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Biocompatible, ionic-strength-sensitive, double-network hydrogel based on chitosan and an oligo(trimethylene carbonate)–poly(ethylene glycol)–oligo(trimethylene carbonate) triblock copolymer

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ABSTRACT: A double-network (DN) hydrogel was prepared through the sequential photopolymerization of oligo(trimethylene carbonate) (TPT)-*block*-poly(ethylene glycol)-*block*-oligo(trimethylene carbonate) diarylate and methacrylated chitosan (CS–MA). The swelling behavior and mechanical properties of the hydrogels were tunable via the control of the concentration of CS–MA. Under physiological conditions, the fracture stress of the DN hydrogel reached 3.4 MPa; this was more than twice that of the corresponding TPT-*block*-poly(ethylene glycol)-*block*-TPT single network (1.6 MPa). At high ionic strength, the fracture stress of the DN hydrogel reached 6.4 MPa. The DN hydrogel exhibited good cytocompatibility, as revealed by a live–dead assay. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 42459.

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INTRODUCTION

Because of its hydrophilic nature and similar elasticity to natural soft tissue, hydrogels have been studied widely as biomaterials, such as contact lenses,[1] biosensors,[2] drug-release matrices,[3] and tissue engineering scaffolds.[4] However, the inferior mechanical properties of chemically crosslinked hydrogels hinders their applications in specific applications, such as scaffolding materials in cartilage tissue engineering, that require adequate mechanical properties; in such cases, a hydrogel with good mechanical properties would appeal to biomaterials scientists.

A double-network (DN) hydrogel, which exhibits fracture strength up to several 10s of megapascals at high water contents,[5] usually consists of two networks: a densely crosslinked rigid and brittle polyelectrolyte first network [i.e., poly(2-acrylamido-2-methylpropanesufonic acid)] and a loosely crosslinked soft and ductile neutral polymer second network (i.e., polyacrylamide). In such a design, the first network is fragmentized under compression into small clusters to dissipate the stress energy; these clusters, which act as the crosslinker for the second network, along with the chains of the second network, can retard crack propagation. Frank *et al.*[6] fabricated an inverse-DN hydrogel using a neutral low-molecular-weight poly(ethylene glycol) (PEG) as the first network and an anionic poly(acrylic acid) (PAA) as the second network. Both of the polymers were relatively fragile, and the enhanced mechanical strength of the PEG/PAA DN hydrogel was attributed to the hydrogen bonds between the PEG and PAA chains.

In this study, we constructed a DN hydrogel based on oligo(trimethylene carbonate) (TPT)-*block*-poly(ethylene glycol)-*block*-TPT and chitosan (CS). TPT is a ductile neutral polymer, and TPT hydrogels have demonstrated excellent mechanical properties and *in vitro* biodegradability in comparison to PEG hydrogels.[7] CS is a bioactive natural polysaccharide and has been extensively studied as a tissue engineering scaffold, antibacterial material, drug-/gene-delivery vehicle, and wound-dressing material.[8–10] TPT-*block*-poly(ethylene glycol)-*block*-oligo(trimethylene carbonate) diarylate (TPT–DA) and methacrylated chitosan (CS–MA) were synthesized and used as the precursor in the fabrication of the DN hydrogel through a two-step photopolymerization process (Figure 1). The effects of the concentration of CS–MA and the ionic strength on the swelling behavior and mechanical properties of this hydrogel were examined. The cytocompatibility of the obtained hydrogel was also evaluated *in vitro*.

EXPERIMENTAL

Materials

PEG with a molecular weight of 20,000 g/mol (Shanghai Chemical Reagent Co., Ltd., China) was precipitated in diethyl ether

