Acta Biomaterialia 22 (2015) 59–69

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Hydrosoluble, UV-crosslinkable and injectable chitosan for patterned cell-laden microgel and rapid transdermal curing hydrogel *in vivo*

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ARTICLE INFO

Article history:
Received 10 November 2014
Received in revised form 16 April 2015
Accepted 19 April 2015
Available online 25 April 2015

Keywords:
Patterned microgels
Injectability
Transdermal photopolymerization
Photolithography
Localized delivery

ABSTRACT

Natural and biodegradable chitosan with unique amino groups has found widespread applications in tissue engineering and drug delivery. However, its applications have been limited by the poor solubility of native chitosan in neutral pH solution, which subsequently fails to achieve cell-laden hydrogel at physiological pH. To address this, we incorporated UV crosslinking ability in chitosan, allowing fabrication of patterned cell-laden and rapid transdermal curing hydrogel *in vivo*. The hydrosoluble, UV crosslinkable and injectable N-methacryloyl chitosan (N-MAC) was synthesized *via* single-step chemoselective N-acylation reaction, which simultaneously endowed chitosan with well solubility in neutral pH solution, UV crosslinkable ability and injectability. The solubility of N-MAC in neutral pH solution increased 2.21-fold with substitution degree increasing from 10.9% to 28.4%. The N-MAC allowed fabrication of cell-laden microgels with on-demand patterns *via* photolithography, and the cell viability in N-MAC hydrogel maintained 96.3 ± 1.3% N-MAC allowed rapid transdermal curing hydrogel *in vivo* within 60 s through minimally invasive clinical surgery. Histological analysis revealed that low-dose UV irradiation hardly induced skin injury and acute inflammatory response disappeared after 7 days. N-MAC would allow rapid, robust and cost-effective fabrication of patterned cell-laden polysaccharide microgels with unique amino groups serving as building blocks for tissue engineering and rapid transdermal curing hydrogel *in vivo* for localized and sustained protein delivery.

1. Introduction

Tissue engineering based on microfabrication, assembly, and *in vitro* culturing of cell-laden three dimensional (3D) extracellular matrix (ECM) analogs has been developed for several decades [1–6]. Serving as cell-laden 3D ECM analogs, patterned cell-laden microscale hydrogels (microgels) could accurately replicate the heterogeneous nature of native cellular environments. For regenerating complex tissues and organs *in vitro*, one reliable strategy is fabrication of cell-laden microgels serving as building blocks, which are then assembled to form complex artificial micro-tissues with specific physiological function *via* bottom-up tissue engineering [7,8]. Various microfabrication 3D patterned cell-laden building blocks with natural and/or synthetic polymer have been widely adopted, such as photolithography [9–11], micromolding [12] and bioprinting [13,14]. So far, the versatile and efficient cell-friendly photolithography allows fabrication of patterned building blocks with advantages of high precision, short time and low costs especially in fabrication of 3D patterned building blocks [15–17]. Various cells were seeded on patterned azido-chitosan hydrogel fabricated by UV lithography, such as cell spheroid microarrays of Hep G2 and NIH/3T3 [18], patterned cardiac fibroblast, cardiomyocyte and osteoblast microarrays on chitosan surfaces [19]. UV irradiation time for chitosan gelation is usually 5–15 min [20,21], thus these patterned chitosan hydrogels are not

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http://dx.doi.org/10.1016/j.actbio.2015.04.026
1742-7061/

suitable to encapsulate cells during UV irradiation, which fail to mimic *in vivo* cell niche microenvironment for biomedical applications.

Injectable hydrogels with biodegradability have *in situ* formability, which allow an effective and homogeneous encapsulation of drugs/cells in a minimally invasive way. Biocompatible, biodegradable and injectable hydrogels *via* chemical, temperature, pH and UV irradiation triggered gelation have been developed for localized drug delivery with advantages of minimal invasion and tunable release behavior of therapeutic drugs [22,23]. Chitosan is a biodegradable and natural biomaterial with amino groups, which has been widely used in tissue engineering [24], localized drug delivery [25] and injectable hydrogels for cancer therapy [26] owing to its biological properties such as biodegradability by lysozyme, biocompatibility, antibacterial activity and hemostatic ability. Injectable chitosan could undergo thermal or pH triggered gelation and could be enzymatically degraded *in vivo* by lysozyme and chitosanase enzymes. However it usually takes a long gelation time for previously reported injectable chitosan hydrogel, for example, 5–60 min for thermo-sensitive chitosan/β-glycerophosphate hydrogel [27,28] and 60 min for pH-sensitive chitosan/polyacrylamide hydrogel [29]. In addition, the main component in chitosan hydrogel is glycerol phosphate or non-biodegradable polyacrylamide component rather than chitosan, which increases the risk of biological toxicity to surrounding tissues. UV crosslinkable hydrogels allow injection of hydrogel precursor and following rapid curing at desired position in target tissue under mild physiological conditions in a spatiotemporal controlled manner [30,31], avoiding obvious pH or temperature shock in biological tissues while maintaining intrinsic structure and bioactivity of drugs or biomolecules.

