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Novel injectable biodegradable glycol chitosan-based hydrogels crosslinked by Michael-type addition reaction with oligo(acryloyl carbonate)-*b*-poly(ethylene glycol)-*b*-oligo(acryloyl carbonate) copolymers

Yuexin Yu,[1,2,3] Chao Deng,[1,2,3] Fenghua Meng,[1,2,3] Qin Shi,[4] Jan Feijen,[1,2,3] Zhiyuan Zhong[1,2,3]

[1]Biomedical Polymers Laboratory, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou 215123, People's Republic of China
[2]Jiangsu Key Laboratory of Advanced Functional Polymer Design and Application, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou 215123, People's Republic of China
[3]Department of Polymer Science and Engineering, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou 215123, People's Republic of China
[4]Department of Orthopaedic Surgery, The First Affiliated Hospital of Soochow University, Suzhou 215006, People's Republic of China

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Abstract: Novel injectable biodegradable glycol chitosan hydrogels were developed based on thiolated glycol chitosan (GC-SH) and water soluble oligo(acryloyl carbonate)-*b*-poly(ethylene glycol)-*b*-oligo(acryloyl carbonate) (OAC-PEG-OAC) triblock copolymers via Michael-type addition reaction. The rheology measurements showed that robust hydrogels were formed rapidly upon mixing aqueous solutions of GC-SH and OAC-PEG-OAC at remarkably low total polymer concentrations of 1.5–4.5 wt % under physiological conditions. The gelation times (varying from 10 s to 17 min) and storage moduli (100 to 4300 Pa) of hydrogels could be controlled by degrees of substitution (DS) of GC-SH, solution pH, and polymer concentration. These glycol chitosan hydrogels had microporous structures, low swelling and slow hydrolytic degradation (stable for over 6 months) under physiological conditions. Notably, these hydrogels were prone to enzymatic degradation with lysozyme. The multiple acryloyl functional groups of OAC-PEG-OAC allowed facile conjugation with thiol-containing biomolecules prior to gelation endowing hydrogels with specific bioactivity. The preliminary cell culture studies revealed that these glycol chitosan hydrogels were cell non-adhesive while Gly-Arg-Gly-Asp-Cys (GRGDC) peptide modified hydrogels could well support adhesion and growth of both MG63 osteoblast and L929 fibroblast cells. These rapidly *in situ* forming enzymatically biodegradable hybrid hydrogels have great potentials in the development of injectable cell-specific bioactive extracellular matrices for tissue engineering. J Biomed Mater Res Part A: 99A: 316–326, 2011.

Key Words: biodegradable hydrogels, injectable, chitosan, Michael addition, tissue engineering

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INTRODUCTION

The past decade has witnessed significant progress in the development of biocompatible hydrogels for various biomedical applications such as cell-based drug delivery, sustained protein delivery and tissue engineering.[1–3] In particular, rapidly *in situ* forming biodegradable chemical hydrogels have received growing attention because they offer several remarkable advantages, e.g., they can be administered in a minimally invasive manner; different bioactive entities including drugs, proteins, and live cells may be readily and homogeneously encapsulated into the hydrogels; they may provide excellent match to complex settings at the implantation site; and unlike physical hydrogels, they are usually stable and mechanically robust.[4,5] In the past years, a few different approaches such as photopolymerization,[6,7] click chemistry,[8,9] enzymatic crosslinking,[10,11] and Michael-type addition reaction[12–15] have been explored for the *in situ* formation of biodegradable chemical hydrogels. Michael-type

Correspondence to: Z. Zhong; e-mail: zyzhong@suda.edu.cn
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addition hydrogels pioneered by Lutolf and Hubbell are especially appealing in that they are formed quickly under physiological conditions without aid of a catalyst and without generation of any byproducts, fully circumventing potentially toxic contaminations. Lutolf, Hubbell, and coworkers reported different types of *in situ* forming degradable poly (ethylene glycol) (PEG) hydrogels based on Michael-type addition of acrylate or vinyl sulfone-functionalized linear or star PEG with cysteine-containing peptides or proteins wherein cell-responsive hydrogels were developed through incorporation of cell-adhesive domains and/or proteolytically degradable peptides.[16–19] Leach and coworker reported preparation of hydrolytically degradable PEG hydrogels from vinyl sulfone functionalized four-arm PEG with PEG-diester-dithiol.[20] Pritchard et al. applied the injectable Michael-type addition PEG hydrogels for sustained release of methylprednisolone sodium succinate.[21]