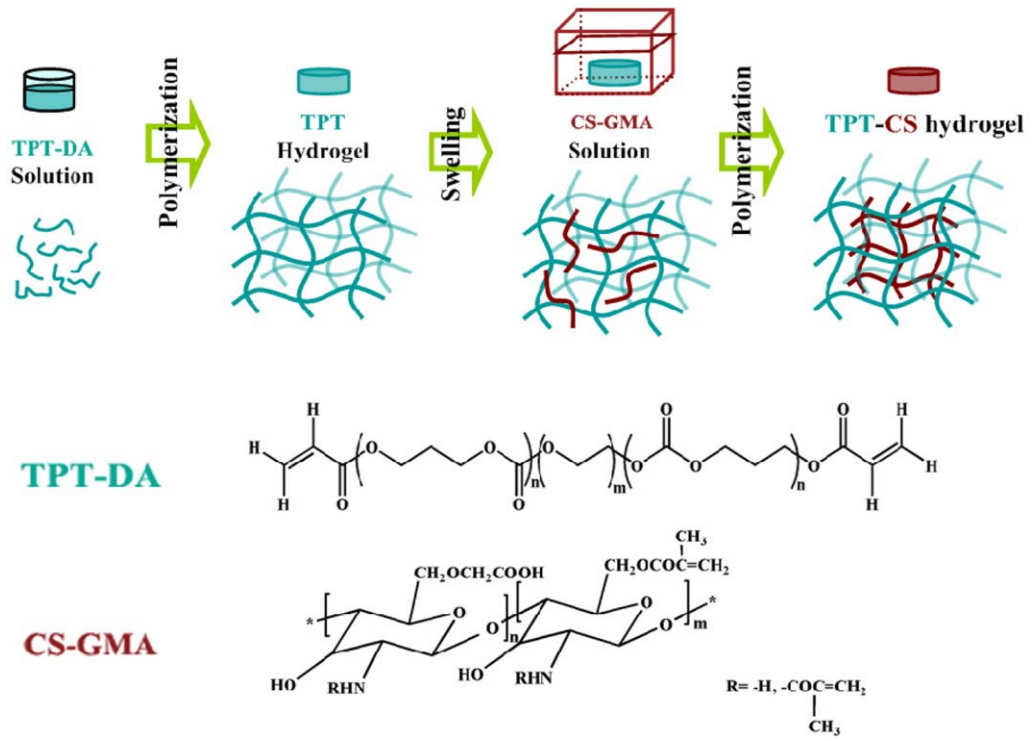

Figure 1. Schematic illustration of the preparation of the DN hydrogel. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and dried *in vacuo*. Trimethylene carbonate (TMC) was synthesized and purified according to the literature.[11] Acryloyl chloride was purified by distilling. Toluene was refluxed over CaH_2 and distilled. Triethylamine was purified according to an established protocol before use. CS with a medium molecular weight was purchased from Sigma. Monochloroacetic acid was recrystallized by chloroform and dried *in vacuo*. All of the other reagents were analytical grade and were used as received.

Swine cartilage chondrocytes (SCCs) were isolated as reported previously.[12] Chondrocyte culture medium (CCM) containing Dulbecco's modified Eagle medium (high glucose, HyClone) and supplemented with fetal bovine serum (10%), penicillin–streptomycin (1%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (10 m*M*), nonessential amino acids (0.1 m*M*), proline (0.4 m*M*), and ascorbic acid (50 mg/L) was used in the cell culture.

Synthesis of the TPT–DA Precursor

TPT was synthesized according to the literature.[7] Typically, TMC, PEG (molecular weight = 20,000), and 0.1% (mol/mol) $Sn(Oct)_2$ was added to a silanized glass ampule and sealed *in vacuo*. The ampule was immersed in an oil bath at 145°C for 48 h. After the ampule cooled down to room temperature, the product was dissolved in CH_2Cl_2, precipitated in excessive diethyl ether, collected, and dried *in vacuo*.

The obtained polymerization product (5.00 g) was dissolved in 200 mL of toluene and dehydrated by azeotropic distillation at 120°C for 4 h. After the reaction mixture cooled down to room temperature, triethylamine (0.270 mL) was added, and the reaction mixture was cooled to 0°C in an ice bath. Acryloyl chloride (0.156 mL) in anhydrous toluene (10 mL) was added dropwise and reacted for 12 h at 45°C. The obtained solution was concentrated and precipitated in diethyl ether three times. Then, the precipitate was collected, dialyzed against distilled water, and freeze-dried. The chemical structure of the product was confirmed via ^{1}H-NMR spectroscopy.

