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Non-cytotoxic, in situ gelable hydrogels composed of *N*-carboxyethyl chitosan and oxidized dextran

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ABSTRACT

A series of in situ gelable hydrogels were prepared from oxidized dextran (Odex) and *N*-carboxyethyl chitosan (CEC) without any extraneous crosslinking agent. The gelation readily took place at physiological pH and body temperature. The gelation process was monitored rheologically, and the effect of the oxidation degree of dextran on the gelation process was investigated. The higher the oxidation degree of Odex, the faster the gelation. A highly porous hydrogel structure was revealed under scanning electron microscopy (SEM). Swelling and degradation of the Odex/CEC hydrogels in PBS showed that both swelling and degradation were related to the crosslinking density of the hydrogels. In particular, the hydrogels underwent fast mass loss in the first 2 weeks, followed by a more moderate degradation. The results of long-term cell viability tests revealed that the hydrogels were non-cytotoxic. Mouse fibroblasts were encapsulated in the hydrogels and cell viability was at least 95% within 3 days following encapsulation. Furthermore, cells entrapped inside the hydrogel assumed round shape initially but they gradually adapted to the new environment and spread-out to assume more spiny shapes. Additionally, the results from applying the Odex/CEC system to mice full-thickness transcutaneous wound models suggested that it was capable of enhancing wound healing.

1. Introduction

Aqueous injectable, in situ gel-forming systems have received much attention in tissue engineering because of several advantages over their preformed counterparts (e.g., scaffolds). First of all, an injectable, in situ gel-forming matrix could be directly introduced through a needle or a catheter and does not require surgical implantation [1]; it is initially a fluid and readily conforms to the surrounding upon introduction. Second, various potential therapeutic agents, including cells, drugs and growth factors, could be incorporated into the matrix by simple pre-mixing. Third, the aqueous in situ gel-forming matrix is completely devoid of residual solvents as compared with many preformed scaffolds [2].

Chitosan, a linear polysaccharide composed of α-1,4-linked 2-amino-2-deoxy-α-D-glucose (*N*-acetylglucosamine), is biocompatible, biodegradable, non-toxic, and bioresorbable [3–6]. Chitosan is a FDA GRAS (Generally Recognized as Safe) material and has been widely utilized in many fields, such as pharmaceutics, tissue engineering, as food additives, textiles, etc. [4,7–10]. However, native chitosan is practically insoluble in organic solvents and can only be dissolved in aqueous dilute acid, thereby, limiting its direct application in tissue engineering. Various chemical modifications have been employed to enhance the aqueous solubility of chitosan; some examples are reductive amination with phosphorylcholine–glyceraldehyde [11], sulfation [12], carboxymethylation [13], carboxyethylation [14], and *N*- or *O*-acylation [9,15,16]. Amongst these, introducing the highly hydrophilic acrylic acid into the chitosan chains through Michael's reaction is both facile and effective to render it soluble in water [14].

In general, polysaccharides biodegrade relatively fast and are not as durable as synthetic polymers; crosslinks could be introduced into the polysaccharide chains for stabilization and thus enhance their durability. There are numerous reports on crosslinking of chitosan to form hydrogels [17–22], where small molecular crosslinkers are generally involved but they have cytotoxicity potential. Furthermore, slow gelation on some of the preparations renders them impractical for applications requiring rapid in situ gelation. Dextran is a naturally occurring bacterial polysaccharide consisting primarily of an α-1,6-linked D-glucopyranose residues with a few percent of α-1,2-, α-1,3- or α-1,4-linked side chains [23,24]. Being biodegradable and non-toxic, dextran has been used as a macromolecular carrier for delivery of drugs or proteins and for separation and purification of biological materials [25]. Oxidizing dextran with periodate is a classic method to

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functionalize dextran and produces an entity with multiple functional aldehyde groups that could serve as a macromolecular crosslinker for those polymers bearing free amino groups to form hydrogels [26]. We previously reported an in situ gelable hydrogel system prepared from *N*-carboxyethyl chitosan (CEC) crosslinked by a macromolecular crosslinker, oxidized dextran (Odex), and characterized the gelation process with rheology [27]. Herein, we further characterized the system; the modification of both dextran and chitosan was described; the structure, cytotoxicity, biodegradability, and gelation mechanism of the gel system were presented and discussed. Our data showed that the gelation time could be modulated and the hydrogel was both non-cytotoxic and biodegradable.

2. Materials and methods

2.1. Materials

Chitosan (deacetylation degree 85%, M_w 750,000), dextran (from *Leuconostoc mesentereoides*, $M_w = 76{,}000$), sodium periodate, sodium hydroxide, and acrylic acid were purchased from Sigma–Aldrich (St. Louis, MO).

