Digest Journal of Nanomaterials and Biostructures Vol. 9, No. 2, April - June 2014, p. 739 - 744

DIFFUSION-ASSISTED SPHERICAL MICROGEL FABRICATION USING IN SITU GELABLE CHITOSAN AND DEXTRAN

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We demonstrated themicrofluidic fabrication of*insitu* gelable chitosan-dextran microgels.In the gelation process, the amine groups of *N*-carboxyethyl chitosanand the aldehyde groups of oxidized dextran undergo a cross-linking process via imine formation.In order to produce spherical microgels, a water-in-oil emulsion was employed in a microfluidic flow-focusing device for microdroplet generation.*N*-carboxyethyl chitosan(3wt%) and oxidized dextran(3wt%) in microdropletswere homogenously mixedby diffusion, resulting in *insitu*cross-linking oftwo hydrogels without the use of additional chemicals.The average value of the Young's modulus of the cross-linked microgel was 1.34±0.21 kPa. For cell applications, the viability of 3T3 cells encapsulated in the microgels was evaluated after0, 3, and 4 days using a live/dead assay. The results demonstrated that the viability was well maintained at around 80% in the culture afterfour days. This demonstrates the biocompatibility of the microgels and fabrication process, which is very promising for applications in injectable tissue constructs.

(Received March 31, 2014; Accepted May 26, 2014)

Keywords: Chitosan, Oxidized dextran; In situ gelable; Microgel; Microfluidic fabrication

1. Introduction

Well-suited combinations of hydrogels at themicrometer scaleare providing numerous exciting possibilities in biological applications and as a result,microgels are widely used in drug delivery and tissue engineering applications[1-9].Microgels can be produced by various fabrication methods such as spray drying, solvent evaporation, and single or double emulsion techniques[10].However, conventional methodsof microgel fabrication utilize non-specific mechanical agitationor a chemical process, which results in polydisperse particles.

One of major fabrication methods used to generate microspheres is spray drying[11-14].The fabrication process consists of the dissolution of polymers in organic solvents, atomization for generation of small drops, and solvent evaporation by vacuum drying for microsphere formation. Although the advantage of this process is the abilityto operate rapidly under aseptic conditions, the process cannotguaranteemicrospheres with acontrolledsize. In solvent evaporation, a core material for microencapsulation is dissolved in a coating polymer solution[15-24]. With simple mechanical agitation and a chemical process, the microencapsulation is formed and the solvent in the core material is evaporated. However, the agitation causes non-uniform microsphere

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generation. In the emulsion technique, two immiscible liquids with different phases,such as water and oil, are mixed[25-31].After mechanical agitation,the dispersed liquid forms microspheres in the continued phase.Then, microgels can be produced by anadditional cross-linking process. Like solvent evaporation, agitation reduces the control of the microgel size.

In this study, we employed a microfluidic flow-focusing device to generate highly monodisperse aqueous microdroplets in an efficient manner[30].The microdroplets consisted of *in situ* gelable polymers made of chitosan and oxidized dextran.The microdropletsunderwent a polymerization reaction to become chitosan-dextran microgels.The viability of the cells encapsulated within the chitosan-dextran microgels was tested to demonstrate the biocompatibility of the microfluidic processing steps andthe polymerization reaction.

2. Experimental Section

Watersoluble *N*-carboxyethyl chitosan (CEC)and oxidized dextran (Odex) were synthesized by modifying a previously described method[32]. Briefly, 0.80 g of chitosan (Sigma-Aldrich, MO, USA) was dissolved in400 mL of deionized distilled water (DW) containing 5.6 mL of acrylic acid. The mixed solution was stirred at a speed of 400 rpm at 50°C for 2 days. The CEC was converted into its sodium salt by adjusting the pH to 10-12 by adding a0.1 M NaOH aqueous solution to the DW containing dissolved chitosan. The mixture was dialyzed using adialysis membrane (MWCO 12000) against DW for 3 days.Then, pure water soluble CEC was lyophilized for the experiments.Dextran was oxidized by introducing aldehyde functionalities and thus, it can serve as a cross-linker for polymerizing CEC. We mixed 320 mL of a 1.25% (w/v) dextran solution (Sigma-Aldrich, MO, USA) and 624 g of a $NaIO_4$ solution dissolved in 80 mL of DW. The mixture was stirred at a speed of 400 rpm at room temperature for 2 days to which we added 2.45 g of polyethylene glycol (Sigma-Aldrich, MO, USA) to quench the unreacted $NaIO_4$. As with the preparation process of CEC, the mixture was then dialyzed using adialysis membrane (MWCO 12000) against DW for 3 days and lyophilized to obtain pure Odex.