Synthesis of the CS–MA Precursor

Carboxymethyl chitosan (CM–CS) was synthesized as follows: CS (4.00 g), sodium hydroxide (21.60 g), and distilled water (80 mL) was added to a 500-mL flask and stirred at 50°C for 3 h. Then, isopropyl alcohol (240 mL) was added to the mixture, and the mixture was refluxed at 50°C for 30 min. Monochloroacetic acid (15 g) in isopropyl alcohol (20 mL) was then added dropwise to the mixture. After it was refluxed for 7 h, the reaction mixture was filtered, and the filter cake was dissolved in water. The solution was centrifuged to remove insoluble reactants, and then, the clear filtrate was precipitated in 80% ethanol. The precipitate was collected and dried *in vacuo* at room temperature.

The obtained product was dissolved in water (400 mL). Triethylamine (20 mL), *N*,*N*-dimethylformamide (200 mL), and glycidyl methacrylate (GMA; 60 mL) were added to the CM–CS solution and stirred for 72 h. Then, the solution was precipitated in excessive ethanol, collected, and dialyzed against distilled water for 72 h.

Preparation of the DN Hydrogel

The TPT–DA precursor was dispersed in phosphate-buffered saline (PBS; pH 7.4) at concentrations of 10% w/v, and 0.04% w/v Irgacure 2959 was added as a photoinitiator. Then, 100 μL of the solution was transferred to a cylindrical mold and exposed

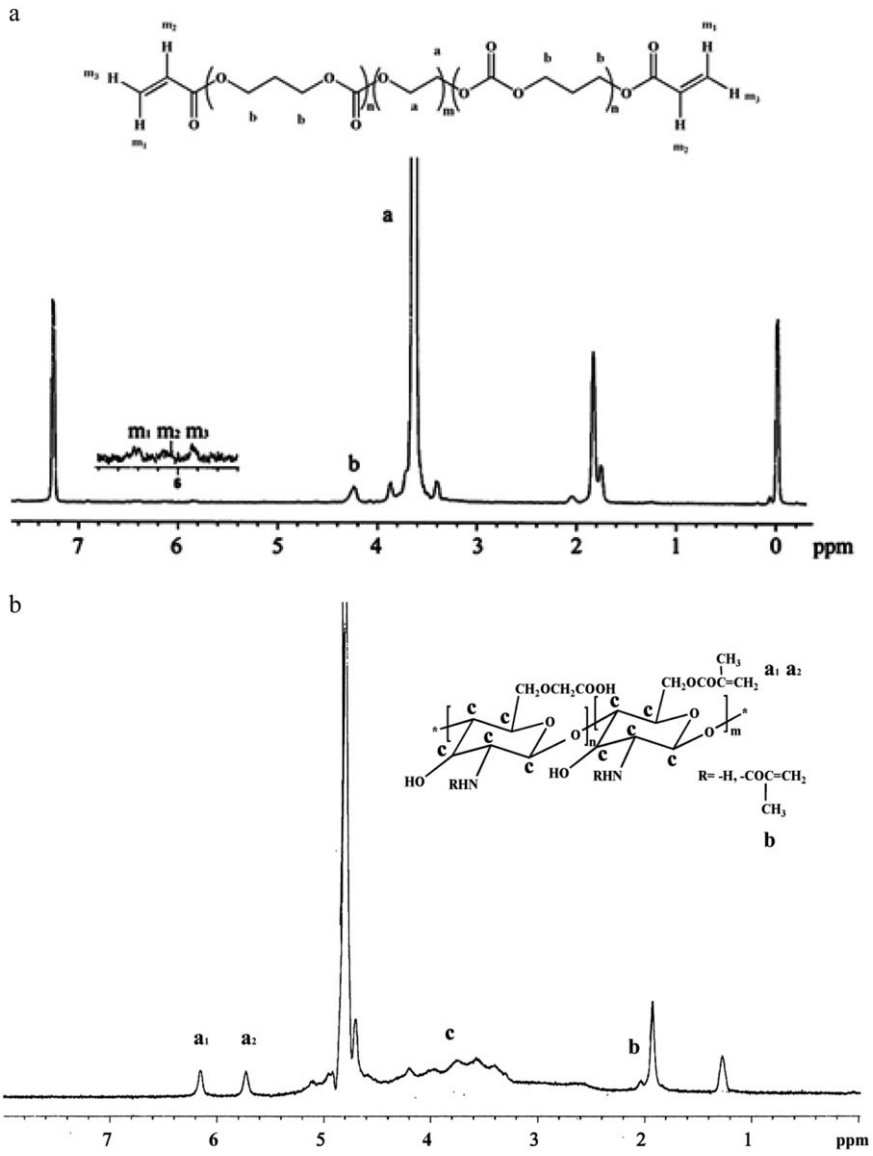

Figure 2. [1]H-NMR spectra of TPT and CS–GMA.

