Microfluidic wet spinning of chitosan-alginate microfibers and encapsulation of HepG2 cells in fibers

Bo Ram Lee,^{1,a)} Kwang Ho Lee,^{1,2,a)} Edward Kang,¹ Dong-Sik Kim,³ and Sang-Hoon Lee^{1,b)}

¹*Department of Biomedical Engineering, College of Health Science, Korea University, Seoul 136-703, South Korea*

²*Department of Mechanical Engineering, Massachusetts Institute of Technology, Massachusetts 02139, USA*

³*Department of Surgery, College of Medicine, Korea University, Seoul 136-703, South Korea*

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The successful encapsulation of human hepatocellular carcinoma (HepG2) cells would greatly assist a broad range of applications in tissue engineering. Due to the harsh conditions during standard chitosan fiber fabrication processes, encapsulation of HepG2 cells in chitosan fibers has been challenging. Here, we describe the successful wet-spinning of chitosan-alginate fibers using a coaxial flow microfluidic chip. We determined the optimal mixing conditions for generating chitosan-alginate fibers, including a 1:5 ratio of 2% (w/w) water-soluble chitosan (WSC) solution to 2% (w/w) alginate solution. Ratio including higher than 2% (w/w) WSC solution increased aggregation throughout the mixture. By suspending cells in the WSC–alginate solution, we successfully fabricated HepG2 cell-laden fibers. The encapsulated HepG2 cells in the chitosan-alginate fibers were more viable than cells encapsulated in pure alginate fibers, suggesting that cross-linked chitosan provides a better environment for HepG2 cells than alginate alone. In addition, we found that the adhesion of HepG2 cells on the chitosan-alginate fiber is much better than that on the alginate fibers. © 2011 American Institute of Physics. [doi:10.1063/1.3576903]

I. INTRODUCTION

Regenerative medicine is attracting considerable interest and is expected to resolve many obstacles of disease treatment.^{1,2} In this research, biodegradable and biocompatible natural biopolymers are crucial to the production of scaffolds,³ and several biopolymers have been developed and widely applied.⁴ Among these, calcium alginate and chitosan have been extensively studied for use in cellular encapsulation, drug delivery, and tissue engineering.^{5,6} Calcium alginate, which is obtained from brown algae, has excellent biocompatibility, low toxicity, and it easily forms a hydrogel in the presence of divalent cations.⁷ Chitosan, which is characterized by excellent biostimulating properties that facilitate the reconstruction and vascularization of damaged tissue, is one of the most suitable scaffold materials for liver tissue because its structure resembles that of glycosaminoglycans, which are natural components of the liver extracellular matrix.⁸ Although these two biopolymers have been used separately in biomedical engineering, each biopolymer carries intrinsic limitations. Alginate lacks the ability to promote specific cell-recognition signals, by which anchorage-dependent cells interact with the matrix. The hydrophilic nature of alginate prevents the absorption of serum proteins, which limits anchorage-sensitive

^{a)}Both authors contributed equally to this paper.

^{b)}Author to whom correspondence should be addressed. Tel.: ++82 2 940 2881. FAX: ++82 2 921 6818. Electronic mail: dbiomed@korea.ac.kr.

cells, such as hepatocytes, from promoting specific cell interactions or performing various cellular functions including migration, proliferation, and specific gene expression.^{9,10} Chitosan, on the other hand, is mechanically weak and difficult to handle and to form a scaffold structure. Furthermore, encapsulation of cells is very challenging.^{11–13} Therefore, a chitosan-alginate composite could address the limitations of the individual biopolymers. An important requirement for the practical application of biopolymers to tissue engineering is the ability to construct desired biopolymer scaffold structures and to load cells into the scaffold in a stable and easy manner. Microscale biopolymer fibers have attracted much attention as scaffolds for guided cell culture or cell delivery.^{14,15} However, the spinning of uniformly sized fibrous structures from the biopolymer and encapsulation of cells in the fiber without damage using a simple method under the mild environment has been challenging.^{16–18} We and other researchers have suggested the production of alginate or chitosan microfibers using microfluidic chips; however, the microfluidic fabrication of chitosan-alginate fibers has not previously been attempted. A two-step method based on a conventional spinning apparatus (first stage: alginate fibers were formed; second stage; fibers were immersed in an acid bath, including partially hydrolyzed chitosan)^{16,19} was tried to produce chitosan-alginate fibers, but this process is complicated, time-consuming, and the uniformity of the distribution of chitosan throughout the alginate fiber has been limited. In addition, encapsulation of cells into these fibers is still very challenging.