Hydrogels based on natural polysaccharides such as hyaluronan, dextran, and chitosan have been considered as one of the most promising matrices for drug delivery and tissue engineering due to their excellent biocompatibility.[22] Prestwich and coworkers reported rapid *in situ* formation of hydrogels from thiolated hyaluronan and PEG diacrylate through Michael-type addition reaction.[23,24] Vermonden and coworkers reported preparation of injectable hyaluronan hydrogels by tandem thermal gelling and Michael-type addition chemistry.[25] We prepared *in situ* forming degradable dextran hydrogels through Michael-type addition reaction between thiolated dextran and PEG tetra-acrylate or dextran vinyl sulfone conjugates,[13,26] and applied for sustained protein release and cartilage tissue engineering.[27,28] It is surprising to note that there is no report on preparation of injectable chitosan-based hydrogels via Michael-type addition reaction. Chitosan, structurally resembling with glycosaminoglycans (GAG) have besides excellent biocompatibility and biodegradability several unique biological properties such as antibacterial activity, mucoadhesivity, wound healing activity, and high affinity to anionic biologically active molecules (such as GAG and proteoglycans).[29–31] Chitosan-based hydrogels have been widely investigated for wound healing, drug delivery, and tissue engineering applications.[32,33] The insolubility of chitosan in water at physiological pH has, nevertheless, hampered its development for injectable *in situ* forming chemical hydrogels. Glycol chitosan (GC) is a water-soluble chitosan derivative retaining key properties of chitosan including excellent biocompatibility, bioadhesive activity, and enzymatic biodegradability. Kwon and coworkers through hydrophobic modification of glycol chitosan developed nanoparticles for *in vitro* as well as *in vivo* drug delivery.[34–36]

In this article, we report on preparation of novel injectable *in situ* forming biodegradable glycol chitosan-based hydrogels via Michael-type addition reaction under physiological conditions, for which thiolated glycol chitosan (GC-SH) and water soluble oligo(acryloyl carbonate)-*b*-poly(ethylene glycol)-*b*-oligo(acryloyl carbonate) (OAC-PEG-OAC) triblock copolymers were designed. OAC-PEG-OAC could be conveniently prepared with multiple acrylate groups by ring-opening polymerization of acryloyl cyclic carbonate (AC) under conditions similar to synthesis of PEG-PAC-PLA triblock copolymers.[37] Moreover, oligocarbonate had advantages over commonly applied oligoesters such as oligolactide by its slow hydrolytic degradation and lacking acidic degradation products. Glycol chitosan is enzymatically degradable *in vivo*, which renders it superior to PEG and dextran. Interestingly, the rheology measurements showed that robust hydrogels were formed rapidly upon mixing aqueous solutions of GC-SH and OAC-PEG-OAC at remarkably low solid concentrations of 1.5–4.5 wt % under physiological conditions. The gelation times, mechanical properties, pore sizes, and enzymatic degradation profiles of GC-based hydrogels were investigated. Moreover, the cell adhesion and growth behaviors of MG63 osteoblast and L929 fibroblast cells on thus formed GC hydrogels, with or without GRGDC functionalization, were also studied.