to 365-nm UV light (30 mW/cm^2) for 5 min to form a TPT single-network hydrogel. Then, the TPT hydrogel was immersed in 2 mL of CS–MA solution in PBS containing 0.04% Irgacure 2959 and various concentrations of CS–MA (0.5, 1, and 2% w/v) for 24 h. The swollen hydrogel was then exposed to UV light for an additional 5 min to form the second network. The DN hydrogel was named TPT–CS$_x$, where x stands for the weight/volume percentage of the CS precursor in the immersion solution.

[1]H-NMR

The [1]H-NMR spectrum was recorded on a Mercury VX-300 spectrometer (Varian) with $CDCl_3$ and D_2O as solvents for TPT–DA and CS–GMA, respectively. Tetramethylsilane was applied as the internal reference.

Surface Morphology

The fully swollen hydrogel was frozen in liquid nitrogen and then lyophilized for 48 h. The lyophilized hydrogel was sputter-coated with gold, and the surface morphology of the hydrogels was observed on a scanning electron microscope (FEI-QUANTA200, Holland)

Swelling Behavior

The as-prepared DN hydrogel was immersed in corresponding PBS solutions with different NaCl concentrations (0, 0.137, 0.5, and 1M) for 48 h and swollen to equilibrium. The sample was weighed (W_s) after the PBS solution on the surface was wiped off and dried *in vacuo*. The dry mass (W_d) of the hydrogel was then measured. The equilibrium swelling ratio (ESR) and water content were calculated with the following equations:

$$ESP = \frac{W_s}{W_d}$$

$$\text{Water content} = \frac{W_s - W_d}{W_s} \times 100\%$$

Three duplicates for each hydrogel were examined, and the results are presented as the mean plus or minus the standard deviation.

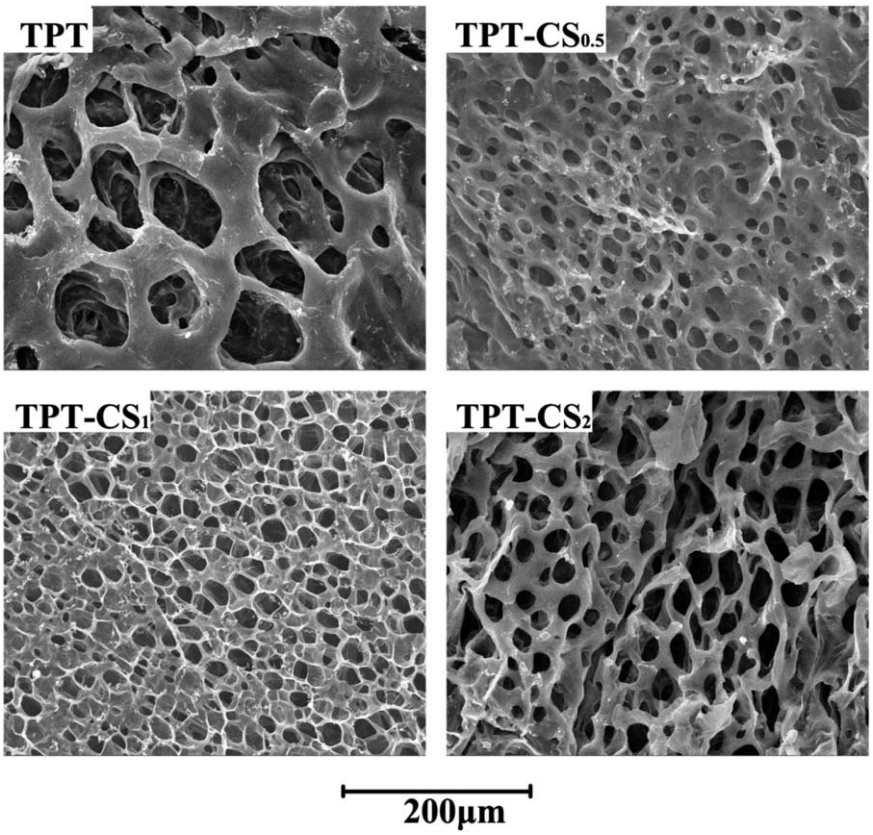

Figure 3. Scanning electron microscopy images of the TPT, TPT–$CS_{0.5}$, TPT–CS_1, and TPT–CS_2 hydrogels.