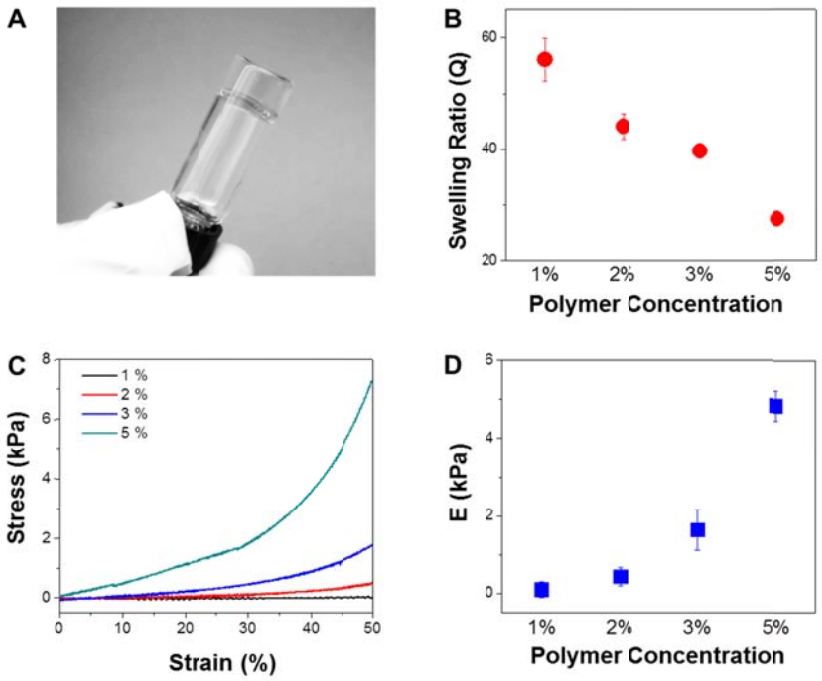

Fig. 1.Characterization of hydrogels formed by mixing equal amounts of CEC and Odex. (A) A photograph showing the CEC-Odex hydrogel in a bottle. (B) Swelling ratios (Q) obtained at various total concentrations. (C) Stress-strain curves of each hydrogel from unconfined compression tests. (D) Elastic modulus (E) determined from the slope of the stress-strain curves.

Fig. 1A shows CEC crosslinked by Odex in a bottle. In the gelation process, the amine groups of CEC and aldehyde groups of Odex undergo a cross-linking process via imine formation. We determined the swelling ratios by measuring the weights of samples placed in PBS for 24 hours and after fully drying them, as shown in Figure 1B. We also determined the mechanical propertiesof the CEC-Odex hydrogels by utilizing an unconfined compression test using amaterials testing system (Instron 5542, Norwood, MA, USA). The compressive modulus, E, was determined by calculating the slope of the linear region in the range of 0–10% strain, as shown in Figure 1C and D.

