Published in final edited form as:

Biomaterials. 2008 October ; 29(29): 3905–3913. doi:10.1016/j.biomaterials.2008.06.025.

Non-cytotoxic, In Situ Gelable Hydrogels Composed of *N*-carboxyethyl Chitosan and Oxidized Dextran

Lihui Weng¹, Alexander Romanov², Jean Rooney², and Weiliam Chen^{1,*}

¹ Department of Biomedical Engineering, State University of New York-Stony Brook, Stony Brook, NY 11794-8181

² Division of Laboratory Animal Research, State University of New York-Stony Brook, Stony Brook, NY 11794-8611

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.

Keywords

Dextran; Chitosan; Hydrogel; Cell encapsulation; Oxidation

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

*Corresponding author: Weiliam Chen, Department of Biomedical Engineering, State University of New York-Stony Brook, Stony Brook, NY 11794-8181, Tel: 631-444-2788, Fax: 631-444-6646, Email: weiliam.chen@stonybrook.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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 pharmaceuticals, 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 phosphorycholine-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 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 were 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 mesenteroides*, 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 μ m 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"TM 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 [28]. 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 6,000 to 8,000) 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 NaIO₄, in 5%, 15%, and 25% molar equivalent, in 100 mL distilled H₂O. The mixture was stirred at 25 °C for 24 h, and an equimolar of diethylene glycol was added to quench the unreacted NaIO₄; the product was dialyzed exhaustively for 3 days against distilled H₂O 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 [29,30]. Briefly, an Odex solution (10 mg/mL in pH 5.2 acetate buffer) was prepared, and a 5-fold 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 5-fold excess of NaBH₃CN. 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 ¹H 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 KNO₃ 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 were 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 ($0.5 \times 0.5 \times 0.2$ cm³) 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). 5 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% CO₂. 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 mm $\times$ 3 mm $\times$ 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 μ L 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 to 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% CO₂ for 10 min. The PBS in the hydrogels was removed by equilibrating the cell embedded hydrogels with fresh culture medium every 20 min for 2 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 μ m thick, prepared by shaving the intact hydrogel with a razor blade) of cell-laden hydrogel was incubated in 200 μ L of a “Live/Dead™” dye solution (2 μ M calcein AM and 4 μ M 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 [31]. Briefly, male mice (5 weeks, Balb/cj strain, Jackson Lab, Bar Harbor, ME) were first anesthetized with isoflurane (5% for induction and 2 to 2.5% for maintenance). After removal of the hair on the dorsal side, a full-thickness excisional wound of diameter of 0.8 to 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 μ L 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 (3M, 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 μ L 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 some selected samples. The histology sections were observed under a Nikon Eclipse E800 microscope and the images were captured and digitized for analyses; the extent of re-epithelialization and granulation tissue formation were determined by Image J (National Institutes of Health). All animal studies were performed following the guidelines prescribed by the Institutional Animal Care and Use Committee of SUNY-Stony Brook (IACUC Protocol # 2006–1320).

2.2.10. Statistical analysis—Statistical analysis was performed using a Student's t-test with a $p < 0.05$ for statistical significance. All values are reported as the mean and standard deviation.

3. Results and discussion

Dextran contains vicinal hydroxyl groups that can readily be modified with periodate to form aldehyde groups. The oxidized dextran bearing aldehyde groups serves as a good macromolecular crosslinker for polysaccharides with free amino groups. As summarized in Table 1, the actual oxidation degrees of periodate treated dextrans with theoretical oxidation degrees of 5%, 15% and 25%, were determined as 4.6%, 7.4%, and 15.8%, respectively, by ^1H NMR; and the HPLC determined molecular weights were around 48.1, 38.5, and 36.8 kDa, respectively. Evidently, increasing oxidation degree resulted in a decrease in the molecular weight of the Odex. The substitute degree of chitosan undergoing Michael's addition was determined as approximately 45% by ^1H NMR. Incorporation of COOH groups into the structure of chitosan partially disrupted the strong hydrogen bonds in native chitosan and thus rendered it water-soluble. A mixture of Odex and CEC readily formed transparent hydrogels, and the gelation time (within several tens of seconds) was dependent upon both the polymer concentration and temperature. Rapid gelation also readily occurred at physiological pH and body temperature. The mechanism was proposed in our previous work [27]; briefly, the aldehyde groups on Odex formed Schiff base with the free amino groups on the CEC, leading to the formation of a 3-D crosslinked network structure. This investigation aimed to study the formation, structure of the hydrogel and its in vitro properties including validation of its non-cytotoxicity. In addition, in vivo validation of its efficacy in enhancing the repair of murine full-thickness transcutaneous dermal wounds was also performed.