There are some challenges associated with chitosan for its applications in tissue engineering and regenerative medicine, such as poor solubility in neutral pH solution and spatiotemporal designability, which subsequently fail to achieve cell-laden hydrogels with controlled architecture and rapid transdermal curing hydrogel *in vivo*. Chitosan is only dissolved in dilute acid aqueous solution, but hardly in water or cell culture medium, which subsequently limits its cell-related applications. To endow chitosan with hydrosoluble ability, various derivatives have been synthesized by chemically grafting with ferulic acid, phosphorylcholine, 4-imidazolecarboxaldehyde and succinic anhydride [32–35]. Furthermore, hydrosoluble chitosan derivatives have been subsequently endowed with UV-crosslinkable ability through covalent linkage with photosensitive components, such as 4-azidobenzoic acid [36], 2-amino ethyl methacrylate [37], or ethylene glycol acrylate methacrylate [38–40]. A water-soluble and UV-crosslinkable chitosan derivative was synthesized by two-step grafting azide and lactose moieties serving as a biological adhesive [36]. Another water-soluble and UV-crosslinkable chitosan derivative was synthesized for supporting neuronal differentiation of encapsulated neural stem cells using two-step chemical modification: synthesis of carboxylmethyl chitosan and subsequent grafting 2-amino ethyl methacrylate on carboxylmethyl chitosan [37]. A UV crosslinkable chitosan was obtained through amidation reaction with EDC/NHS activation between amino groups of chitosan and carboxyl groups of N-methacryloyl glycine [41]. However this chitosan derivative could be dissolved in acetic acid solution instead of water/cell culture medium. Besides, these synthesis protocols normally involve multistep chemical modification, long UV irradiation time for gelation (usually 3–15 min) and coupling agent (EDC/NHS activation) [20,21].

To achieve hydrosoluble, UV crosslinkable and injectable chitosan for patterned cell-laden microgels and rapid transdermal curing hydrogels, we facilely synthesized a hydrosoluble, UV crosslinkable and injectable N-MAC by single-step chemoselective N-acylation between amino group and methacrylic anhydride without using any coupling agents or catalysts. The methacryloyl groups in N-MAC not only allow well solubility in neutral pH solution but also endow it with UV-crosslinkable ability. Patterned cell-laden N-MAC microgels with on-demand regular geometric shapes and complex logos were fabricated *via* UV lithography for tissue engineering. Finally, injectable and rapid transdermal curing N-MAC hydrogels *in vivo* were developed *via* skin-penetrable UV crosslinking strategy within mice subcutaneous space for localized protein delivery.

2. Materials and methods

2.1. Materials

Chitosan (CS, viscosity average molecular weight $M_\eta = 3.4 \times 10^5$, degree of deacetylation = 91.4%) was purchased from Qingdao Hecreat Bio-tech company Ltd. Methacrylic anhydride (MA, 94%), photoinitiator Irgacure 2959 (I2959) and fluorescein isothiocyanate (FITC) labeled dextran were purchased from Sigma–Aldrich. Sodium bicarbonate was supplied by Sinopharm Chemical Reagent Co. Dialysis tubing with molecular weight cut off range 8000–14,000 was supplied by Solarbio (USA).

2.2. Synthesis of N-MAC

N-MAC was synthesized by single-step chemoselective N-acylation between CS and MA. Typically, MA was added dropwise to 1% (w/v) CS acetic acid solution in which the ratio of anhydride to amino groups was 0.5, 1, 2 and 4, respectively. The reaction was carried out at 60 °C for 6 h. The resulting solution was neutralized and diluted 10-fold with 10% (w/v) sodium bicarbonate solution. The N-MAC was dialyzed against deionized water for 4 days to remove the unreacted reagent. The snow sponge was obtained by lyophilization.

2.3. Characterization of N-MAC

^{1}H NMR was recorded on a Bruker NMR (ADVANCE III, 400 MHz) with D_2O as solvent. The degree of substitution (DS) of N-MAC was calculated by ratio of integrated area of the $H_c \sim H_f$ peaks at 2.52–4.18 ppm to that of the methylene (H_g) peaks at 5.46 and 5.68 ppm according to the Eq. (1).

$$DS = \frac{A_{H(5.5\&5.7)}/2}{A_{H(2.5-4.1)}/5} \times 91.4\% \quad (1)$$

where $A_{H(5.5\ \&\ 5.7)}$, $A_{H(2.5-4.1)}$ were the area of methylene protons peak (H_g) at 5.46 and 5.68 ppm, the ring protons ($H_c \sim H_f$) peak of GlcN residues at 2.52–4.18 ppm respectively. FTIR spectra were recorded on a Perkin-Elmer Spectrum One by the KBr pellets method. The measurement was carried out at 298 K ranging from 500 to 4000 cm^{-1}. X-ray diffraction (D/max-2550, Rigaku) was used to investigate the crystalline of CS and N-MAC. The water solubility of N-MAC was evaluated from turbidity. N-MAC (400 mg) was dissolved in deionized water (10 mL). Following stepwise addition of deionized water, the transmittance of solution was recorded on UV–vis spectrometer using a 1 cm quartz cuvette at 600 nm. Images of N-MAC solution and hydrogels were taken by Canon IXUS 210. ESEM image of N-MAC hydrogel was carried out on a Helios NanoLab 600i with operating voltage of 10.0 kV. The compress testing was carried out with Texture Analyzer TA.XT plus (Stable Micro System, UK) in MARMALADE mode. All tested samples were prepared in cylindrical with height of 10 mm and diameter of 20 mm. The probe (P/0.5) with 5 kg load cell was compressed into the sample to a depth of 2.5 mm at the test-speed of 1 mm/s.

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