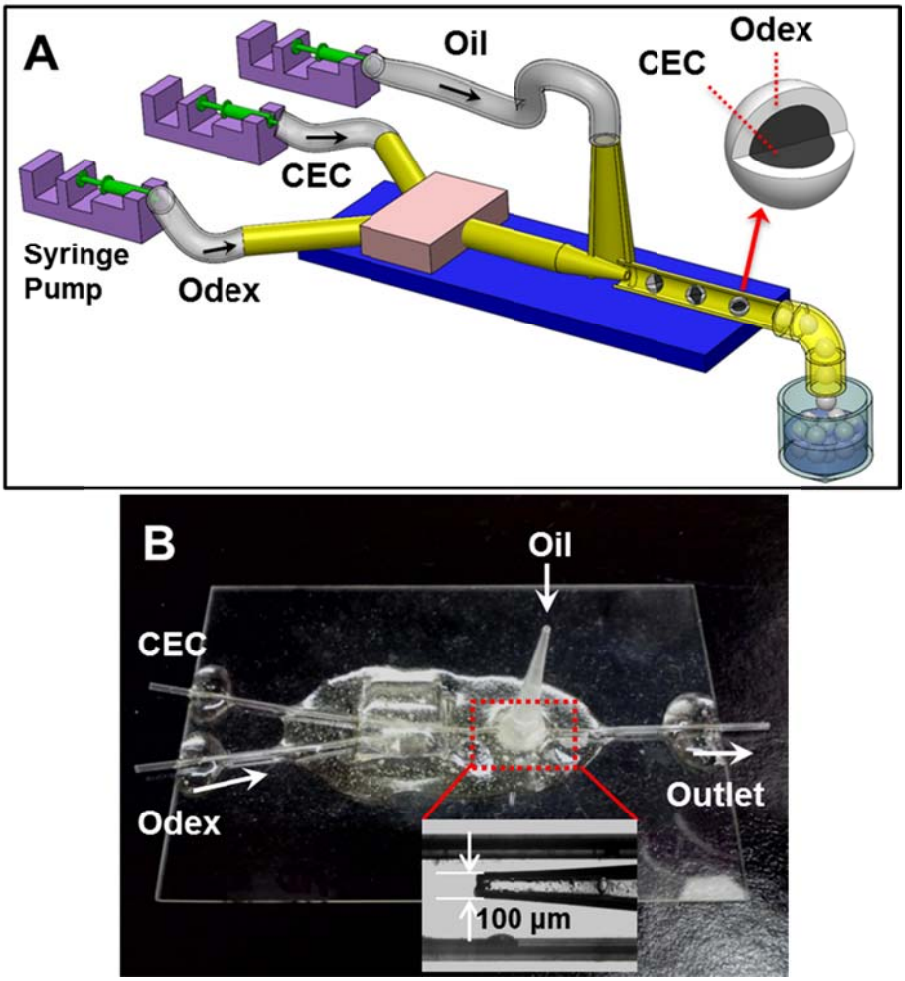

Fig. 2.Themicrofluidic flow-focusing device used to generate generating microdroplets. (A) Schematic of the flow-focusing process using two aqueous phases (CEC and Odex) and an oil phase. The oil flow focuses aqueous flow into the outlet orifice, resulting in droplet generation. (B) A photograph showing the device fabricated with glass capillaries. The device consists of two inlets for infusing the CEC and Odex pre-polymer solutions, one inlet for oil, and one outlet for the exiting microspheres.

Fig. 2A and B show the schematic and coaxial flow focusing device whichconsistsof three inlets for the two hydrogels (CEC and Odex) and oil, a main channel for microdroplet generation, and one outlet. In the main channel, one of two glass capillaries (World Precision Instruments, Inc., FL, USA) with inner diameters of 580 µm was manually pulled under a localized hot thermal condition to reach an inner diameter of 100 µm at the end of the capillary such that the smaller capillary could be easily slid into the larger one. All capillaries were glued onto a 2" x 3" glass slide using epoxy. A PDMS block carved to embed glass capillaries was bonded using plasma treatment and epoxy. Then, two holes on the bottom of a 0.1-10 µL pipette tip were cut with a razor blade such that the tip fit and could be permanently glued over the connection point of the

two capillaries at the center. All inlets were connected with two syringe pumps (Harvard Apparatus, MA, USA) via plastic tubing. Two 5 mL glass syringes filled with each pre-polymer solution were loaded into thesyringe pump.