3.1. Rheological analysis

Fig. 1 shows the variation of elastic (G') and viscous modulus (G'') as well as the complex viscosity (η^*) versus time for a 2.5% Odex-III/CEC (ratio 5:5) preparation after mixing. At the very beginning, with G' lower than G'' , the system exhibited the behavior of a viscous fluid, corresponding to an Odex/CEC sol. Both moduli elevated rapidly as gelation proceeded, whereas the buildup rate of G' was much higher than that of G'' due to the occurrence of crosslinking through Schiff base formation between the aldehyde residues of Odex and the amino residues of CEC. Different rates of elevation of G' and G'' led to their crossover ($t = t_{\text{gel}}$, where t_{gel} is defined as the gel point), indicative of transition of the Odex/CEC system from a viscous behavior dominated liquid-phase to an elastic behavior dominated solid-phase [32]. Eventually, both moduli leveled off, implying the formation of a well-developed three-

dimensional network. η^* of the system also underwent a similar transformation, a rapid buildup at the beginning followed by leveling off over time. For a 2.5% Odex/CEC system, t_{gel} was approximately 79 s, which was very fast. The gelation speed could be modulated by adjusting the concentrations of Odex/CEC [27].

The dependence of gelation time on the oxidation degree of Odex was investigated and the results were summarized in Table 1. While the Odex solutions reacted with the same 2.5% CEC solution, the gelation time decreased rapidly with an increase in the theoretical oxidation degree of the Odex from 5% to 25%, indicating that gelation was strongly correlated with the abundance of aldehyde groups, due to the fact that a higher rate of Schiff base crosslink formation occurred in the Odex/CEC mixed systems with a higher content of aldehyde groups on the Odex. Because of the similarity in structure of the Odexs of three oxidation degrees and their incremental physicochemical properties, the systematic investigations conducted using Odex-III was selected as a representative example for further description.

3.2. Morphology of the Odex/CEC hydrogels

From gross observation, the Odex/CEC hydrogel was transparent and yellowish in color, as shown in Fig. 2A. The intensity of the yellowish color of the hydrogel increased gradually with storage. Fig. 2B depicted the SEM image of a fractured, lyophilized hydrogel prepared from a 2.5% Odex-III/CEC (ratio 5:5) solution mixture, revealing a highly porous and interconnected interior structure with an average pore size of approximately 50 μm . It could be inferred that the hydrogel has a high water retention capacity, and both small and macromolecules could diffuse freely into it.

3.3. Swelling analysis

Fig. 3 showed the relationship between the swelling ratio q of the Odex/CEC hydrogels prepared from a 2.5% Odex-III solution and a 2.5% CEC solution, and the Odex content in the hydrogels. First, q decreased rapidly from 36.7 to 25.3 with an increase in the Odex content from 30% to 50%; thereafter, a slight decline from 25.3 to 23.2 in q was observed with the Odex content increased from 50% to 60%; and finally, q increased from 23.2 to 26.5 with a further increase in Odex content from 60% to 70%. This was attributed to the variation of the crosslinking density in the hydrogels, with an elevation of Odex content. As summarized in Table 1, when the Odex content was in the range of 50% to 60%, the theoretical ratio of the CHO on the Odex to the NH_2 on the CEC was close to 1 (i.e., mixing the 2.5% Odex and 2.5% CEC solution in equal volume). Therefore, it can be inferred that the crosslinking density increased with an increase in the Odex content from 30% to 50% when the NH_2 groups were in excess, leading to a decrease in the swelling ratio. When the crosslinking density reached its maximum in the range of 50% and 60%, q exhibited values approaching its minimum. A further increase in Odex content resulted in an excess of CHO groups, thus a lower crosslinking degree assuring a higher swelling ratio.

