Preparation of carboxymethyl cellulose based microgels for cell encapsulation

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Abstract. Biocompatible and biodegradable carboxymethyl cellulose (CMC) has been modified with 4-hydroxybenzylamine (CMC-Ph) in order to prepare CMC-based microgels through the horseradish peroxidase/hydrogen peroxide enzymatic reaction. CMC-Ph was identified as a blend, and the amount of the grafted 4-hydroxybenzylamine per 100 units of CMC was between 17 and 23 according to the molecular weight of CMC. Through a special designed co-flowing microfluidic device, CMC-Ph microgels were prepared with the radius from 100 to 500 μm *via* adjusting the flow rates of the disperse phase and the continuous phase, respectively. The chondrocytic cell line ATDC5 was encapsulated in the CMC-Ph microgels. The cell-laden microgels were cultured for up to 40 days, illustrating the biocompatibility of CMC-Ph and the microfluidic approach through the enzymatic crosslinking reaction primarily. CMC-Ph showed a great promise to encapsulate the cells for further fabrication of the injectable scaffolds.

Keywords: biocompatible polymers, polymer synthesis, polymer gels, carboxymethyl cellulose, microfluidic

1. Introduction

Osteoarthritis, characterized by progressively degeneration or loss of articular cartilage, is the most common form of arthritis. Tissue engineering has shown great promise as a strategy to develop biological substitutes to restore, replace or regenerate the defective tissue [1]. Scaffolds made of polymeric biomaterials offer support for cell attachment, proliferation and differentiation [2, 3]. Encapsulation of living cells within a semi-permeable membrane is a simple one-step procedure with characteristics of homogenous cell distribution and excellent cell viability [4, 5]. Its unique self-assembled capability makes it suitable for injectable scaffolds

for *in situ* tissue regeneration. Recent advances in microfluid designs have brought the field of microfluidics to the forefront of the preparation of micro- or nano-gels for cell encapsulation [6–8].

Many naturally derived biomaterials have been used for encapsulation, such as sodium alginate [9] and agarose [10]. However, these biomaterials have limited ability to support cell attachment, growth and differentiation, resulting in low cell viability and growth [11]. Carboxymethyl cellulose (CMC) is a water-soluble, biodegradable and biocompatible derivative of cellulose. Its hydrophilic carboxylic or hydroxyl groups serve as active sites for preparing CMC gels. Physical-crosslinking CMC gels *via*

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supermolecular [12] and ionic [13] interaction are simple to be produced, but usually questioned by their reversibility.

Chemical-crosslinking provides CMC gels a more stable three-dimensional network. For example, divinyl sulfone [14], epichlorohydrin [15], aldehydes [16, 17], fumaric acid [18] and citric acid [19] have been used as crosslinkers to form CMC and CMC composite gels. Monomers with double bond, such as *N*-isopropyl acrylamide [20] and partially neutralized acrylic acid/rectorite [21], have been initiated by ammonium persulfate and coupled onto CMC backbones *via* methylene bisacrylamide (crosslinker). These chemical crosslinkers are limited for the applications in biomedical or pharmaceutical areas because of their toxicity or the stimulatory reaction to the encapsulated bioactive molecules or live cells. A bio-based carbodiimide crosslinker has been used to prepare chitosan/CMC microgels [22], indicating a very low efficiency of crosslinking reaction. Another synthetic route is to form CMC gels *via* functional groups of both components without any crosslinker, for example, hydrazide-functionalized CMC/aldehyde-functionalized dextran, suggested by Kesselman *et al.* [23]. They have introduced the two reactive streams into a continuous oil solution simultaneously through two separate inlets of a microfluidics device. However, the premixing of the reactive streams before the formation of droplets often resulted in the blockage of small opening of the microfluidics.

Enzymatic reaction has been extensively studied owing to its low toxicity, mild reaction, stereochemistry, and high reaction velocity, high enantio-, regio- and chemo-selectivity [24]. Horseradish peroxidase/hydrogen peroxide (HRP/H₂O₂) is a common enzymatic system, where peroxidases (oxidoreductases) catalyzes the oxidation of donors using H₂O₂, resulting in polyphenols linked at the aromatic ring by C–C and C–O coupling of phenols [25]. DeVolder *et al.* [26] have prepared hydrogels of alginate grafted with pyrrole groups through a HRP-activated crosslinking reaction for drug release system. We designed a special microfluidic device to prepare monodisperse CMC-based microdroplets [27]. In this work, we synthesized 4-hydroxybenzylamine modified CMC (CMC-Ph) with different molecular weight and prepared CMC-Ph microgels through an enzymatic reaction. The properties of

CMC-Ph that influenced the formation of microgels were studied. Moreover, cells were encapsulated in CMC-Ph microgels to study the biocompatibility of CMC-Ph and the microfluidic approach through the enzymatic crosslinking reaction.