NIH-3T3 fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose, Invitrogen, NY, USA) supplemented by 10% fetal bovine serum (FBS, Invitrogen, NY, USA) and 1% penicillin-streptomycin (Invitrogen, NY, USA). The cells were incubated under5% CO_2at37°Cand passaged every 4 days while the media was refreshed every 2 days. Live⁄dead® viability cytotoxicity kits(Invitrogen, NY, USA) wereused to evaluatethe encapsulated cell viability at day 1. The live/dead assay of 0.5 μL/mL calcein-AM (green) and 2 μL/mL ethidium homodimer-1 (red) was diluted in phosphate buffered saline (PBS). Cell encapsulated microgels in the assay were incubated at 37°C for 15 minutes and washed with PBS three times. The greenfluorescent live cells and redflorescent dead cells were imaged using an inverted fluorescence microscope.

3. Results and discussion

To confirm the hydrogel formation, the same amounts of CEC and Odex were mixed at various total concentrations and the mechanical properties of the resulting hydrogels were measured (Fig. 1). As expected, increasing the concentration of CEC and Odex resulted in an increased elastic modulus and decreased swelling ratio, which areindicative of the cross-linking reaction between CEC and Odex. We measuredthe Young's moduliand swelling ratios of the CEC-Odex hydrogels with polymer concentrations of 1%, 2%, 3%, and 5% (Fig. 1BD). The Young's modulus values of the 1%, 2%, 3%, and 5% CEC-Odex hydrogels were0.10±0.05, 0.44±0.13 kPa, 1.34±0.21 kPa, and 4.84±0.25 kPa, respectively. Based on the results, the 1% CEC-Odex hydrogel was nearly at the limit of forming gels and 3% was suitable for encapsulating cells.

We infused CEC and Odex pre-polymer solutions (3 wt%) through two inlets of the device to form a coaxial flow and mineral oil with 20 wt% Span®80 (Sigma-Aldrich, MO, USA) as a surfactantwas introduced through the inlet port at the center of the device. The two pre-polymer solutions were pumped into the device at a flow rate of 500 μL/hr. Mineral oil was perfused into the device at a flow rate of 1,500 μL/hr. LowviscosityOdex was located in the outer regionsof the droplets, and comparatively viscous CEC was located in the inner regions of the droplets (Fig. 2A). As shown in Fig. 3A, microdroplets with diameters of120 μm were continuously generated by focusing the coaxial flow. Reducing and increasing the flow rate of the coaxial flow ata fixed oil flow rate produced smaller and bigger droplets.

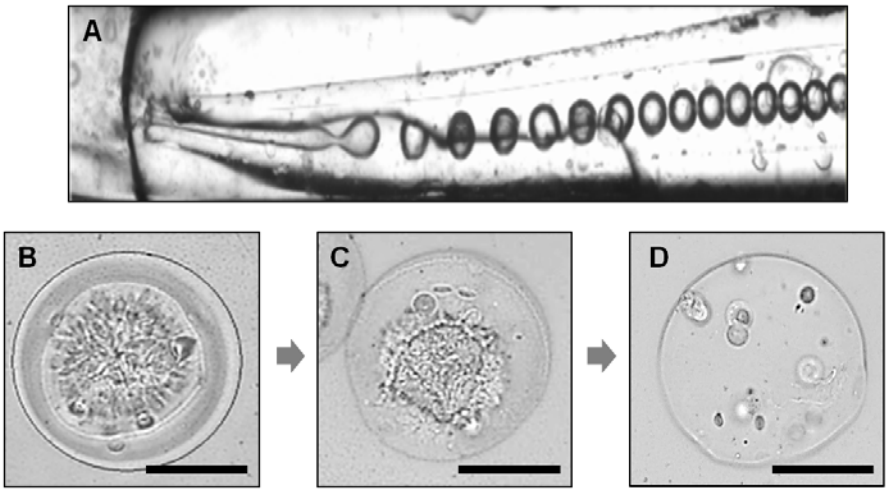

Fig. 3.Microfluidic generation of CEC-Odex microgels. (A) A snapshot image of the microdroplet generation. (B, C, and D) A series ofimages of CEC and Odex forming a droplet which shows that two different hydrogels were cross-linked and became homogeneous by diffusion. Scale bar: 50 μm.