3.4. In vitro hydrolytic degradation of the Odex/CEC hydrogel

In vitro degradation of 2.5% Odex-III/CEC hydrogels (ratio 3:7, 5:5, and 7:3) was conducted in a 0.01 M PBS by examining the weight loss of the hydrogels at 37 $^{\circ}\text{C}$. As shown in Fig. 4, all hydrogels underwent fast mass loss in the first 2 weeks, followed by a more moderate degradation thereafter, implying that the Odex/CEC hydrogels were degradable hydrolytically. Apparently, degradation of the hydrogels depended on the relative amount of Odex and CEC used in their preparation. The 7:3 Odex-III/CEC hydrogel exhibited a considerably faster degradation profile as compared to its counterparts formulated using the other two ratios, with the 5:5 Odex-III/CEC hydrogel having the lowest degradation rate. In general, the degradation behavior of hydrogels is dependent upon the crosslinking density [33]; greater extent of crosslinking typically produces more stable hydrogels. As alluded to previously, the 5:5 Odex-

III/CEC hydrogel has the highest crosslinking density and therefore, degraded considerably slower. The 7:3 Odex-III/CEC hydrogel degraded slower than the 3:7 Odex-III/CEC hydrogel, although their theoretical crosslinking density were comparable. This indicated that not only the hydrolytic susceptibility of the Schiff base but also the degradation of both Odex and CEC are important in breaking down of the hydrogel.

3.5. Cytotoxicity and cell viability analysis

The cytotoxicity and cell viability of the Odex-III/CEC (3:7, 5:5, and 7:3) hydrogels were evaluated by performing MTS assay. Cells were seeded at the same initial density in the wells, with or without hydrogel, on day 0. The cell viability was examined at day 3, 7, 12 and 30, respectively, and the results were depicted in Fig. 5. There was no significant difference between the results from cells exposed to hydrogels and the control ($p < 0.05$) after less than one week of incubation, suggesting the hydrogels did not affect cell growth; thus, their non-cytotoxicity. It was noticed that on days 3 and 7, the medium of the 3:7 Odex/CEC hydrogel showed a slightly lower absorbance than those of the other two hydrogels (5:5 and 7:3) as well as the control, which could be attributed to the large amount of COO^- on the hydrogel matrix in the presence of excess amounts of CEC. Longer-term cell culture results (one month) showed that the cells started to proliferate in the same manner comparable to that of the controls, further confirming the non-cytotoxic nature of the hydrogels; it could also be inferred that the degradation byproducts of the hydrogel were nontoxic as well. Moreover, the hydrogels were clearly broken down steadily with the increase of culture time with cells (data not shown), this was likely due to the collective effect of hydrolytic degradation (see Fig 4) and enzymes produced by the fibroblasts hastening the degradation of the hydrogel's polymer network structure [34].