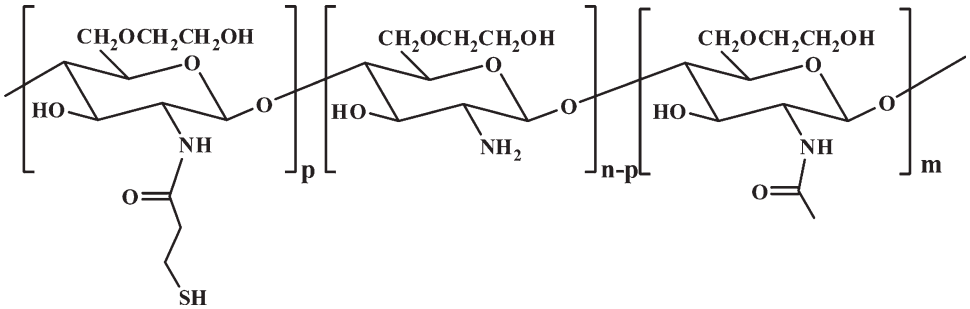
MATERIALS AND METHODS

Materials

Toluene and dichloromethane (DCM) were dried by refluxing over sodium wire and CaH_2, respectively, and distilled prior to use. Poly(ethylene glycol) (PEG, $M_n = 6$ kDa, Alfa Aesar) was dried by azeotropic distillation from dry toluene. 3,3′-dithiodipropionic acid (DTP, 99%, Alfa Aesar), glycol chitosan [GC, $M_w = 80$ kDa, degree of deacetylation (DD) = 90%, Wako], 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 98%, Alfa Aesar), *N*-hydroxysuccinimide (NHS, 98%, Alfa Aesar), 1,4-dithio-DL-threitol (DTT, 99%, Merck), and Zinc bis [bis(trimethylsilyl) amide] (97%, Aldrich) were used as received. GRGDC was purchased from ChinaTech Peptide(Suzhou) Co., Ltd. Acryloyl cyclic carbonate (AC) was synthesized according to our previous report.[38]

Preparation of thiolated glycol chitosan (GC-SH)

GC-SH derivatives were prepared with different degrees of substitution (DS) by reacting GC with varying amounts of 3,3′-dithiodipropionic acid (DTP) followed by reduction with DTT. In a typical example, a solution (5 mL) of DTP (0.056 g, 0.27 mmol), EDC (0.16 g, 0.81 mmol), and NHS (0.91 g, 0.81 mmol) in methanol was added to a solution of GC (0.10 g, 0.49 mmol) in deionized water (10 mL) and methanol (5 mL). The pH of the reaction mixture was adjusted to pH 5.0 with 4.0*M* HCl and the mixture was stirred for 48 h at room temperature (r.t.). Then, the solution pH was adjusted to pH 8.5 with 4.0*M* NaOH and treated with DTT (0.82 g, 5.3 mmol) for 24 h. The reaction mixture following adjusting its pH to 3.5 with 4.0*M* HCl was purified by ultrafiltration (MWCO 3000) against aqueous HCl salt solution (pH 3.5, 100 m*M* NaCl) once and deionized water for three times. GC-SH was obtained as white solid after lyophilization. The amount of free thiol groups in GC-SH was determined by Ellman's test. Yield: 70.9–80.4%. ^{1}H-NMR (400 MHz, D_2O): δ 1.98 (NHCOCH_3), 2.38–2.78 (m, —CH_2CH_2SH), 2.97–3.16 (m, GC methine proton linked to amino group), 3.27–4.10 (m, GC methylene and methine protons next to ether or hydroxyl group), 4.46 (s, GC anomeric proton).



SCHEME 1. Structure of thiolated glycol chitosan (GC-SH).

Synthesis of OAC-PEG-OAC triblock copolymer

OAC-PEG-OAC triblock copolymers were synthesized by ring-opening polymerization of acryloyl cyclic carbonate (AC) using PEG (M_n = 6 kDa) as an initiator. In a typical example, in a glove box under nitrogen atmosphere, to a solution of PEG (1.5 g, 0.25 mmol) and AC (0.45 g, 2.2 mmol) in DCM (7 mL) was quickly added a stock solution of zinc bis [bis (trimethylsilyl) amide] (96 mg, 0.22 mmol) in DCM. The reaction vessel was sealed and the polymerization was allowed to proceed under stirring at 30°C for 72 h. The resulting OAC-PEG-OAC copolymer was isolated by twice precipitation from diethylether and drying *in vacuo* at r.t. for 48 h. Yield: 75.2–80.0 %. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 1.06 (s, —CCH_3), 3.64 (s, methylene protons of PEG), 4.11 (s, —CH_2OCO), 5.81–6.48 (m, —CH=CH_2).