In this paper, we report the production of chitosan-alginate microfibers and encapsulation of living HepG2 cells using a microfluidic chip. There are several factors to producing chitosan-alginate fibers stably and to encapsulating living cells safely including the mixing ratio of both biopolymer and flow conditions; we proposed the proper conditions for this end. Generally, encapsulation of cells in chitosan is very restricted because of the lack of solubility in neutral pH solution. The chitosan is usually dissolved in an aqueous acetic acid that is harmful to the encapsulated cells, which makes chitosan encapsulation difficult. To address this problem, we used the water-soluble chitosan. But the mechanical weakness of pure water-soluble chitosan hinders the stable production of cell-encapsulated microscale fiber. Using the mixture of alginate-chitosan, we successfully encapsulated HepG2 cells in the fibers and confirmed their viability over a 1 week culture. The microfluidic method demonstrated that the fiber size could be controlled under atmospheric conditions and stable encapsulation of cells was achievable. The chemical and mechanical properties of fibers were characterized by measuring the attenuated total reflectance-Fourier transformed infrared spectrum (ATR-FTIR) and the stress-strain relations. HepG2 cells were seeded and cultured on the fiber surfaces, and they adhered to the chitosan-alginate fibers better than to the alginate fibers, proving the effect of chitosan for good cell-extracellular matrix interaction. The aggregation of cells was also observed over time. HepG2 cells were successfully encapsulated within the chitosan-alginate fibers, and their behaviors and viability were measured after 1 week of culturing.

II. MATERIALS AND METHODS

A. Materials

To produce a 2% (w/w) water-soluble chitosan solution, 2 g of high molecular weight water-soluble chitosan powder (WSC) (Kittolife) was dissolved in 98 g of de-ionized water solution. A 2% (w/w) sodium alginate solution was also prepared by dissolving alginic acid powder (Sigma Aldrich) in de-ionized water. A 100 mM calcium chloride (Samchun Pure Chemical Co., Ltd.) solution was used to induce gelation upon mixing with the WSC and alginate solutions. The filter was obtained from Adventec, Inc., and the polydimethylsiloxane (PDMS) was purchased from Dow Corning. The borosilicate capillary glass micropipettes, which were used to focus the flow, were obtained from Sutter Inc.

B. Production of chitosan-alginate microfibers

Continuous chitosan-alginate microfibers were obtained using a microfluidic device.¹⁹ The schematic for the microfluidic device is shown in Fig. 1(a). It was fabricated by combining the

FIG. 1. (a) Schematic for the microfluidic device and (b) detailed view of fiber generating part.

PDMS platform and the pulled borosilicate glass tip. A detailed view of the microfluidic device is shown in Fig. 1(b). The inner diameter of the glass tip was $100\ \mu\text{m}$. The continuous microfibers were formed at the interface between the sample and sheath solutions. The solutions containing 2% (w/w) WSC and 2% (w/w) alginate, mixed at a ratio that varied from 1:1 to 1:5, were introduced into the sample inlets, optimizing the mixing ratio to produce the qualified fibers. When the WSC and alginate solution were initially mixed, they aggregated due to ionic interactions. The aggregated suspension was removed by filtering through a cellulose acetate membrane (pore size: $0.2\ \mu\text{m}$) before wet spinning. The cylindrical chitosan-alginate microfibers, which distributed chitosan uniformly, were obtained by inducing gelation of the filtered WSC-alginate solution upon introduction to the calcium chloride solution at the sheath inlet.

C. Analysis of chitosan-alginate fibers

The composition of the WSC-alginate mixture before and after gelation was characterized by ATR-FTIR spectrometry (PerkinElmer series 2000, Beaconsfield, UK). To this end, three types of samples were prepared: (1) a WSC solution, (2) an alginate solution, and (3) a gelled matrix formed by mixing the WSC-alginate solution with CaCl_2 . Each spectrum was formed by coaddition of six scans at a resolution of $4\ \text{cm}^{-1}$, allowing detection of a frequency range from 4000 to $380\ \text{cm}^{-1}$. The microstructure of the chitosan-alginate fibers was examined by field emission scanning electron microscopy (FE-SEM) (JEOL). The samples were dehydrated in 70%, 80%, 90%, and 100% ethanol for 20 min each, and then dried overnight in a vacuum drier.¹⁹ The chitosan-alginate microfibers, which were fabricated under a variety of flow rates, were collected. The fiber diameters were measured by optical microscopy in the aqueous solution to prevent the distortion. The elastic properties of the chitosan-alginate and calcium alginate fibers were evaluated. Elasticity was measured by fixing one end of a length of fiber [mean length of 14 cm (diameter of $100\ \mu\text{m}$)] on a glass plate and applying force to the other end by hanging a series of adhesive masses sequentially. During the measurement, distilled water (DI) water was sprayed around specimens to prevent dehydration of the fibers. The percentage elongation of each fiber was then measured.