3.6. Cell encapsulation in hydrogels

To further validate the non-cytotoxicity of the Odex/CEC hydrogels and in concert with exploring its potential as an in situ gelable biomaterial for directly applying to dermal wounds, cell encapsulation was performed using dermal fibroblast as a model cell. As shown in Fig. 6, cells residing inside the hydrogel initially assumed a round shape (Fig. 6A), which was apparently very different from the pattern of the cells residing on the 2D culture dish (Fig. 6F). Initially, the cells did not adhere to the Odex/CEC hydrogel, which could be due to its abundance of COO^- and thus, negative charges, on the hydrogel network. However, there was no evidence of cell cytotoxicity, typically manifested as excessive cell deaths. After 1 week of incubation, some of the cells started to adhere to the Odex/CEC hydrogels, as indicated by their assumption of oval or rod shape (Fig. 6B, C, and D). Fig. 6C was an image of living cells. Clearly, cells were spreading out and cell clusters started to form, indicating proliferation. To further examine the morphology of the cells entrapped inside the hydrogels, cell-laden hydrogels were fixed with 70% ethanol followed by staining with crystal violet. The results were depicted in Fig. 6B and D at a higher magnification. The nuclei were readily recognizable with cells assuming a spread-out pattern (marked with “▼”), which was several times of their original sizes (round, unattached). This suggested that the cells were adapting to or modifying the hydrogel and became more tolerant of the new environment surrounding them after several days of incubation. As depicted in Fig. 2B, the Odex/CEC hydrogel showed a highly porous internal structure with a pore size sufficiently large for accommodating fibroblasts and their change in conformation. Fibroblasts are known to secrete and deposit extracellular matrix (ECM) on polymer matrices [35,36]. The presence of ECM rendered the Odex/CEC hydrogel more amenable to cell growth, functioning and cell-cell interactions [37]. Furthermore, the cell density steadily increased with time (Fig. 6E), implying that the cells were proliferating in the confined space formed by the hydrogel network. Continual increase in cell number accelerated the break down of the hydrogel, presumably due to the increased degradative enzyme produced

by the fibroblasts, further confirming the biodegradability of the hydrogel network (data not shown).

Live/DeadTM staining was performed to differentiate the living cells stained with calcein-AM (green) versus the dead cells stained with ethidium homodimer (red) under fluorescence microscopy. Fig. 7 depicted the stained cells residing in the 2.5% Odex-III/CEC (ratio 5:5) hydrogels. On day 3 (Fig. 7A), the cells maintained their characteristic round shape, and nearly 95% cells were alive (as indicated by the green color). On day 7 (Fig. 7B), some of the cells started to spread out (marked with “*”) and this was in good agreement with the results shown in Fig. 6. Collectively, these results suggested that the viability of cells was not affected after being embedded in the Odex/CEC hydrogel for one week.

3.7. Acceleration of wound healing in murine transcutaneous full-thickness wounds

Wound healing is a dynamic process that typically evolves from its initial inflammatory response to completely resolution and thus, healing [38]. The capacity of an implantable to maintain a moist environment has been implicated as an important factor in accelerating the wound repair process. Hydrogels, with their high water contents and retention capacity, appear to be optimal media to enhance wound healing [39], and thus, much interest has been focused on developing hydrogel-based wound dressings from biomaterials [39–42]. In situ forming hydrogels are capable of fully conforming to wound beds and are thus advantageous over performed scaffolds.