Compression Test

The hydrogel was immersed in PBS with a predetermined ionic strength for 48 h before the mechanical test. The test was performed on an Instron 3342 universal testing system (Instron, Norwood, MA) equipped with a model 2519-104 force transducer at a compression rate of 1 mm/min. The compressive modulus was calculated from the slope of the initial linear region of the stress–strain curve.[11] The toughness was calculated from the area integration of the stress–strain curve to the fracture points. Five duplicates were measured for each sample, and the results are presented as the mean plus or minus the standard deviation.

Three-Dimensional Photoencapsulation of SCCs

SCCs at the third passage were used to evaluate the cell viability in the hydrogels during the two-step photopolymerization process according to the literature.[12] SCCs were dispersed at a density of 1×10^6 cells/mL in a mixture solution of TPT–DA

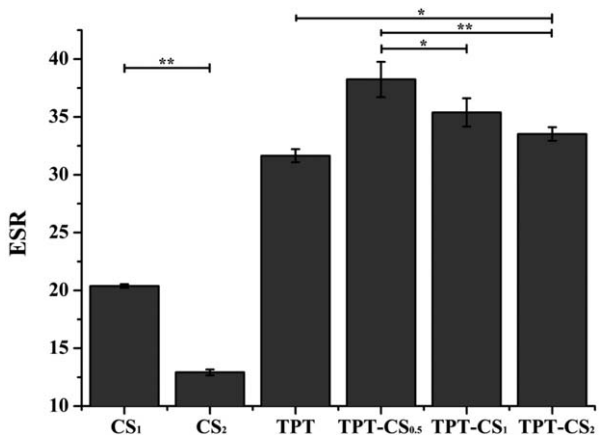

Figure 4. Effects of the CS concentration on the ESR of the TPT–CS DN hydrogels. $^*p \leq 0.05$. $^{**}p \leq 0.01$.

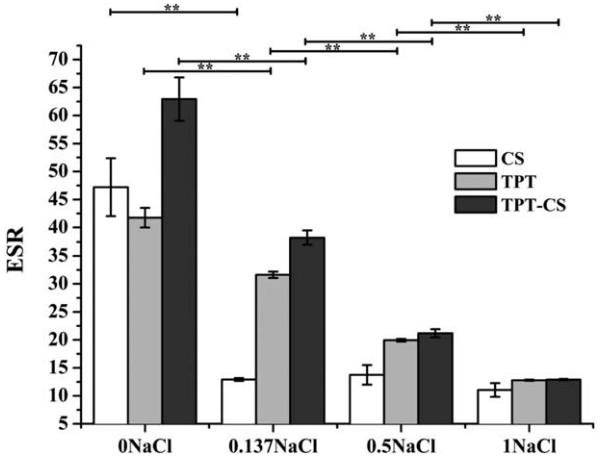

Figure 5. ESRs of CS, TPT, and TPT–CS in PBS solutions with different ionic strengths (NaCl concentration controls). $^{**}p \leq 0.01$.

Table I. Mechanical Properties of the TPT, CS, and TPT–CS Hydrogels

Sample name	Fracture stress (MPa)	Fracture strain (%)	Toughness (kJ/m^3)	Modulus (kPa)
TPT	1.680 ± 0.820	93.87 ± 4.29	39.41 ± 15.24	1.47 ± 0.40
$CS_{0.5}$	—	—	—	—
CS_1	0.016 ± 0.003	35.77 ± 9.08	1.44 ± 0.31	6.15 ± 6.61
CS_2	0.025 ± 0.016	24.28 ± 8.23	2.64 ± 1.97	69.52 ± 54.02
TPT-$CS_{0.5}$	3.360 ± 1.362	96.51 ± 2.93	77.83 ± 33.37	0.536 ± 0.23
TPT-CS_1	1.580 ± 0.473	94.43 ± 2.64	41.89 ± 9.95	1.51 ± 0.55
TPT-CS_2	0.026 ± 0.0138	80.94 ± 3.90	3.05 ± 1.04	1.43 ± 0.33