Preparation of (GRGDC)OAC_3-PEG-OAC_3(GRGDC)

GRGDC peptide functionalized OAC-PEG-OAC was prepared through Michael-type addition of OAC_3-PEG-OAC_3 (20.6 mg, 2.9 μmol) with GRGDC (2.9 mg, 5.7 μmol) in 1.5 mL of PB (pH 8.0, 10 m*M*) at r.t. for 3 h under a nitrogen atmosphere. The free GRGDC peptide if present was removed by extensive dialysis (MWCO 3500). (GRGDC)OAC_3-PEG-OAC_3 (GRGDC) was obtained as a white solid following lyophilization. Yield: 98.5%. ^{1}H-NMR (400 MHz, DMSO-d_6): δ 0.95 (s, —CCH_3), 3.50 (s, methylene protons of PEG), 4.17 (s, —CH_2OCO), 5.91–6.40 (m, —CH=CH_2), 1.23–1.78, 2.66, 6.98, 7.51, 8.48 (GRGDC).

Characterization

^{1}H-NMR spectra were recorded on an INOVA 400 MHz nuclear magnetic resonance instrument using deuterated chloroform ($CDCl_3$), deuterated water (D_2O), or deuterated dimethyl sulphoxide (DMSO-d_6) as solvents. The chemical shifts were calibrated against residue solvent signals of $CDCl_3$, D_2O and DMSO-d_6. The molecular weight and polydispersity of the copolymers were determined by a Waters 1515 gel permeation chromatograph (GPC). The measurements were performed using THF as eluant at a flow rate of 1.0 mL/min at 30°C and a series of polystyrene as standards. The number of free thiol groups of GC-SH was determined by the Ellman test. The absorption at 412 nm was recorded using a U-3900UV-VIS spectrophotometer (HITACHI, Tokyo, Japan). Morphology of hydrogels was characterized using scanning electron microscopy (Quanta200F D9048, FEI, The Netherlands). The hydrogel samples were lyophilized and mounted on metal stubs.

Rheological analysis

Rheological analysis was carried out with RS6000 (ThermoFisher, Germany) using parallel plates (20 mm diameter) configuration at 37°C in the oscillatory mode. GC-SH and OAC-PEG-OAC aqueous solutions in phosphate buffer (PB, pH 7.4, 100 m*M*) were quickly mixed and applied to the

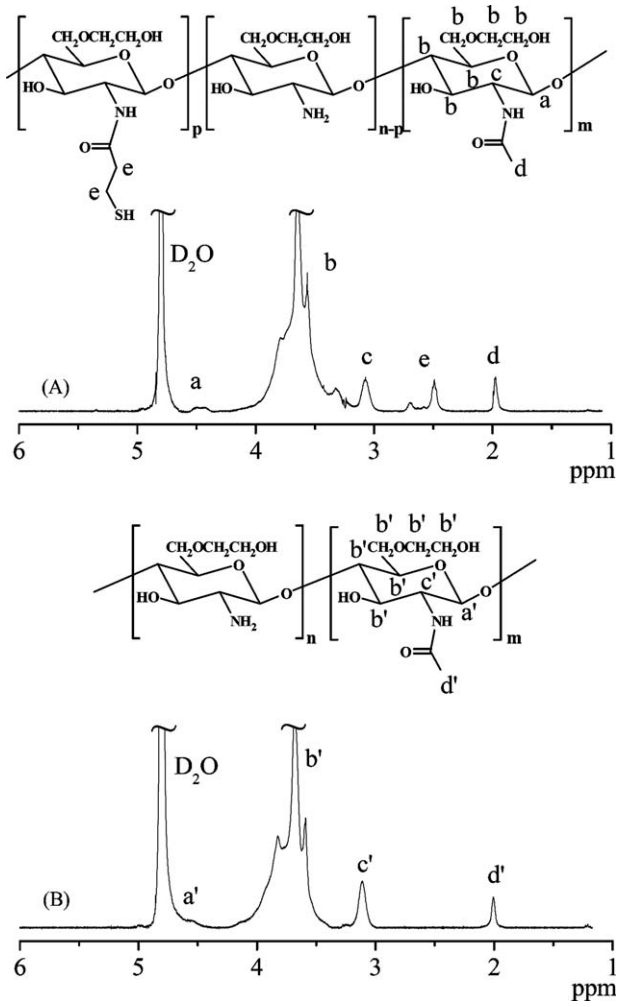


FIGURE 1. ^{1}H-NMR (400 MHz, D_2O, pH 1.5) of GC-SH (Table I, Entry 3) (A) and unmodified GC (B).