2. Experimental

2.1. Materials

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (NHS), HRP (250 units/mg), lecithin, 4-hydroxybenzylamine, 1-hydroxybenzotriazole hydrate (HOBt) and 2-(4-morpholino)ethanesulfonic acid (MES) were obtained from Qiyun Biotech (China). Aqueous H₂O₂ (30%, w/w) and liquid paraffin were purchased from Dalu Chemical Reagent (China). All reagents were of analytical grade and used as received.

CMC with M_w of 1.0×10^5 (CMC₁₀), 2.0×10^5 (CMC₂₀) and 3.0×10^5 (CMC₃₀) were purchased from Jingchun Chemical Reagent (China). CMC-Ph was synthesized according to our previous study [27]: Briefly, CMC and 4-hydroxybenzylamine were dissolved in MES buffer (50 mM, pH 6.0) at 1.0 and 0.6%, respectively. NHS, HOBt and EDC were added at a weight ratio of 1:0.26:0.68:0.70 (CMC:NHS:HOBt:EDC). The solution was magnetic stirred for 24 hours and dialyzed against deionized water using an ultrafiltration membrane (molecular weight cut-off = 3500 Da) at 25°C for 4 days. The resultant polymer solution was enriched by a rotary evaporator (100 rpm) at 50°C and lyophilized.

2.2. Preparation of CMC-Ph microgels

A co-flowing microfluidic device with an inner diameter of 260 μm and an outer diameter of 510 μm was used for preparing CMC-Ph microparticles. The continuous liquid paraffin phase was prepared as follows: In brief, 250 mL of liquid paraffin containing 1.25 mL H₂O₂ was magnetically stirred for 12 hours at 25°C and centrifuged at 2000 rpm for 10 minutes. Lecithin was dissolved at 3.0% (w/v) into the upper liquid paraffin containing H₂O₂. CMC-Ph aqueous solution containing HRP as the dispersed phase was injected into the microfluidic device using a micro-syringe pump (Baoding Longer TS-1B/W0109-1B, China), and the continuous phase was injected into an inlet in a perpendicular direction. The particles flew along the channel and finally collected.

2.3. Preparation of cell-laden CMC-Ph microgels

The lyophilized CMC-Ph was sterilized by exposure to epoxyethane vapor. CMC-Ph (~0.2 g) was dissolved in 4 mL of Dulbecco's modified Eagle medium (DMEM, Hyclone, USA). HRP was then dissolved at 1 mg/mL in DMEM, and the solution was kept at 37°C. The chondrocytic cell line ATDC5 (Sigma, USA) of sixth passage was trypsinized at a density of 1×10^7 cells/mL. 1 mL of cell suspension was added into the CMC-Ph solution containing HRP, which injected as the dispersed phase at a rate of 50 $\mu\text{L}/\text{min}$. The continuous liquid paraffin phase was injected at a rate of 10 mL/min. The resulted microgels were collected and centrifuged at 2000 rpm for 5 min. Phosphate buffer saline (PBS, pH = 7.4, Gibco, USA) was added to the tube, followed by centrifuging at 2000 rpm for 5 min twice. The collected cell-laden CMC-Ph microgels were removed into 6-well cell culture dishes (Corning, USA). Cells enclosed in microgels were incubated in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, USA) in a humidified atmosphere at 37°C under 5% CO_2 . The medium was exchanged for fresh medium every 2 days, and a new cell culture dish was replaced every 4 days till cell-culturing for 40 days. The morphology of the cell-laden CMC-Ph microgels was evaluated under an inverted light microscope (Nikon ECLIPSE TS100, Japan).

2.4. Characterization

A Bruker (Germany) Vertex 70 Fourier transform infrared spectrometry (FTIR) was used to obtain infrared analyses of 4-hydroxybenzylamine, CMC and CMC-Ph using KBr pellet method. The spectra comprised 64 scans at a resolution of 1 cm^{-1} in $4000\sim 400 \text{ cm}^{-1}$ spectral range.