Chitosan is both biocompatible and bacteriostatic [43] and has been shown to promote wound healing [44,45]. In this study, chitosan was modified to form CEC. Unlike native chitosan that is only soluble in acidic condition, CEC is soluble at physiological pH and mixing it with Odex at the same pH produces a chitosan containing hydrogel precursor (Fig. 1) capable of fully-conforming to the application site before congealing. As shown above, the hydrogel formed is both highly porous (Fig. 2), non-cytotoxic to dermal fibroblasts and does not hinder their proliferation (Fig. 5,6 and 7). Therefore, a further investigation of the application of this system in wound healing was conducted. As alluded to previously, compared to other ratios, the 5:5 of Odex-III: CEC has the highest theoretical crosslinking density and the resulted hydrogel is more stable (Fig. 4). Hence, the precursor of Odex-III/CEC mix (5:5) was applied to mouse full-thickness transcutaneous wound models ($n = 7$ per group) to form hydrogel in situ. Fig. 8 shows a set of typical wound beds shortly after the surgical procedure and closure with TegadermTM. The healing patterns after 7 days post-implantation were examined. From gross observation, the wound beds of the group treated with Odex/CEC hydrogel were considerably smaller as compared to those of the controls treated with PBS (result not shown). The histological sections of two typical wound beds were depicted in Fig. 9. Fig. 9A depicted the histology of a typical wound bed received the Odex/CEC hydrogel; whereas, its counterpart received PBS was depicted in Fig. 9B. The residue of the hydrogel implanted after 7 days can be seen lack of initial integrity in Fig. 9A, implying the biodegradation of the hydrogel. Granulation tissues were present in the dermis of both groups, with infiltrated inflammatory cells more abundant in the group treated with the Odex/CEC hydrogel (see the insets). The extent of wound healing was quantified as % granulation tissue formation and % re-epithelialization/epidermal hyperplasia. The % granulation tissue formation of the wound bed received the hydrogel was $84.9 \pm 9.0\%$ (Fig. 9A) as compared to the $46.3 \pm 8.3\%$ of the control (Fig. 9B) ($p < 0.001$). Granulation tissue fills the wound bed and set the stage for re-epithelialization [39]. Application of the Odex/CEC hydrogel resulted in 100% re-epithelialization, as compared to the counterpart treated with PBS ($54.3 \pm 8.4\%$, $p < 0.001$). Our finding supports further development of the Odex/CEC hydrogel as an in situ gelable wound dressing for promoting wound healing.

4. Conclusions

A versatile, non-toxic, in situ crosslinkable, biodegradable polymer hydrogel has been successfully prepared from dextran and chitosan without incorporating any extraneous crosslinking agents. The gelation time of the Odex/CEC system can be modulated by adjusting the oxidation degree of dextran. The hydrogel formed has a highly porous interior structure with both their swelling behavior and degradability dependent upon the relative contents of Odex and CEC. The results of in vitro cell culture studies reveal that the hydrogel is non-cytotoxic and biodegradable. Mouse fibroblasts encapsulated in the hydrogel, despite not adhering initially, are alive inside the hydrogel and eventually adapt to the environment presumably by deposition of extracellular matrix. Furthermore, applying the Odex/CEC hydrogels to mice full-thickness transcutaneous wound models demonstrates good efficacy of accelerating wound healing. These results show that this material could be adapted as an in situ gelable wound dressing.

Acknowledgements

The investigation was supported by a grant from the National Institutes of Health (DK068401), with partial support was also provided by an Innovative Technology Development grant from the Center for Biotechnology of SUNY-Stony Brook. The authors would like to thank Mr. Christopher Judd of Engelhard, Inc. for his assistance in HPLC analyses.