TABLE I. Synthesis of Thiolated Glycol Chitosan (GC-SH)

Entry	Molar feed ratio of carboxyl groups of DTP to amino groups of GC	DS (^{1}H-NMR)[a]	DS (Ellman's test)[b]	SH (μmol/g) (Ellman's test)[b]	Yield (%)
1	0.56/1	8.9	6.9	300	80.4
2	0.89/1	12.9	10.0	430	72.2
3	1.11/1	15.2	14.1	600	70.9
4	1.33/1	19.5	17.8	750	72.2

[a]Degree of substitution (DS), defined as the average number of DTP or free thiol groups per 100 disaccharide units, estimated from ^{1}H-NMR by comparing integrals of signals at δ 2.38–2.78 and 1.98.
[b]Determined by Ellman's test.

test platform of RS. 6000. The evolution of storage modulus (G') and loss modulus (G'') was recorded as a function of time. A gap of 0.5 mm, a frequency of 1 Hz and a strain of 1% were applied to maintain the linear viscoelastic regime. A solvent trap was used to avoid water evaporation. The gelation time, defined as the time point where $G' = G''$, was determined in triplicate.

Hydrogel preparation and gel content

Hydrogels were prepared in vials by quickly and thoroughly mixing aqueous solutions of GC-SH and OAC-PEG-OAC triblock copolymers in phosphate buffer (PB, pH 7.4, 100 m*M*) at 37°C. To determine the gel content, 0.50 mL of GC-SH/OAC-PEG-OAC hydrogel samples was lyophilized and weighed (W_d). The samples were extensively extracted with deionized water (over one week) to a constant weight. The remaining hydrogels were, then, lyophilized and weighed (W_s). The gel content was expressed as $W_s/W_d \times 100\%$.

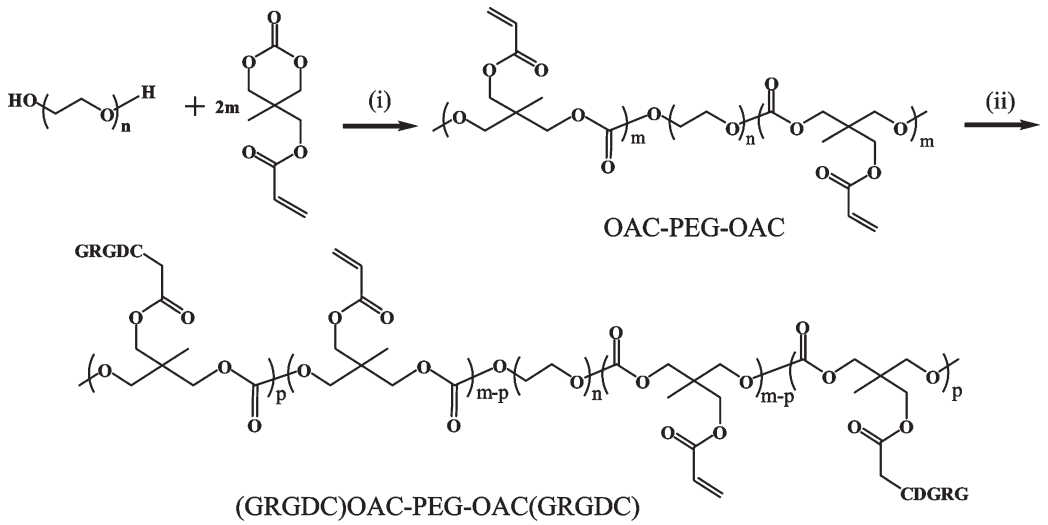
Swelling and enzymatic degradation of hydrogels