^1H nuclear magnetic resonance (NMR, Drx-400 Bruker, Germany) spectra were achieved at 400 MHz using deuterated water (D_2O) as solvent in the presence of tetramethylsilane as an internal standard. Graft density of phenols in CMC-Ph was calculated by measuring the absorbance at 275 nm of CMC-Ph solutions using a Thermo (USA) Evolution 300 UV-VIS spectrometer. The absorbance being measured was compared with a 4-hydroxybenzylamine standard curve. The graft density of phenols then calculated from the ratio of phenols to 100 repeat units of CMC. The data was the mean of five samples.

Molecular weight and polydispersity index (PDI) of CMC and CMC-Ph were determined by a Water (USA) 515–410 gel permeation chromatography (GPC). Weight-average (M_w) and number-average (M_n) molecular weight were expressed with respect to polyethyleneglycol standards.

Rheological behavior of CMC-Ph solutions with different concentrations was measured using a TA (USA) ARES/RFS rotational viscometer at 16 and 30°C, respectively. The solutions were prepared by mass using a XS105DU balance (Mettler Toledo, Switzerland) with a precision of 10^{-5} g. The employed shear rate varied from 0.01 to 250 s^{-1} , and viscosity and stress were identified.

Stereomicroscope images of the microdroplets in the liquid paraffin were obtained with a SMZ-DM200 stereomicroscope (Optec, China) with a digital CCD camera to estimate the size in number-average diameters and coefficients of variation (CV, defined as the ratio of standard deviation to the mean) by analyzing images of 100 particles in liquid paraffin phase.

3. Results and discussion

3.1. CMC-Ph

3.1.1. FTIR spectrum

In Figure 1a, the absorption bands at 1450, 1501, 1561 and 1611 cm^{-1} were ascribed to the benzene skeleton vibration. The peaks at 842, 1261 and 1386 cm^{-1} were attributed to the C–H out-of-plane bending vibration of benzene ring, C–O stretching vibration and O–H in-plane bending vibration of phenol, respectively. The absorption peak at 1642 cm^{-1} was associated to N–H scissoring vibration of free amine. The broad association peak at 3423 cm^{-1} would be the stretching vibration of C–H, O–H and N–H, in which a hydrogen bond formed between a hydrogen atom and O–H group or N–H group. In FTIR spectrum of CMC-Ph (Figure 1b, curve (2)), the absorption bands at 1045, 1323 and 1415 cm^{-1} were attributed to the C=O stretching vibration, in-plane bending vibration of O–H and C–H scissoring vibration of methylene of CMC, respectively (Figure 1b, curve (1)). The characteristic absorption bands of 4-hydroxybenzylamine were traced, including the C–H out-of-plane bending vibration of benzene ring (827 cm^{-1}), C–O strong stretching vibration of phenol (1257 cm^{-1}), benzene skeleton vibration (1504 and 1456 cm^{-1}) and N–H scissoring vibration of free amine (1644 cm^{-1}). The

Figure 1. FTIR spectra of 4-hydroxybenzylamine (a), CMC (b-(1)) and CMC-Ph (b-(2))

band with a peak at 1579 cm^{-1} was the IR absorption of the —O— stretching vibration of CMC (1592 cm^{-1} , Figure 1b, curve (1)) and the benzene skeleton vibration (1561 and 1611 cm^{-1}).

3.1.2. ¹H NMR spectra

Figure 2 illustrates ¹H NMR spectra along with schematics of their chemical structures, on which proton assignments are indicated. 4-Hydroxybenzylamine (Figure 2a) showed chemical shifts (δ) at 6.60 and 7.06 ppm of aromatic protons (a) and (b), respectively, and at 3.81 ppm of aliphatic protons (c). The spectra of CMC (Figure 2b) and CMC-Ph (Figure 2c) showed δ of anomeric protons at 4.15 and 4.42 ppm, and sugar ring protons between 3.11 and 3.99 ppm for both macromolecules [28]. The signals at 7.21 and 6.84 ppm of CMC-Ph spectrum were attributed to the aromatic protons of 4-hydroxybenzylamine being grafted with CMC (CMC-g-Ph, 1a and 1b). The strong inductive effect being caused by the neighboring carbonyl groups decreased the electron density of the benzene ring, so that δ of aromatic protons decreased compared with those of 4-hydroxybenzylamine (Figure 2a, (a) and (b)). The signals at 1.79–2.02 ppm (2a), 0.96–1.19 ppm (2b) and 2.75 ppm (2c) were ascribed to the byproduct being formed between CMC and EDC (CMC-EDC). EDC and phenolic groups of 4-hydroxybenzylamine reacted to form CMC-g-Ph-EDC, resulting in a change in proton shift of the aromatic protons at 7.51 and 7.35 ppm (3a and 3b) [29].

