response, as well as the release of growth factors to expedite the healing process.

This was the first study on CNT-Alg nanocomposite gel, whereby cell viability and histopathological evaluation were performed using only one type of prepared CNT-Alg gel, CNT-Alg-4. As CNT-Alg gel will be applied to the living tissue, the ease of application or handling of CNT-Alg gel into tissue defects as well as metabolism in living tissue will be important factors to consider. These factors would be further studied and their findings revealed in the next series of our experiment.

In summary, the present study showed that CNT-Alg gel could be prepared using water-soluble carbon nanotubes. Further, results of the present study indicated that the prepared gel has potential for use as a scaffold or drug delivery system for tissue re-
generation. However, to realize its usefulness in biomedical applications, known drawbacks related to the binding and release behavior of growth factors in CNT-Alg gel must be addressed.

ACKNOWLEDGEMENTS

This study was supported by a Grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 17592057).

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