Imitation Proteoglycans Improve Toughness of Double Network

Hydrogels

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Abstract:

Although tough double network hydrogel (DN gel) has been fabricated for years, the practical applications haven't achieved. One of the reasons is the limited polymer choices. The recent researches indicated that the addition of the Proteoglycans (PG), a heavily glycosylated protein, can make soft DN gel tough. However, the extract of natural PG and synthesis of its substitute are expensive and time consuming. In this work, chondroitin sulfate coated carbon nanotube (CS-T), a nanostructure biomimic PG, was synthesized to make soft hydrogel tough. 295 % and 360 % enhancements have been found on compressive strength and toughness after adding small amount of CS-T, respectively. As the PG plays a critical role in the cartilage to resist compression and maintain microstructure, this novel nanostructure paves an affordable way to produce substitutes for load-bearing tissue and multi-functional hydrogel.

Key words: biomaterials, mechanical testing, chemical synthesis, nanostructures.

1. Introduction

Hydrogels are water-swollen, three-dimensional crosslinked macromolecular networks. Due to their good biocompatibility, responsiveness to various stimuli, low surface friction, and environmental friendliness [1-5], the hydrogels, especially made from the biopolymers like agar, hyaluronan, alginate, and collagen, are considered innovative materials for bio-applications such as scaffolds and tissues [6-8]. However, for years, the low toughness nature of the hydrogel

restricts their potential applications as tissue substitute [9]. In order to improve their mechanical properties, several special hydrogel structures have been synthesized, like slide-ring, nanocomposite, tri-block copolymers, hydrophobic modified, tetra-PEG gels, and macromolecular microsphere composite hydrogels [10-12]. Among them, one of the most notable approaches is the double-network gel (DN gel) structure which presents both rubber-like robustness and high water content. The optimized DN gels show excellent hardness (modulus: 0.1-1.0 MPa), strength (tensile fracture stress: 1-10 MPa; strain 100-3000%), and toughness (tearing energy: 100-4500 J/m²) which are comparable to or even superb than the natural cartilage [13-17]. However, very limited polymers can be used to fabricate tough DN gel because of its unique structure.

Tough DN gel consists of two polymer networks with contrasting physical properties: a densely crosslinked strong polyelectrolyte network (rigid and brittle skeleton: N1) in low concentrations and a sparsely crosslinked neutral polymer network (soft and ductile substance: N2) in high concentrations [18, 19]. Because of the highly charged N1 extends the DN gel, the material of the N1 is required to be polyelectrolyte. Although more and more charged biopolymers were used to fabricate DN gel [20, 21], neutral biopolymers are still not applicable for DN gel. In this case, an approach to introduce more biopolymers into DN gel is demanded for variable applications.

During recent years, several approaches have been carried out. Suekama et al. have produced tough DN hydrogels by using chemically modified methacrylate chondroitin sulfate as the N1 [22]. However, its disadvantages, like complicated modifying process and unfavorable changes in the biochemical properties caused by the chemical crosslinking, restrict its practical applications. The so-called molecular stent method is a more preferred method due to its simple

processing and little influence on biopolymers [23, 24]. In this method, a natural bio-polyelectrolyte is dispersed within the N1 which is made by unmodified, chemical inert and neutral biopolymer. The bio-polyelectrolyte enhances osmotic pressure to extend and toughen the N1 network gel while the N2 network prevents hydrogel breaking. Although previous researches have shown some promising results [23, 24], in order to have DN gel with excellent mechanical properties, a special bio-polyelectrolyte, chondroitin sulfate proteoglycans (PG), is required which is expensive to purchase and difficult to extract. The addition of other synthesized polyelectrolytes and bio-polyelectrolytes, like chondroitin sulfate (CS) and sodium hyaluronate, exhibited very limited mechanical improvement and serious leaking due to their low charge density and small structures, respectively [24]. A simple method to produce biomimic PG is required for clinic applications.

In the PG, large numbers of the CS are anchored onto a protein backbone to form a large structure with high charge density. Herein, chondroitin sulfate coated carbon nanotube (CS-T), biomimic by the PG, is fabricated. Large numbers of the CS are anchored onto functionalized carbon nanotube (CNT). The limited modification on the CS (1 to 2 sites within ~150k Da structure) ensures its bio-properties while the functionalization on the CNT offers large amounts of reaction sites. Due to the large numbers of CS attaching and the charge repelling between CS chains, this structure is spread into micrometer size and is capable to entangle with multiple N1 chains, which can prevent leaking. As the CS is much cheaper than the PG and the synthesis processing of CS-T is simple, this structure is promising to fabricate tough hydrogel with different functions by using the most suitable polymers.