3.1.3. Graft density of phenols

The CMC-Ph aqueous solution showed a specific absorbance with a peak at 275 nm of 4-hydroxy-

benzylamine. We also tested the dialysis solution at four days, the UV absorption was not detected, so that the UV absorption of the CMC-Ph solution would be ascribed to the grafted 4-hydroxybenzylamine. Based on the standard curve, the amount of the grafted 4-hydroxybenzylamine per 100 units of CMC (grafting density) was calculated (Table 1). It fell in the range of 17–23, and increased as M_w of CMC increased. Usually, the flexibility of polymer increased with the increasing molecular weight, so that CMC with higher M_w or M_n was favorable to collide effectively with small molecules *via* changing conformation, leading to a higher reaction probability of CMC with 4-hydroxybenzylamine.

3.1.4. Molecular weight

The molecular weight and the polydispersity (PI) of the molecular weight were also shown in Table 1. M_w , M_n and PI of CMC-Ph were higher than those of CMC. The difference of molecular weight between CMC and CMC-Ph increased as the molecular weight of CMC increased, perhaps owing to a slight cross-linking between hydroxyl groups of 4-hydroxybenzylamine and carboxyl groups of CMC under EDC/NHS reactive system.

Table 1. Grafting density and molecular weight of CMC-Ph

	Grafting density	M_n ($\times 10^{-4}$)	M_w ($\times 10^{-4}$)	Polydispersity
CMC ₁₀	0	4.47	9.86	2.2
CMC ₂₀	0	6.20	24.53	4.0
CMC ₃₀	0	10.20	31.52	3.1
CMC ₁₀ -Ph	17.4	4.44	17.43	3.9
CMC ₂₀ -Ph	17.8	9.42	48.08	5.1
CMC ₃₀ -Ph	22.5	17.36	87.50	5.0

Figure 2. ^1H NMR spectra of 4-hydroxybenzylamine (a), CMC (b) and CMC-Ph (c) with protons assigned

3.1.5. Viscosity

Rheological behavior of the CMC-Ph solution with the different molecular weight has been determined by analyzing the influence of shear rate on viscosity. Flow curve was a straight line passing through the origin, and the stress increased lineally with the shear rate at 30°C , respectively (Figure 3 (a_2), (b_2), (c_2)). An increase in the molecular weight produced

an increase in the viscosity (Figure 3). For example, the viscosity was 5.4, 14.8 and $25.5 \text{ mPa}\cdot\text{s}$, respectively for $\text{CMC}_{10}\text{-Ph}$, $\text{CMC}_{20}\text{-Ph}$ and $\text{CMC}_{30}\text{-Ph}$ at 30°C . As the molecular weight of CMC-Ph increased, the intermolecular volume decreased and thus the molecular interaction of CMC-Ph increased. The CMC-Ph with high molecular weight possessed low ability to rearrange and move past each other, so

Figure 3. Viscosity of 1.0% CMC-Ph solution being measured at 16°C: (a₁) CMC₃₀-Ph; (b₁) CMC₂₀-Ph; (c₁) CMC₁₀-Ph, and at 30°C: (a₂) CMC₃₀-Ph; (b₂) CMC₂₀-Ph; (c₂) CMC₁₀-Ph

that the internal resistance to flow increased. Therefore, the viscosity increased with the increasing molecular weight of CMC-Ph.

At 16°C, the viscosity of CMC₃₀-Ph decreased significantly with the increasing shear rate. When the shear rate was 215 s⁻¹, the viscosity was 9.9, 19.1 and 26.6 mPa·s, respectively for CMC₁₀-Ph, CMC₂₀-Ph and CMC₃₀-Ph. In general, an increase in temperature allows quicker molecules motion and thus less energy is needed to flow CMC-Ph solution, resulting in a decrease in the viscosity. When the shear rate was 215 s⁻¹, the difference between viscosity measuring at 16 and 30°C was 4.45, 4.31 and 1.08 mPa·s for CMC₁₀-Ph, CMC₂₀-Ph and CMC₃₀-Ph, respectively. The less difference of CMC₃₀-Ph was perhaps owing to the decreasing viscosity as the shear rate increased. As the shear rate was 215 s⁻¹, the viscosity ratio of CMC₃₀-Ph to CMC₁₀-Ph at 16°C was 2.7, which reached 4.7 when temperature went up to 30°C.