precursor (10%, g/mL) and Irgacure 2959 (0.04%, g/mL) in sterile PBS. Then, the cell suspension was transferred into a cylindrical mold and exposed to 365-nm UV light for 5 min to form a cell-laden single-network hydrogel. The cell-laden single-network hydrogel was immediately transferred to a 24-well plate with 2 mL of CCM containing CS–MA (0.5%, g/mL) and Irgacure 2959 (0.04%, g/mL) and incubated at 37°C in a 5% CO_2 atmosphere for 24 h. The swollen cell-laden hydrogels were exposed to UV light for 5 min to form the second network. The cell-laden DN hydrogel was then incubated in CCM at 37°C in a 5% CO_2 atmosphere.

Live–Dead Assay

A live–dead assay was performed to investigate the SCC viability in the DN hydrogel after the two-step photopolymerization process. Slices of the cell-laden DN hydrogel were incubated in a live–dead assay fluorescent dye solution containing calcein–AM (0.25 μL) and ethidium homodimer-1 (1 μL) in Dulbecco's modified Eagle medium (0.5 mL) for 10 min. Then, the slices were rinsed with PBS and observed under a fluoroscope microscope (Olympus-LX71 inverted microscope with a U-RFL-T fluorescence lamp). The viability of the SCCs was presented by live cells stained with green dye and dead cells stained with red dye. Widely recognized PEG hydrogels were investigated as the control.

RESULTS AND DISCUSSION

Synthesis of the Precursor

The chemical structure of the TPT–DA precursor was confirmed by ^{1}H-NMR [Figure 2(a)]. The peak at 3.6 ppm (—CH_2—CH_2—O—, 4H) belonged to the methylene protons in PEG segments. The peaks at 4.2 and 2.0 ppm (—CO—O—CH_2—, 2H, and —COO—CH_2—CH$_2$—CH_2—COO—, 2H) were assigned to the methylene protons in the oligo-TMC segment. The degree of polymerization of TMC was calculated to be 4.1. Multiple peaks appearing at 5.8–6.4 ppm were attributed to the protons on the acryloyl group. The degree of acrylation of the TPT precursor was calculated to be 59%.

CS is insoluble under physiological conditions; grafting carboxymethyl groups onto CS can improve its solubility and help it retain its biological activity.[13,14] The chemical structure of the CS–GMA precursor was confirmed by ^{1}H-NMR [Figure 2(b)]. The peaks at 5.8 and 6.2 ppm were the characteristic peaks of vinyl protons, and the peak at 2.0 ppm was assigned to the methyl group in the *N*-acetyl group. The peaks at 2.5–4.2 ppm were attributed to the protons on the polysaccharide backbone.

Swelling Behavior

Figure 3 shows the morphologies of the hydrogels. Both the single and DN hydrogels exhibited three-dimensional penetrative porous structures. The DN hydrogels possessed smaller porous sizes compared to that of the single TPT hydrogel. As an important parameter of the hydrogel, ESR was dependent on the crosslinker type,[15] crosslinking density,[16] and swelling medium.[17] In this study, the effects of the concentration of CS–MA and the ionic strength on the ESR of the DN hydrogels were investigated in detail.

At a fixed concentration of the first TPT network, the effect of the concentration of CS precursor on the ESR of the final DN network was examined. Those DN hydrogels were defined as TPT–CS_x. ESR of the CS single-network hydrogels decreased with increasing concentration of CS–MA (Figure 4). This was attributed to the increased crosslinking density at higher concentrations of CS precursor. However, the introduction of the CS second network into the TPT network initially (TPT–$CS_{0.5}$) led to a significantly increased ESR; this was attributed to the electrorepulsion between carboxymethyl groups. A further increase in the concentration of CS–MA led to an increase in the crosslinking density of the second network, which counteracted with the electrorepulsion between carboxymethyl groups. Thus, ESR of the TPT–CS DN hydrogels declined. Compare with the TPT and CS single-network hydrogels, the TPT–CS DN hydrogel showed a higher ESR; this was also attributed to the increased number of hydrophilic moieties. The water contents of the TPT–CS DN hydrogels were all above 97% and were higher than that of natural cartilage tissue.[5]