D. Cell preparation

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO), and 1% antibiotics containing 10 000 units (GIBCO)

FIG. 2. (a) Optical images of the chitosan-alginate fibers and (b) SEM image of fiber's surface.

penicillin and streptomycin at 37 °C under 5% CO₂ and 95% atmospheric air. Cells were detached using a solution containing 0.25% trypsin EDTA 1X (GIBCO) for 2–3 min at 37 °C. The viability of the encapsulated cells was tested by incubating the cell-loaded chitosan-alginate or alginate fibers in a CO₂ incubator for 7 days. Subsequently, aliquots of the fibers were removed every 2–3 days, treated with LIVE/DEAD assay reagents (LIVE/DEAD[®] Viability/Cytotoxicity Kit, Molecular Probe) for 20 min at 37 °C, and evaluated by fluorescence microscopy (AxioVision4, Germany) to determine cell viability. The viability was calculated by the ratio of live cell/(dead+live cell)

E. HepG2 seeding on the fiber surfaces and encapsulation

Cell adhesion, proliferation, and viability on the surface of and within the chitosan-alginate or calcium alginate microfibers were evaluated and compared. HepG2 cells were seeded on the surface of chitosan-alginate to investigate the cellular behavior in the presence of chitosan. The cells were also encapsulated within both fibers. Approximately 2×10^6 cells in 1 ml medium were seeded and cultured on the fibers. For encapsulation, confluent cells were resuspended in the filtered WSC-alginate solution after trypsinization. The suspension density was adjusted to 1×10^6 cells/ml. By injection of the WSC-alginate–cell solution into the sample or sheath inlets, cell-loaded chitosan-alginate fibers were generated. To prevent the fibers from degrading, 1 ml calcium chloride solution was added to 5 ml medium. Every 3 days the culture medium was replaced by fresh medium. Experiments were performed over a period of 7 days, and the morphology of the cells cultured on the fibers was examined by fluorescence microscopy (AxioVision4, Germany).

III. RESULTS

A. Fabrication of chitosan–alginate fibers and characterization

The chitosan-alginate fibers were fabricated successfully, as shown in Fig. 2. To allow for the combination of chitosan-alginate, we found that the 1:5 mixing ratio of 2% (w/w) water-soluble chitosan (WSC) solution to 2% (w/w) alginate solution was appropriate. When the ratio was higher, the aggregation of both biomaterials increased. In contrast, the fiber fabrication was unstable at a mixing ratio lower than 1:5 (results were not shown). The diameters of the fibers varied with the flow rates of sample and sheath flows, and the results are plotted in Fig. 3. For each fixed sheath flow rate (10, 15, or 20 ml/h), the core flow rate varied from 4 to 16 ml/h. The fiber diameter increased almost linearly with increasing core flow rate at each fixed sheath rate. The mechanical properties of the chitosan-alginate fibers were evaluated by measuring the stress–strain relation (Fig. 4). The mean value for the Young's modulus of the chitosan-alginate fibers was 0.84

FIG. 3. Diameter of the chitosan-alginate fibers as a function of the core and sheath flow rates.

MPa, whereas the calcium alginate fibers had a mean Young's modulus of 1.03 MPa. In contrast, the chitosan-alginate fibers exhibited higher tensile strength of 0.85 MPa compared to 0.66 MPa for the calcium alginate fibers. Such mechanical property may be caused from the high ionic interaction between the amino groups of chitosan and carboxyl groups of alginate.

FIG. 4. Stress-strain curves of the chitosan-alginate fibers and calcium alginate fibers.

FIG. 5. FTIR analysis (a) WSC, (b) calcium alginate, and (c) WSC-alginate+CaCl₂ solution.

B. Chemical characterization of chitosan–alginate fiber

The chemical integrity of the individual ingredients in the chitosan–alginate fibers and the possible interactions among them were analyzed by ATR-FTIR spectroscopy. Figure 5 shows the ATR-FTIR spectra of (a) WSC, (b) calcium alginate, and (c) WSC-alginate after filtering and cross-linking with calcium chloride. ATR-FTIR spectra of WSC contained two strong vibrations at 1622 and 1517 cm^{-1} corresponding to the vibrations of amides I and II, respectively. A peak at 1151 cm^{-1} indicated an amino group. The calcium alginate displayed two significant peaks corresponding to the carboxylate groups at 1594 cm^{-1} (antisymmetric stretching) and 1416 cm^{-1} (symmetric stretching). An apparent change in the spectrum was observed after WSC-alginate was cross-linked with calcium chloride, and the amide I and II bands were replaced by a new band (1590 cm^{-1}) due to the Coulombic forces of interaction between the amino groups of the WSC and the carboxylic acid groups of the alginate.