M. DUNNI (clone III8C) murine dermal fibroblast CRL-2017 and McCoy's 5A medium were purchased from ATCC (Manassas, VA, USA). Fetal Bovine Serum (FBS) was acquired from Hyclone (Logan, UT, USA) and Penicillin–Streptomycin (Pen–Strep) solution was purchased from Gibco (Grand Island, NY, USA). Cell culture inserts (polycarbonate, 6.5 mm diameter, 0.2 μm pore size) were purchased from NUNC (Rochester, NY, USA). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay kit (CellTiter 96s) was obtained from Promega (Madison, WI). "Live/Dead™" staining kit was purchased from Molecular Probes (Eugene, OR, USA).

2.2. Methods

2.2.1. Synthesis of CEC and Odex

CEC was synthesized according to a previously reported method [14]. Briefly, in 50 mL of water containing 1.88 mL of acrylic acid, 1 g of chitosan was dissolved and the mixture was maintained at 50 °C and stirred constantly for 3 days. Thereafter, the pH of the reaction mixture was adjusted to 10–12 with a 10 N NaOH aqueous solution in order to convert the carboxylic acid to its sodium salt. The mixture was then dialyzed (MWCO 6000–8000) extensively against water for 3 days and then lyophilized to obtain pure CEC. Odex was prepared by first dissolving 5 g of dextran in 400 mL of distilled water, followed by the addition of a desired amount of $NaIO_4$, in 5, 15, and 25% molar equivalent, in 100 mL distilled H_2O. The mixture was stirred at 25 °C for 24 h, and an equimolar of diethylene glycol was added to quench the unreacted $NaIO_4$; the product was dialyzed exhaustively for 3 days against distilled H_2O followed by lyophilization to obtain pure Odex (yield, 65–80%). The oxidation degree of Odex was determined by quantifying the aldehyde groups formed by *tert*-butyl carbazate via carbazone formation [28,29]. Briefly, an Odex solution (10 mg/mL in pH 5.2 acetate buffer) was prepared, and a fivefold excess *tert*-butyl carbazate in the same buffer was added and the mixture was allowed to react for 24 h at ambient temperature followed by addition of a fivefold excess of $NaBH_3CN$. After another 12 h, the reaction product was precipitated thrice with acetone and the final precipitate was dialyzed thoroughly against water followed by lyophilization. Odex preparations with theoretical oxidation degrees of 5, 15 and 25% were coded as Odex-I, Odex-II, and Odex-III, respectively. The degree of oxidation (i.e., abundance of aldehyde groups) was assessed by ^{1}H NMR by integrating the peaks at 1.3 ppm (*tert*-butyl) against 4.8 ppm (anomeric proton of dextran) (Varian 400). The molecular weights of Odex (dissolved in water, concentration: 0.5 mg/mL) were determined by HPLC (Waters 600e pump and controller, 715 injector, 410 differential refractometer, USA) coupled to Waters Ultrahydrogel 2000, 1000, and 500; 300 mm × 7.8 mm columns connected in series, using 0.1 M KNO_3 as a mobile phase at a flow rate of 0.8 mL/min with temperature maintained at room temperature. Dextran standards (Fluka Chemie AG, Switzerland) in the molecular range of 12–80 kDa were used as calibration standards.

2.2.2. Preparation solutions and hydrogels

Desired amount of Odex with theoretical oxidation degrees of 5, 15 and 25%, and CEC was dissolved in PBS (0.01 M, pH = 7.4) to form 2.5% (w/v) solutions, respectively. The solutions were stored at 5 °C before testing. Odex/CEC hydrogels were prepared by mixing Odex solutions with CEC solutions in desired ratios at room temperature, and the mixtures were gently stirred for 10 s to allow homogenous mixing. Then, the mixtures were kept at 37 °C for 12 h for gel formation.

2.2.3. Rheological measurements

Rheological measurements were performed on a rheometer (Physica MCR 301, Anton Paar, Hertford Herts, UK) under a dynamic model by applying a small oscillatory strain to the sample. A couette geometry (bob $d = 26.687$ mm, $h = 39.994$ mm, cup $d = 28.930$ mm) was used. The measurement starts at $t = 60$–90 s. For time sweeping tests, the storage moduli G' and loss moduli G'' of a 2.5% Odex/CEC (5:5) mixed system were monitored as a function of time at a frequency of 1 rad/s and a shear strain of 2% at a constant temperature of 37 °C.

2.2.4. Assessment of the morphology of the hydrogel by scanning electron microscopy

Lyophilized fractured pieces of Odex-III/CEC hydrogels ($0.5 \times 0.5 \times 0.2$ cm^3) were secured on an aluminum board with copper tapes and sputtered with gold. Both surface and cross-sectional morphologies were recorded with a field-emission scanning electron microscope (SFEG Leo 1550, AMO GmbH, Aachen, Germany) at 20 kV.