Because CS bore carboxymethyl groups, the second network, as a polyelectrolyte, could be affected by the environmental ionic strength in terms of the ESR and mechanical properties (Figure 5). ESR of the hydrogels was examined in PBS with concentrations of NaCl of 0, 0.137, 0.5, and 1 M. ESR of the TPT single-network hydrogels showed a monotonous decrease as the ionic strength increased. The TPT chain contained a significant number of ether bonds. The hydrogen bond interaction between oxygen in ether bond and water resulted in a good hydrophilicity of TPT.[18] The strong ionic strength could shield the hydrogen bond interaction and thus reduce the hydrophilicity of

Table II. Effect of the Ionic Strength on the Mechanical Properties of the DN Hydrogel

Sample name	Fracture stress (MPa)	Fracture strain (%)	Toughness (kJ/m^3)	Modulus (kPa)
0NaCl	1.560 ± 0.881	87.88 ± 5.36	37.16 ± 18.67	1.45 ± 0.49
0.137NaCl	3.360 ± 1.362	96.51 ± 2.93	77.83 ± 33.37	0.54 ± 0.23
0.5NaCl	3.640 ± 0.570	94.39 ± 3.41	94.14 ± 5.74	2.40 ± 0.93
1NaCl	6.375 ± 1.594	100.93 ± 4.43	206.10 ± 78.75	2.98 ± 0.78

[a] The number before "NaCl" indicates different NaCl concentrations, which is 0, 0.137, 0.5, and 1M for 0NaCl, 0.137NaCl, 0.5NaCl, and 1NaCl, respectively.

TPT[19] and ESR of the hydrogel. As for the CS single-network hydrogel, it possessed a high ESR when the concentration of NaCl was 0 M. Because of the shielding of the electrorepulsion between the COO^- groups by Na^+ ions, the ESR of the CS hydrogel decreased in PBS with 0.137 to 1.0*M* of NaCl. As a result, the TPT–CS DN hydrogel decreased with increasing ionic strength.

Mechanical Properties

As reported in the literature, the molar ratio of the brittle first network to the ductile second network had a great influence on the mechanical properties of the DN hydrogel. As the amount of the ductile second network increased, the mechanical properties of the DN hydrogel increased.[5] The compressive properties of the TPT, CS, and TPT–CS hydrogel with different concentrations of CS–GMA precursor are presented in Table I. Compared with corresponding TPT and CS single-network hydrogels, the TPT–CS DN hydrogel with 0.5% CS–GMA showed remarkable fracture stress and toughness. The fracture stress of TPT–$CS_{0.5}$ (3.36 ± 1.36 MPa) was nearly twice that of the TPT hydrogel (1.68 ± 0.82 MPa). In the TPT–CS DN hydrogel, the TPT network was the neutral ductile network, and the CS network was the brittle one. When the CS concentration increased to 1 and 2%, the fracture stress, fracture strain, and toughness of TPT–CS decreased; this was a similar trend to that of the reported DN hydrogels.

Changes in the environmental ionic strength have been shown to be an effective for modulating the physical properties of hydrogels.[20] In this study, the effect of the ionic strength on the