2. Materials and Methods

2.1 Materials

Chondroitin sulfate (CS, 220k Da), allylamine, nitric acid, allyl glycidyl ether (AGE), acrylamide (AA), N, N'-methylenebis (acrylamide) (MBAA), sodium borate buffer, sodium borate buffer, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), and ammonium persulfate (APS) were purchased from Sigma Aldrich.

2.2 Allyl Glycidyl Ether modification Chondroitin sulfate (AGE-CS)

In order to add reaction site to the CS, the AGE was used. AGE, an epoxide and vinyl containing monomer, can react with amines at an alkaline pH. Briefly, AGE was added into 10 mg/ml of CS in sodium borate buffer (SBB, 0.1M, pH 9.4) to form 1000:1 molar ratio of AGE to CS [25, 26]. After stirring this solution for 24 h, the AGE modified CS (AGE-CS) was purified by dialysis (12kDa MWCO) for 7 days.

2.3 Allyl Functionalized CNT (ACNT)

The functionalization of the CNT is similar to our previous research [27]. Briefly, CNT was reflux at 120 °C for 8 hr with 7M nitric acid to form Oxidized CNT (O-CNT). O-CNT was washed with hydrochloride acid and water for times. The allyl groups were added onto CNT through amide reaction with allylamine catalyzed by EDC.

2.4 Chondroitin sulfate coated Carbon Nanotube (CS-T)

The Chondroitin Sulfate was coated onto Carbon Nanotube through a free radical reaction. Briefly, after tip-sonicate 5 ml of 1 mg/ml ACNT for 30 min, 100 ml of the 10 mg/ml purified AGE-CS solution was added. 5 mg of the APS was then added into this solution after nitrogen gas bubbling. The mixture was stirred, heated at 65°C, and protected by the N₂ atmosphere for 10 h. The CS-T was purified by dialysis for 3 days and collected by

centrifugation. The PCS (poly (allyl glycidyl ether modified Chondroitin sulfate)) was synthesized similar with CS-T without adding ACNT. The synthesis is presented in Fig. 1.

Fig. 1. The scheme of fabrication Chondroitin Sulfate coated Carbon Nanotube (CS-T) through double bond reaction.

2.5 Double Network hydrogels (DN gel)

Table 1. Sample, Stent, and Compositions of DN gels

Sample	Stent	wt% ^(a)	x1-y1/x2-y2 ^(c)
Control	-	-	1-2/4-0.02
D(CS1)	CS	1	1-2/4-0.02
D(CS3)	CS	3	1-2/4-0.02
D(CS6)	CS	6	1-2/4-0.02
D(PCS1)	PCS	1	1-2/4-0.02
D(PCS3)	PCS	3	1-2/4-0.02
D(PCS6)	PCS	6	1-2/4-0.02
D(CS-T1)	CST	1	1-2/4-0.02
D(CS-T3)	CST	3	1-2/4-0.02
D(CS-T6)	CST	6	1-2/4-0.02
D(CS6+ACNT)	CS+ACNT	6+0.03 ^(b)	1-2/4-0.02

Note: ^(a) the wt% of the stent is the weight ratio of stent in the dried weight of the first network (N1). ^(b) 6 wt% of CS with 0.03 wt% of ACNT was added to form D(CS6+ACNT). ^(c) In x1-y1/x2-y2, x and y denote the AA monomer concentration (M) and MBAA crosslinker density (mol%) with respect to the monomer, respectively; the subscripts 1 and 2 denote the N1 and N2 network, respectively.