2.2.5. Swelling analysis

Swelling studies were performed on Odex-III/CEC hydrogels (ratio 3:7, 4:6, 5:5, 6:4 and 7:3, Odex:CEC) in 0.01 M PBS at 37 °C. The weights of lyophilized hydrogels were recorded (W_d) prior to immersion in PBS. After 48 h of incubation, the hydrogels were blotted with tissue paper to remove excess water and weighed (W_s). The swelling ratio (q) was calculated by $q = (W_s - W_d)/W_d$.

2.2.6. Hydrolytic degradation

Degradation was performed on hydrogels prepared from blending 2.5% Odex-III and 2.5% CEC aqueous solutions in different ratios (3:7, 5:5, 7:3). Five groups of samples ($n = 3$) were prepared for a 5-week degradation study, with each group being utilized per week/time-point. The hydrogels were incubated with 5 mL of PBS at 37 °C, and the PBS was changed daily. Degradation of the hydrogels was monitored weekly by tracking their weight loss after lyophilization.

2.2.7. Cytotoxicity of hydrogels and their degradation byproducts

Cell toxicity assays were carried out in 96-well plates (seeding density: 1×10^5 cells/mL) on Odex-III/CEC hydrogels comparable to those used in the swelling analysis. Co-culture was performed using a model mouse dermal fibroblast with McCoy's 5A medium containing 10% FBS and 1% Pen–Strep solution maintained at 37 °C under a humidified atmosphere of 5% CO_2. Cell viability studies were performed using MTS assays to verify the non-cytotoxicity of both hydrogels and their degradation byproducts. To avoid any potential error introduced by transferring the hydrogel for performing assay, a non-contact method was employed to evaluate the cytotoxicity of the Odex/CEC hydrogels. Briefly, sterilized Odex/CEC hydrogel pieces, tailored to $2 \times 3 \times 2$ mm, were first deposited in culture inserts and immersed in culture wells pre-seeded with cells ($n = 3$ per group). Cell viability was determined on days 0, 3, 7, 12 and 30, respectively. For each time point, 20 μL of MTS solution was added to the culture medium, and monolayer cultured cells were used as controls. After incubating at 37 °C for 1 h, the absorbance of the solutions was determined at 490 nm.

2.2.8. Cell encapsulation in the hydrogel

Cell encapsulation was performed with the same cell line at passages of 5–10. In a 48-well plate, pre-sterilized 2.5% Odex-III in PBS (0.25 mL), 2.5% CEC in PBS (0.25 mL) and fibroblasts were mixed and deposited into each well to reach a final cell density of 1×10^5 cells/mL. The mixture was incubated at 37 °C for gelation under a humidified atmosphere of 5% CO_2 for 10 min. The PBS in the hydrogels was removed by equilibrating the cell embedded hydrogels with fresh culture medium every 20 min for two consecutive hours. Finally, 0.5 mL of cell culture medium was added to each well, and it was changed every other day. To observe cell morphology and proliferation inside the hydrogels, images of cells were acquired in situ with a QCapture 5 imaging software (Surrey, Canada) through a fluorescent microscope (Olympus IX-71). The viability of cells, in direct contact with hydrogels, was verified by Live/Dead™ staining. Briefly, a fresh cross-section (approximately 200 μm thick, prepared by shaving the intact hydrogel with a razor blade) of cell-laden hydrogel was incubated in 200 μL of a "Live/Dead™" dye solution (2 μM calcein-AM and 4 μM EthD-1) for 10 min, and it was observed under a fluorescent microscope.

2.2.9. Efficacy assessment of the hydrogel to accelerate wound healing in a murine full-thickness transcutaneous dermal wound model

The efficacy of the Odex/CEC hydrogel formulation was evaluated in a mouse transcutaneous full-thickness dermal wound model that we have been consistently utilizing [30]. Briefly, male mice (5 weeks, Balb/cj strain, Jackson Lab, Bar Harbor, ME) were first anesthesized with isoflurane (5% for induction and 2–2.5% for maintenance). After removal of the hair on the dorsal side, a full-thickness excisional wound of diameter 0.8–1 cm was created surgically. The hydrogel precursors (CEC 1.75% and Odex 1.75% (w/v) both dissolved in PBS and autoclaved) were mixed and approximately 100 μL of the mixture were deposited into the wound bed created. The hydrogel-filled wound bed was then covered and sealed by an overlay of Tegaderm™ dressing (3 M, St. Paul, MN), followed by the application of a Band-Aid™ (fabric, 1″ wide, Johnson & Johnson, New Brunswick, NJ) to fully secure the wound site. For controls, the wound beds were likewise treated with 100 μL of sterile PBS and then covered by Tegaderm™ dressing followed by Band-Aid™. Seven animals were used per group and they were euthanized after 7 days. The entire wound bed in conjunction with the tissue adjoining the implants were excised, preserved in neutral buffered formalin, processed and paraffin embedded. The cross-sections prepared were stained with H&E. Mason-Trichrome staining was performed on