The two hydrogels in the droplets were mixed and crosslinked by diffusion from Odex to CEC. The Odex with aldehyde groups is a crosslinker for polysaccharides with free amino groups. The Odex pre-polymer solution in themicrodroplets was steadily mixed with a CEC pre-polymer solution by diffusion. As a result, microdroplets were gradually crosslinked from the outer area to the inner area to form microgels without any additional polymerization process. Fig. 3(B-D) shows the initial, medium, and final stages of the crosslinking processes in a single microgel,which are dependent on diffusion. Microgels were fully crosslinked within 5min and the crosslinked microgels were spherical and transparent. After crosslinking, the diameters of the microgels shrunk from 120 to 100 μm due to aldehyde groups forming a Schiff base with free amino groups. Microgels collected in the 24-well plate were centrifuged and separated from the mineral oil layer. The oil layer was removed and then media was added and changed twice.

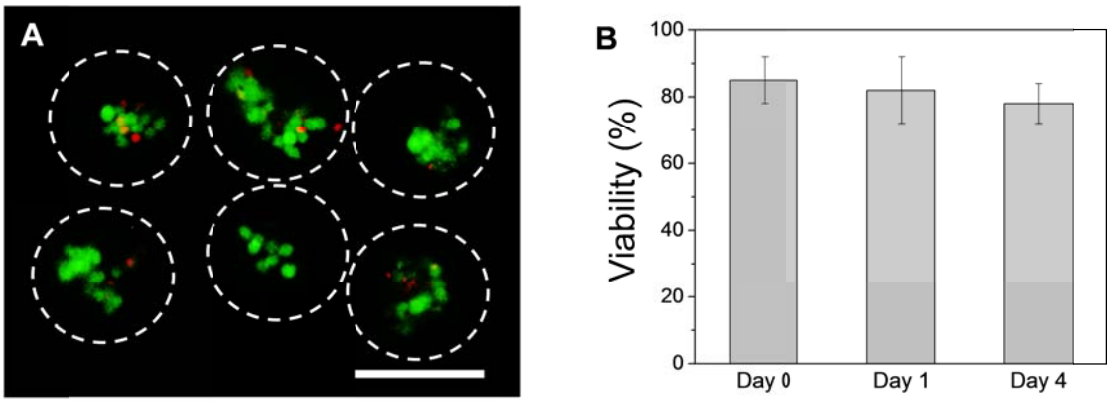

Fig. 4.Evaluation of the cell viability inside the microgels.(A) The viability of encapsulated fibroblasts wasevaluated by fluorescent labeling with calcein-AM (green, live) and ethidium homodimer-1 (red, dead). (B) The viability was well maintained bythe microgel formation over the course of four days of culturing.Scale bar: 100 μm.

To evaluate the biocompatibility of the microgels, 3T3 fibroblasts were encapsulated within the microgels by introducing cells in the CEC solution (Fig. 4). Then, the viability was measured by fluorescently labeling the cells. The viability measured immediately after microgel formation showed a high cell viability (> 80%), demonstrating the biocompatibility of the fabrication process. In addition, the viability was well maintained after culturing for four days, demonstrating thatthe microgels provided suitable environmental conditions for the encapsulated cells.

4. Conclusions

We fabricated cell-laden microgels using a microfluidic flow-focusing geometry. *In situ* gelation between chitosan and oxidized dextran allowed efficient and biocompatible cell-laden microgel synthesis without the need for an additional cross-linking process. Therefore, we expect that the microfluidics approach presented in this study can be successfully utilized to generate injectable tissue constructs for a wide range of biocompatible applications.

Acknowledgments

This paper was supported by research funds of Chonbuk National University in 2013.This research was supported by Basic Science Research Program through the National Research Foundation of Korea(NRF) funded by the Ministry of Education(NRF-2013R1A1A2058875).This

study is (also) financially supported from RCOE(Regional Center of Excellence) program managed by KITECH(Korea Institute of Industrial Technology).

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