In this work, DN gels were synthesized via a two-step sequential free-radical polymerization with molecular stent [24]. Different DN gels with different molecular stent (D(CS) with CS, D(PCS) with PCS, and D(CS-T) with CS-T) were prepared. In the first step, 1 M AA, 4 mol% of the MBAA, and 0.1 mol% thermo initiator (APS) were dissolved in pure water with different wt% of different stents. The mixture was then polymerized at 60°C for 10 h to form the N1 network gel: N1. In the second step, N1 was soaked for 1 d in AA swelling solution (contained 4 M AA, 0.02 mol% MBAA, and 0.01 mol% APS). Then the swollen N1 was polymerized again at 60 °C for 10 h. The polymerized DN gels were submerged in pure water for 2 d to remove unreacted reagents. In order to investigate the influence of stent structure, 6 wt% CS and 0.03 wt% CNT were added into the DN gel. The compositions of different DN

gels are shown in Table 1. In order to invest the influence of additional stents, the control sample was fabricated using the same process without adding any stent.

The swelling degree, q , of the hydrogel was calculated by using the expression:

$$Q = \frac{w_{swell}}{w_0} \times 100\%, \text{ where } w_{swell} \text{ and } w_0 \text{ denote the weights of the swollen and the as prepared}$$

hydrogel, respectively. 5 sets of w_{swell} and w_0 were measured for each sample.

The mechanical properties of the DN gels were investigated by unconfined uniaxial compressive measurements with 10% strain per min speed. 5 cylinders with 10 mm diameter and 5 mm thickness were fabricated and tested for each sample using a commercial universal test machine (SHIMADZU, AGS-X). The engineering compressive stress was calculated by $\sigma_e = load/\pi R^2$, where R is the original radius of the cylindrical specimen. The engineering stress is defined as the change in the height relative to the original height: $\varepsilon_e = 100\% \times (h_0 - h)/h_0$. Stress and strain between $\varepsilon_e = 0$ and 10 were used to calculate the Young's modulus. The compressive toughness is defined as $U_T = \frac{\int_0^{\varepsilon_b} F ds}{\pi R^2}$, where F is the compressive load, s is the compressive displacement to the corresponding strain, and the ε_b is stress at break[28]. The molecular weights of CS and PCS were tested by the light scattering method with 5 different concentrations for linear fitting (Malvern, DLS ZS90). The morphologies of the ACNT and CS-T were imaged by TEM using 75 kV voltage (Transmission Electronic Microscopy, HITACHI T8100). The chemical structures were studied by FTIR using ATR mold (Fourier Transform Infrared Spectroscopy, Nicolet iS10). The UV-vis absorption spectrum is measured on Infinite M1000 Pro plate reader by solution method (Tecan).

2.6 Leaking

1 g N1 gel of each sample (S(CS6), S(PCS6), and S(CTS6), S denoted as single network hydrogel) were polymerized. Instead of merging them into the AA monomer solution, these hydrogels were swollen in 50 ml water for 7 days. The water in each beaker was analyzed by the UV absorption. The relative concentrations of CS in the water were measured by their peak intensity around 260 nm in their UV absorption spectra.

3.7 In Vitro Studies

A brain cancer cell line U87-MG (ATCC, HTB-14) was used to test hydrogel's biocompatibility. 3×10^4 cells/well of U87-MG cells were seeded in a 24-well polystyrene plate and cultured in 350 μ l of growth medium composed of Eagle's Minimum Essential Medium (EMEM) with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin and streptomycin for 24 hours. Then the medium was replaced by another 350 μ l of culture medium in which was immersed with hydrogels (2mm in diameter and 2mm in thickness) for 7 days. After 24, 48, or 72 h, hydrogel was removed and the number of cells is quantified using a crystal violet staining method[29].

3. Results

3.1 Allyl Glycidyl Ether functionalized Chondroitin Sulfate

The AGE-CS was first investigated by the FTIR spectra (Fig. 2 (a)). The peaks of CS included: S=O (1230 cm^{-1}), C-O-S ($812, 927, 730\text{ cm}^{-1}$), -OH ($3200\text{-}3600\text{ cm}^{-1}$), amide/carboxyl (1621 cm^{-1}), C-C (1130 cm^{-1}), C-H (1550 cm^{-1}), C-O (1052 cm^{-1}) [30, 31]. The shoulder peaks at 912 (epoxide), 1094 (ether stretching), 2857 ($\text{CH}_2=\text{CH}_2$ stretching), and 1647 (alkyl stretching) cm^{-1} in the FTIR spectrum of AGE-CS indicated the successful attachment of AGE onto CS [25, 32].