References

1. Shu XZ, Liu Y, Palumbo FS, Luo Y, Prestwich GD. In situ crosslinkable hyaluronan hydrogels for tissue engineering. *Biomaterials* 2004;25:1339–48. [PubMed: 14643608]
2. Gutowska A, Jeong B, Jasionowski M. Injectable gels for tissue engineering. *Anat Rec* 2001;263:342–9. [PubMed: 11500810]
3. Noble L, Gray AI, Sadiq L, Uchegbu IF. A non-covalently cross-linked chitosan based hydrogel. *Inter J Pharm* 1999;192:173–82.
4. Majeti NV, Kumar R. A review of chitin and chitosan applications. *React Funct Polym* 2000;46:1–27.
5. Boucard N, Viton C, Agay D, Mari E, Roger T, Chancerelle Y, Domard A. The use of physical hydrogels of chitosan for skin regeneration following third-degree burns. *Biomaterials* 2007;28:3478–88. [PubMed: 17482258]
6. Chang YY, Chen SJ, Liang HC, Sung HW, Lin CC, Huang RN. The effect of galectin 1 on 3T3 cell proliferation on chitosan membranes. *Biomaterials* 2004;25:3603–11. [PubMed: 15020134]
7. Dutkiewicz JK. Superabsorbent materials from shellfish waste - A review. *J Biomed Mater Res* 2002;63:373–81. [PubMed: 12115771]
8. Kurita K, Kaji Y, Mori T, Nishiyama Y. Enzymatic degradation of β -chitin: susceptibility and the influence of deacetylation. *Carbohydr Polym* 2000;42:19–21.
9. Felix L, Hernandez J, Arguelles-Monal WM, Goycoolea FM. Kinetics of gelation and thermal sensitivity of *N*-isobutyl chitosan hydrogels. *Biomacromolecules* 2005;6:2408–15. [PubMed: 16153075]
10. Welsh ER, Schauser CL, Qadri SB, Price RR. Chitosan cross-linking with a water-soluble, blocked diisocyanate. *Biomacromolecules* 2002;3:1370–4. [PubMed: 12425678]
11. Tiera MJ, Qiu XP, Bechaouch S, Shi Q, Fernandes JC, Winnik FM. Synthesis and characterization of phosphorylcholine-substituted chitosans soluble in physiological pH conditions. *Biomacromolecules* 2006;7:3151–6. [PubMed: 17096545]
12. Zhang C, Qu G, Sun Y, Wu X, Yao Z, Guo Q, et al. Pharmacokinetics, biodistribution, efficacy and safety of *N*-octyl-*O*-sulfate chitosan micelles loaded with paclitaxel. *Biomaterials* 2008;29:1233–41. [PubMed: 18093646]
13. Lu G, Kong L, Sheng B, Wang G, Gong Y, Zhang X. Degradation of covalently cross-linked carboxymethyl chitosan and its potential application for peripheral nerve regeneration. *Eur Polym J* 2007;43:3807–18.