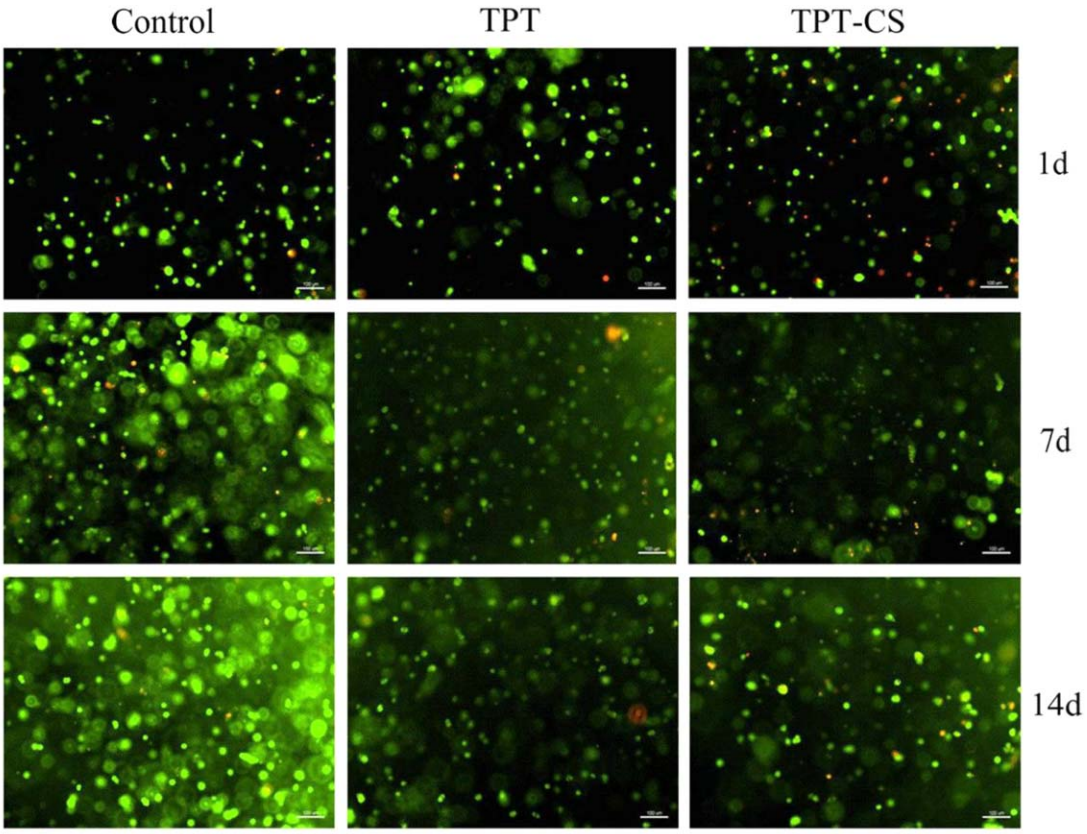

Figure 6. Live–dead assay of SCCs photoencapsulated in hydrogels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mechanical properties of the hydrogel was examined (Table II). At the same CS concentration, TPT–$CS_{0.5}$ showed a notable difference in its compression properties under increased ionic strength conditions. With increasing ionic strength, the fracture stress, fracture strain, and toughness of TPT–$CS_{0.5}$ increased gradually. For example, the highest fracture stress reached 6.38 ± 1.60 MPa in 1*M* NaCl. Under a strong ionic strength, the charges on the CS chain were shielded by Na^+ ions. The CS polyelectrolyte chain underwent a rod-to-coil conformational transition and became more flexible.[21,22] In this case, the hydrogel network became more flexible and could effectively dissipate energy.

Cell Viability in Three-Dimensional Encapsulation

Cell viability during the encapsulation process is of critical importance to the final outcome of both *in vitro* culture and *in vivo* implantation of the cell-laden constructs. In the fabrication of the cell-laden DN hydrogel, SCCs experienced contact with different precursor solutions, photoinitators, and two-step UV irradiation. The cell viability after the fabrication process was one of the major concerns. The TPT–$CS_{0.5}$ DN hydrogel was used to evaluate the viability of SCCs, that is, the cytocompatibility of the fabrication process. No surprisingly, SCCs in TPT–$CS_{0.5}$ DN hydrogels showed a relatively high number of dead cells immediately after 24 h of encapsulation, probably because of the two-step UV exposure.(Figure 6). However, after 7 and 14 days of culturing, the viability of SCCs in the TPT–CS hydrogels revealed no significant difference compared with the control groups; this implied cell proliferation in the long-term cultures. These results suggest that these hydrogels had comparable cytotoxicity to widely recognized PEG hydrogels in long-term cultures and could support SCC survival and proliferation.

CONCLUSIONS

Biocompatible DN hydrogels were prepared with a biodegradable PEG-based triblock copolymer as the first network and CS as the second network. The concentration of the CS precursor and the ionic strength were shown to be two essential factors in the control of the physical properties of the DN hydrogels. The mechanical properties of the hydrogels increased with the ionic strength. This strong and ionic-strength-sensitive hydrogel proved to be cytocompatible in three-dimensional photoencapsulation. Therefore, this DN hydrogel shows great potential as a scaffolding material for cartilage tissue engineering.

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