The molecular weights of AGE-CS and PCS were measured by DLS which also indicated the successful attachment (Fig. 2(b) and (c)). The reciprocal of the intersection at X axis and the linear fitting line indicated the MW of the tested polymer. As calculated, the MW of AGE-CS was 16 kDa while the MW of PCS was 5431 kDa. On average, ~350 AGE-CS were polymerized into a small bottle brush polymer through the double bond on the AGE. Due to the large negative charge and steric hinder effect, larger PCS can't be achieved by directly polymerizing AGE-CS.

The FTIR spectra and the MW measured by DLS exhibited the AGE has been attached onto CS and the double bond on the AGE is activatable for polymerization.

Fig. 2. The FTIR spectra of AGE, CS, and AGE-CS (a), and the molecular weights of AGE-CS (b) and PCS (c).

3.2 Allyl Functionalized Carbon Nanotube

The FTIR spectra of O-CNT and ACNT were compared in Fig. 3. In the O-CNT, the peak at 3444 cm⁻¹ indicated the -OH moieties and -OH in carboxyl groups. The peaks around 1740 cm⁻¹ and 1620 cm⁻¹ represented the C=O bands and ketone in carboxyl functional groups, respectively. Peaks ranging of 900-1300 cm⁻¹ represented the C-O bonds in various chemical surroundings. A band at 1620 cm⁻¹ was due to aromatic and unsaturated structural of C=C bonds and the bands at 1380 cm⁻¹ and 1460 cm⁻¹ were due to OH in-plane deformation and C-O moiety in C-O-C, respectively[33, 34]. In the ACNT, the 1380 cm⁻¹, which represent the C=C bonds are

increased. The nitrogen groups in the allylamine was confirmed by the peaks at 800 cm^{-1} and 1560 cm^{-1} , which represented the primary amine groups and the stretching of amine groups, respectively[27]. The morphology of the ACNT was presented by TEM image in Fig. 3 (b).

Fig. 3. (a) FTIR spectra of ACNT and O-CNT and the (b) TEM image of the ACNT.

3.3 Chondroitin Sulfate coated Carbon Nanotube

The attachment of the CS on CNT was confirmed by TEM images and FTIR spectrum. The CNT as the backbone is wrapped within the CS chains. Because the TEM samples were prepared by dropping and drying. The large extended CS-T structure was spread to form a large 2D thin sheet on the grid due to the large number of small CS chains attached onto the CNT. The FTIR spectrum also exhibited the existence of peaks for CS ($\text{S}=\text{O}$: 1230 cm^{-1} , $\text{C}-\text{O}-\text{S}$: $980, 720\text{ cm}^{-1}$) and CNT ($-\text{COOH}$: 1740 cm^{-1} and 1620 cm^{-1} , $\text{C}-\text{O}-\text{C}$: 1380 cm^{-1} and 1460 cm^{-1}).

Fig. 4. The TEM images (a) and the FTIR spectrum (b) of CS-T.

3.4 Mechanical Properties of DN gel

The mechanical properties of the DN gel with different molecular stents were measured by the compression test as seen in Fig. 5. Small amount of the CS in the DN gel presents the lowest elongation-at-break and strength performance. This might be the result of CS leaking during the swelling in the AA solution which causes inhomogeneous AA monomer concentration in the hydrogel. The inhomogeneity of the N2 is causing from the leakage of the CS. This comes from the different swelling ratio at places with/without CS swelling when the N1 in the monomer solution of N2. The Control exhibited the smallest swelling ratio because of the very little charging of the N1 PAA. Due to the higher charging of the CS compared with the N1, the higher N2 monomer is absorbed at the place with the CS. Due to the inhomogeneous leaking of the CS, the final N2 network is not inhomogeneous. As the second network of PAA integrates the hydrogel, the inhomogeneous N2 PAA makes the DN gel vulnerable.

The Yong's modulus of hydrogels is mainly come from the crosslink density. Due to the low crosslink density of the N2, samples has small swelling perform higher modulus, like the Control. On the other hand, the osmotic pressure comes from the CS can also provide mechanical support[35]. The higher concentration of the CS, the better mechanical properties hydrogel can achieve. The Yong's modulus of DN gel is from the balance of these two factors.