14. Jiang H, Wang Y, Huang Q, Li Y, Xu C, Zhu K, et al. Biodegradable hyaluronic acid/*N*-carboxyethyl chitosan/protein ternary complexes as implantable carriers for controlled protein release. *Macromol Biosci* 2005;5:1226–33. [PubMed: 16307434]
15. Badawy MEI, Rabea EI, Rogge TM, Stevens CV, Smagghe G, Steurbaut W, et al. Synthesis and fungicidal activity of new *N*, *O*-acyl chitosan derivatives. *Biomacromolecules* 2004;5:589–95. [PubMed: 15003025]
16. Zhu A, Shan B, Yuan Y, Shen J. Preparation and blood compatibility of phosphorylcholine-bonded *O*-butyrylchitosan. *Polym Int* 2003;52:81–5.
17. Yin L, Fei L, Cui F, Tang C, Yin C. Superporous hydrogels containing poly(acrylic acid-co-acrylamide)/*O*-carboxymethyl chitosan interpenetrating polymer networks. *Biomaterials* 2007;28:1258–66. [PubMed: 17118443]
18. Roughley P, Hoemann C, DesRosiers E, Mwale F, Antoniou J, Alini M. The potential of chitosan-based gels containing intervertebral disc cells for nucleus pulposus supplementation. *Biomaterials* 2006;27:388–96. [PubMed: 16125220]
19. Berger J, Reist M, Mayer JM, Felt O, Peppas NA, Gurny R. Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications. *Europ J Pharm Biopharm* 2004;57:19–34.
20. Chen L, Tian Z, Du Y. Synthesis and pH sensitivity of carboxymethyl chitosan-based polyampholyte hydrogels for protein carrier matrices. *Biomaterials* 2004;25:3725–32. [PubMed: 15020148]
21. Crompton KE, Prankerd RJ, Paganin DM, Scott TF, Horne MK, Finkelstein DI, et al. Morphology and gelation of thermosensitive chitosan hydrogels. *Biophys Chem* 2005;117:47–53. [PubMed: 15905019]
22. Cana HK, Denizlia BK, Gunera A, Rzaev ZMO. Effect of functional crosslinking agents on preparation and swelling properties of dextran hydrogels. *Carbohydr Polym* 2005;59:51–6.
23. Nowakowska M, Zapotoczny S, Sterzel M, Kot E. Novel water-soluble photosensitizers from dextrans. *Biomacromolecules* 2004;5:1009–14. [PubMed: 15132694]
24. Mehvar R. Dextrans for targeted and sustained delivery of therapeutic and imaging agents. *J Controlled Release* 2000;69:1–25.
25. Ferdous A, Akaike T, Maruyama A. Inhibition of sequence-specific protein-DNA interaction and restriction endonuclease cleavage via triplex stabilization by poly(L-lysine)- graft-dextran copolymer. *Macromolecules* 2004;37:3239–48.
26. Draye J, Delaey B, Voorde Andre Van de, An Van Den Bulcke, Bogdanov B, Schacht E. In vitro release characteristic of bioactive molecules from dextran dialdehyde crosslinked gelatin hydrogels films. *Biomaterials* 1998;19:99–107. [PubMed: 9678856]
27. Weng L, Chen X, Chen W. Rheological properties of in situ crosslinkable hydrogels from dextran and chitosan derivatives. *Biomacromolecules* 2007;8:1109–15. [PubMed: 17358076]
28. Jiang H, Wang Y, Huang Q, Li Y, Xu C, Zhu K, et al. Biodegradable hyaluronic acid/*N*-carboxyethyl chitosan/protein ternary complexes as implantable carriers for controlled protein release. *Macromol Biosci* 2005;5:1226–33. [PubMed: 16307434]
29. Maia J, Ferreira L, Carvalhoc R, Ramosd MA, Gil MH. Synthesis and characterization of new injectable and degradable dextran-based hydrogels. *Polymer* 2005;46:9604–14.
30. Jia XQ, Burdick JA, Kobler J, Clifton RJ, Rosowski JJ, Zeitels SM, et al. Synthesis and characterization of in situ cross-linkable hyaluronic acid-based hydrogels with potential application for vocal fold regeneration. *Macromolecules* 2004;37:3239–48.
31. Wang Z, Pan H, Yuan Z, Chen W, Pan Y. Assessment of dermal wound repair after collagen implantation with optical coherence tomography. *Tissue Eng C* 2008;14:1–11.
32. Winter HH, Chambon F. Analysis of linear viscoelasticity of a crosslinking polymer at the gel point. *J Rheol* 1986;30:367–82.
33. Lee KY, Bouhadir KH, Mooney DJ. Degradation behavior of covalent crosslinked poly(aldehyde guluronate) hydrogels. *Macromolecules* 2000;33:97–101.
34. Weng L, Pan H, Chen W. Self-crosslinkable hydrogels composed of partially oxidized hyaluronan and gelatin: In Vitro and In Vivo responses. *J Biomed Mater Res A* 2008;85:352–65. [PubMed: 17688243]

35. Tamada Y, Ikada Y. Fibroblast growth on polymer surface and biosynthesis of collagen. *J Biomed Mater Res* 2004;28:783–9. [PubMed: 8083246]
36. Halfter W, Liverani D, Vigny M, Monard D. Deposition of extracellular matrix along the pathways of migrating fibroblasts. *Cell Tissue Res* 1990;262:467–81. [PubMed: 1706644]
37. Mo XM, Xu CY, Kotaki M, Ramakrishna S. Electrospun P(LLA-CL) nanofiber: a biomimetic extracellular matrix for smooth muscle cell and endothelial cell proliferation. *Biomaterials* 2004;25:1883–90. [PubMed: 14738852]
38. Schwentker A, Vodovotz Y, Weller R, Billiar TR. Nitric oxide and wound repair: role of cytokines? *Nitric Oxide* 2002;7:1–10. [PubMed: 12175813]
39. Balakrishnan B, Mohanty M, Umashankar PR, Jayakrishnan A. Evaluation of an in situ forming hydrogel wound dressing based on oxidized alginate and gelatin. *Biomaterials* 2005;26:6335–42. [PubMed: 15919113]
40. Yusof N, Hafiza AHA, Zohdi RM, Bakar ZAM. Development of honey hydrogel dressing for enhanced wound healing. *Rad Phys Chem* 2007;76:1767–70.
41. Garcia Y, Wilkins B, Collighan RJ, Griffin M, Pandit A. Towards development of a dermal rudiment for enhanced wound healing response. *Biomaterials* 2008;29:857–68. [PubMed: 18031807]
42. Rayment EA, Dargaville TR, Shooter GK, George GA, Upton Z. Attenuation of protease activity in chronic wound fluid with bisphosphonate-functionalised hydrogels. *Biomaterials* 2008;29:1785–95. [PubMed: 18241915]
43. Liu XF, Guan YL, Yang DZ, Li Z, Yao KD. Antibacterial action of chitosan and carboxymethylated chitosan. *J Appl Polym Sci* 2001;79:1324–35.
44. Ueno H, Mori T, Fujinaga T. Topical formulations and wound healing applications of chitosan. *Adv Drug Deliv Rev* 2001;52:105–15. [PubMed: 11718934]
45. Berger J, Reist M, Mayer JM, Felt O, Gurny R. Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. *Eur J Pharm Biopharm* 2004;57:35–52. [PubMed: 14729079]