C. Cell behavior studies and viability test

HepG2 cells were seeded on the chitosan–alginate fibers and the calcium alginate fibers. Generally, more cells adhered to the chitosan–alginate fibers than to the calcium alginate fibers, as expected. Interestingly, the cells on the chitosan–alginate fibers showed increase in the average area of cell cluster over time. Figures 6(a) and 6(b) show images of cells on the chitosan–alginate and calcium alginate fibers. Higher clusters of cells were observed on the chitosan–alginate fibers. We quantified the average area of HepG2 clusters on these fibers at 3, 5, and 7 days, as indicated in Fig. 6(c). The size distributions of the HepG2 clusters on the chitosan–alginate fibers on each day were described by a Gaussian function [Fig. 6(d)].

HepG2 cells were encapsulated in the fibers and cultured for 1 week. The fiber-encapsulated cells at days 3, 5, and 7 were assayed for viability using the LIVE/DEAD staining test. Figure 7 shows fluorescent images of cells stained with the LIVE/DEAD assay reagent; viable cells are shown in green, and dead cells are shown in red. The viability of HepG2 encapsulated within the alginate fibers significantly decreased after 7 days, whereas the cells encapsulated in the chitosan–alginate fibers mostly survived. To the encapsulated cells, the environment is not suitable because the delivery of nutrient and oxygen delivery is limited comparing to cells on the surface. The

FIG. 6. Culture on fibers (live cell; green and dead cell: red): (a) chitosan–alginate, (b) calcium alginate; the scale bar indicates 50 μm (c) average area, and (d) distribution of clusters on the chitosan–alginate fibers.

dense alginate structure may hinder the proliferation, which may be the stress to cells. In contrast, the cells on the fiber are exposed to better environments, which may be the main cause of higher viability. The percent viability as a function of time for each fiber is plotted in Fig. 8. The chitosan–alginate fibers showed a cell viability that was five times larger than that of the alginate fibers after 7 days. Furthermore, HepG2 cell proliferation was observed in the chitosan–alginate fibers over the course of 7 days. These results demonstrate that the chitosan in the fiber plays a key role in providing a proper environment for encapsulated HepG2 cells, and the chitosan–alginate may be used as a good scaffold material for liver regeneration.

FIG. 7. Encapsulation of (a) chitosan–alginate and (b) calcium alginate (live cell; green and dead cell: red); the scale bar indicates 100 μm .

FIG. 8. Viability of HepG2 cells in chitosan-alginate and alginate fibers.

IV. DISCUSSION

Chitosan-alginate fibers were successfully wet-spun using a microfluidic chip, and their diameters were controlled by regulating the core and sheath flow rate. The effects of chitosan on the fibers' cell culture properties were confirmed by seeding HepG2 cells on the surface of the fibers. Calcium alginate is limited in its ability to promote the culturing of anchorage-sensitive cells on its surface. However, the chitosan-alginate fibers showed enhanced adhesion of anchorage-dependent HepG2 cells. On the chitosan-alginate fibers, the cells actively self-aggregated, which is a typical property of HepG2 cells and hepatocytes. This result indicated that the chitosan, which was cross-linked to the alginate, played a key role in enhancing the interactions between the cells and the fiber surface. The use of alginate for cell culturing has been limited due to a lack of cell anchorage and several modifications of alginate have been reported.^{20,21} In a previous study, we fabricated pure chitosan fibers to enhance the cell-fiber interaction.¹⁹ The production and handling of fine chitosan fibers less than 100 μm in diameter have been challenging due to the mechanical limitations of chitosan. However, the chitosan-alginate produces thinner fibers ($<100 \mu\text{m}$) that interact better with HepG2 cells and can be spun using a microfluidic chip. The blending of WSC-alginate may compensate for several limitations of the individual components. The first successful encapsulation of HepG2 cells is a promising result for broad applications in tissue engineering or analyzing cellular behavior in three-dimensional (3D) structure. The encapsulation of HepG2 cells in pure chitosan fibers was challenging due to the harsh fiber fabrication processes. But cells mixed in the WSC-alginate solution allowed for the successful fabrication of cell-laden fibers. The encapsulated HepG2 cells in the WSC-alginate fibers were more viable than cells in the alginate fibers, and this reflected the fact that the cross-linked chitosan provided a better environment for HepG2 cells than the alginate-only fibers. By controlling the cell density and aligning the fibers on a frame, as reported previously, 3D control over the spatial location of cells may be achieved. This technique may facilitate the uniform distribution of cells, which has been a critical requirement for tissue engineering.

V. CONCLUSION

In summary, chitosan-alginate fibers were hydrodynamically produced under mild conditions without using complicated processes, and the mass production may be feasible by the use of multiple chips. The presence of cross-linked chitosan in the fibers promoted interactions between the HepG2 cells and the fibers. The spinning of cell laden chitosan-alginate fibers was successfully performed and the HepG2 cells in the fibers showed better viability than the calcium alginate fibers over time. The chitosan-alginate fibers produced by microfluidic spinning method will be essential for use in guided cell culture, cell carriers for cell therapy, or scaffolding for tissue regeneration.

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