D(PCS) and D(CS-T) show higher elongation-at-break as well as higher compressive strength than the D(CS). The uniformly dispersed PCS and CS-T became the molecular stent in the hydrogel, which entangled the N1 chains and improve the osmotic pressure. During the swelling in the monomer solution, the N1 was extended and became tough with the help of stents. Large amounts of the AA monomers flowed into the swollen N1 gel and form ductile N2 network during polymerization. This phenomenon results in a homogeneous structure which has tough first network and ductile second network. Although both the D(PCS) and D(CS-T) show high elongation-at-break and strength, the D(CS-T) presented better results as its higher swelling degree ensured more AA monomer flowing to form homogeneous in the gel. The more homogeneous integration of the D(CS-T) promises the better mechanical performance during test.

Fig. 5. The compression test of (a) D(CS), (b) D(PCS), and (c) D(CS-T). (d) The comparison of gels with different stents. The comparison of swelling degree (e) and strength (f) with different DN gels.

Table 2. The swelling ratio, elongation-at-break, and strength of different DN gels.

Sample	Q (%)	Elongation-at-break (%)	Young's Modulus (kPa)	Strength (MPa)	Toughness (kJ/m ³)
CS-T6	452 ± 64	88 ± 2.8	131.6 ± 7.0	7.36 ± 0.60	1007 ± 189
CS-T3	282 ± 75	89 ± 0.7	98.5 ± 16.3	6.10 ± 0.96	887 ± 156
CS-T1	235 ± 41	89 ± 0.5	120.6 ± 4.7	4.38 ± 0.52	488 ± 19
PCS6	340 ± 51	86 ± 1.1	97.3 ± 19.9	5.45 ± 0.40	740 ± 68
PCS3	291 ± 32	86 ± 1.4	100.4 ± 6.3	4.79 ± 0.55	518 ± 54
PCS1	253 ± 44	79 ± 0.5	85.3 ± 13.9	2.16 ± 0.75	368 ± 41
CS6	306 ± 57	91 ± 1.2	109.1 ± 6.4	1.97 ± 0.64	361 ± 15
CS3	274 ± 52	72 ± 2.2	119.1 ± 15.2	0.46 ± 0.09	94 ± 13
CS1	250 ± 101	65 ± 4.3	122 ± 31.7	0.26 ± 0.08	74 ± 28
CS6+ACNT	388 ± 88	83 ± 10.1	74.1 ± 28.4	1.75 ± 0.69	146 ± 21
Control	211 ± 9	84 ± 0.6	150.6 ± 7.2	1.86 ± 1.03	219 ± 49

3.5 Leaking

The leaking of the CS6, PCS6 and CS-T6 in N1 gel was analyzed by UV absorption spectra. The peak intensity of the CS around 260 nm was used to measure the relative concentration of the CS in 50 ml swelling solution. As can be seen in the Fig. 6, ~15 times more of the CS were leaked from the S(CS6) and S(PCS6) compared with the S(CS-T6) after 7 days of

swelling. These results indicated that the large structure of the CS-T can efficiently entangle with the N1 and prevent the leaking.

Fig. 6. (a) The UV-vis spectrum of CS in water, and (b) The amount of CS leaked from S(CS6), S(PCS6), and S(CS-T6) after 7 days of swelling.

3.6 Biocompatibility

Cell culture experiments with U87-MG cells were carried out to evaluate the biocompatibility of the hydrogels. Proliferations at 24, 48, and 72 h after culturing cells with the Control and D(CS-T6) are shown in Fig. 7. The D(CS-T6) supported proliferations of U87-MG cells, while fewer cells were detected after culturing with Control. Also, the proliferative rate of D(CS-T6) is 10 times higher than the Control (17120 cell/hr of VBDN to 1760 cell/hr of BDN). These results indicated that the D(CS-T6) has a better biocompatibility than the Control due to the existence of CS-T.

Fig. 7. The biocompatibility tests of Control and D(CS-T6).

4. Conclusions

In this work, a biomimetic structure (CS-T) was synthesized to perform as a molecular stent in double network hydrogel. Similar with the proteoglycans, the CS-T has a backbone carbon nanotube anchors large amount of chondroitin sulfate chains. With the aid of CS-T, double network hydrogel made by neutral polymers performs high mechanical properties. This achievement not only presented an affordable way to synthesis proteoglycans, but also ensures tough hydrogel with different functions by using the most suitable polymer.

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