Fig. 1. Time evolution of storage modulus (G') and loss modulus (G'') for 2.5% (w/v) Odex-III/CEC (ratio 5:5) at 37°C. Insert: The time where G' and G'' crossover is denoted as t_{gel} (the gelation point).

Fig. 2. Photograph (A) and SEM image (B) for the 2.5% (w/v) Odex-III/CEC (ratio 5:5) hydrogels. Scale bar: 50 μm.

Fig. 3. Dependence of swelling ratio of Odex-III/CEC hydrogels (ratio 5:5) on the Odex content in the hydrogel.

Fig. 4. Hydrolytic degradation of 3:7, 5:5, and 7:3 Odex-III/CEC hydrogels.

Fig. 5. MTS assay of the cell viability with 3:7, 5:5, and 7:3 Odex-III/CEC hydrogels compared to monolayer cells control by the indirect contact method.

Fig. 6. Morphology of cells encapsulated in the Odex-III/CEC (ratio 5:5) hydrogel. (A): day 3 (C) 1 week, (E) 2 week, (B) and (D): 1 week stained with crystal violet; (F) 2-D control. ↑ cell assuming round shape; ▼ spread out cells; **H**: hydrogel.

Fig. 7. Live/Dead™ staining of the cells encapsulated in the Odex-III/CEC hydrogel. (A) day 3; (B) 1 week. ▼ live cells; ↑ dead cells; *: spread out cells, **H**: hydrogel.

Fig. 8. Wound beds of mice covered by Tegaderm™. (A) PBS treated, and (B) Odex/CEC hydrogel treated.

Fig. 9.

Acceleration of wound healing by Odex/CEC hydrogel in mouse transcutaneous full-thickness dermal wounds. **(A)** A representative wound bed implanted with hydrogel with both robust epidermal hyperplasia and granulation tissue formation. Part of the implanted hydrogel was fractured and neo-epidermal tissue apparently developed transversely along the side of the hydrogel fragment. **(B)** A representative wound bed treated with PBS (no hydrogel). Moderate epidermal hyperplasia and granulation tissue formation were observed. The images were captured under 20× magnification, whereas the insets were captured under 200× magnification.

Table 1
Oxidation degree of oxidized dextran determined from ¹H NMR and number average of molecular weight (M_n) estimated by GPC.

Oxidized Dextran	Theoretical oxidation (%)	Obtained degree of oxidation from ¹ H NMR (%)	M _n (kDa)	Gelling time (s) ^b
Odex-I	5	4.6	48.1	359 ± 5
Odex-II	15	7.4	38.5	103 ± 4
Odex-III	25	15.8	36.8	79 ± 4

^a Calculated by integrating the anomeric proton peak at δ 4.8 ppm and the tert-butyl proton peak at δ 1.3 ppm.

^b Gelling time for 2.5 % solutions of Odex and 2.5% CEC solutions in 0.01 M PBS at 37 °C.