3.2. CMC-Ph microgels

Prior to the microfluidic preparation of CMC-Ph microgels, we examined the gelation of the disperse fluid as the presence of H₂O₂. The CMC-Ph gels were formed after a contact time of 1 min. In the microfluidic device, the viscous disperse fluid was extruded into the immiscible continuous liquid flowing in the same direction. The disperse fluid flowed and snapped off at the orifice. H₂O₂ in the continuous liquid surrounded the CMC-Ph droplets containing HRP. Thereafter, diffusion of H₂O₂ from the continuous fluid to the disperse phase triggered the gelation reaction to bind the phenols groups.

Because H₂O₂ was separated from HRP, the enzymatic reaction cannot occur before the droplets formation, thus avoiding the blockage of the inner opening. The concentration of H₂O₂ in the continuous phase (0.82 mmol/L) would not bring severe harmful effect on the encapsulated contents, but it was enough for gelation of the microdroplets. The CMC-Ph microgels were intact while immersed in DMEM for 40 days. This diffusion-controlled cross-linking was very important for improving the integrity and encapsulation efficiency of microparticles.

We prepared the CMC-Ph microdroplets with different molecular weight while using the 1% CMC-Ph containing HRP (1 mg/mL). As the flow rate of the disperse phase (Q_d) was fixed at 10 μL/min, the effect of the flow rate of the continuous phase (Q_c) on the radius of microdroplets was shown in Figure 4a. The increased flow rate of the continuous phase produced a stronger shear force, thus resulted in a decreasing radius of the CMC-Ph microdroplets.

Figure 4. Dependence of flow rates on the size of the microdroplets obtained by using 1.0% CMC₁₀-Ph (triangle), CMC₂₀-Ph (circle) and CMC₃₀-Ph (square): (a) effect of Q_c on the radius of microdroplets at a constant Q_d (10 μL/min); (b) effect of Q_d on the radius of microdroplets at a fixed Q_c (10 mL/min)

When using a fixed Q_c (10 mL/min), the radius of the CMC-Ph microdroplets increased with an increasing flow rate of the disperse fluid (Figure 4b). In general, the CMC-Ph solution with higher molecular weight produced larger microdroplets due to higher viscosity.

3.3. Cell-laden CMC-Ph microgels

The injectable microsphere scaffolds should support cells adhesion, migration, and proliferation, and more important, maintain the differentiated phenotype of the cells within the scaffold. The cell-laden microgels would facilitate gas exchange, nutrient diffusion, and waste metabolism. ATDC5 is a prechondrogenic stem cell line, and reproduces the differentiation stages of chondrocytes during endochondral bone formation [30]. The ATDC5-laden microgels being cultured up to 40 days were shown in Figure 5. The microgels presented round morphology that was very important for mechanical stability under the compressive forces in the body. The living cells (light dots) were distributed separately in the microgels (Figure 5a). Some of the cell-laden microgels broke with the damaged border. The microgel did not maintain the round morphology any more (Figure 5b). Some of the cells released from the broken microgels and can stick to the cell culture dishes at 40 days of culturing (Figure 5c), showing high viability.

4. Conclusions

4-Hydroxybenzylamine modified CMC with different molecular weight was synthesized through EDC/NHS coupling agents. Uniform CMC-Ph microparticles were obtained in the co-flowing microfluidic devices. The radius was tuned in the range of (100~500) μm by changing the flow rates of the disperse phase and the continuous phase, respectively. The cells encapsulated in CMC-Ph microgels were still living at 40 days of culturing. The microfluidic approach to the preparation of the cell-laden microgels will provide a potential method of fabricating scaffolds for tissue engineering, especially in the defect with an irregular-shape and/or a minimally invasive approach. The CMC-Ph microgels with the different molecular weight along with the different encapsulating content may also been used to prepare the injectable microsphere scaffolds, having a

Figure 5. Photographs of the cell-laden microgels up to 40 days of culturing: (a) living cells distributed separately at 40 days; (b) broken cell-laden microgels at 5 days; (c) cells from the broken microgels sticking to the dishes at 40 days

gradient mechanical property, a gradient encapsulating content, and a gradient releasing property.

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