Hydrogel samples (0.50 mL) were prepared in vials according to the procedures described above and accurately weighed (W_i). Subsequently, 5 mL of phosphate buffered saline (PBS, pH 7.4, 150 m*M* NaCl) was added on the top of hydrogels followed by incubation at 37°C with constant shaking (150 rpm). At regular time intervals, the medium was removed carefully and the hydrogels were weighed (W_t). The swelling ratio was determined from the equation $W_t/W_i \times 100\%$. The medium was refreshed once a week. The experiments were performed in triplicate.

For enzymatic degradation studies, hydrogel samples (0.50 mL) prepared as described above were accurately weighed (W_i). Then, 3 mL of PBS (pH 7.4, 150 m*M* NaCl) containing 2 mg/mL of lysozyme was added on the top of hydrogels followed by incubation at 37 °C with constant shaking (150 rpm). At regular time intervals, the medium was removed carefully and the hydrogels were weighed (W_t). The swelling ratio was determined from the equation $W_t/W_i \times 100\%$. The medium (containing 2 mg/mL of lysozyme) was refreshed once a day. The experiments were performed in triplicate.

Preliminary cell culture studies

The hydrogels were prepared by quickly applying a mixed solution of GC-SH and OAC-PEG-OAC in PB (pH 7.4, 100 m*M*) on coverslips. The hydrogel-coated coverslips were put



SCHEME 2. Synthesis of OAC-PEG-OAC and (GRGDC)OAC-PEG-OAC(GRGDC). Conditions: (i) zinc bis[bis(trimethylsilyl)amide], CH_2Cl_2, r.t., 3 d; (ii) GRGDC, phosphate buffer (pH 8.0, 10 m*M*), r.t., 3 h.

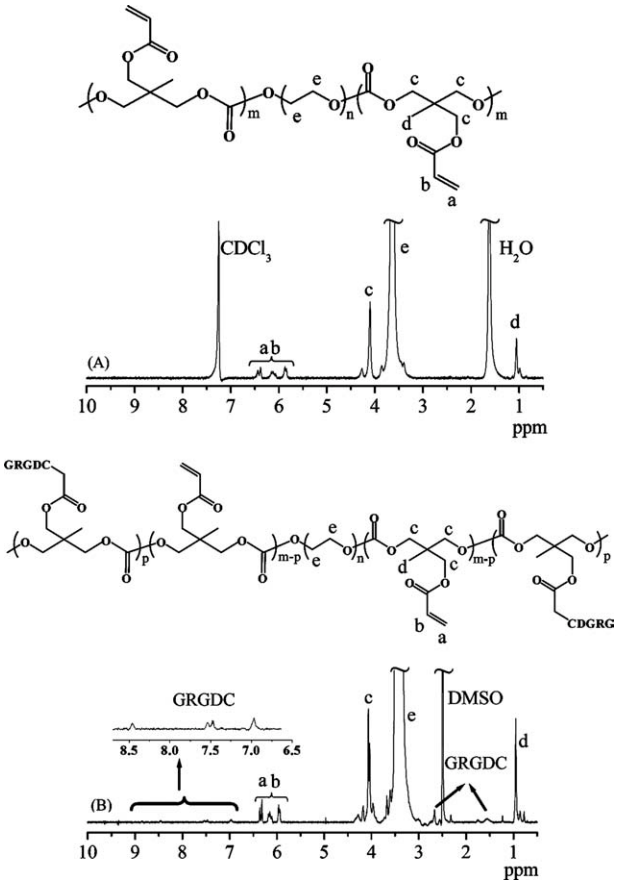


FIGURE 2. ^{1}H-NMR (400 MHz) spectra of OAC-PEG-OAC (Table II, Entry 1, $CDCl_3$) (A) and (GRGDC)OAC_3-PEG-OAC_3(GRGDC) (DMSO-d_6) (B).

into a 24-well cell culture plate followed by sterilization with cobalt radiation. MG63 osteoblast and L929 fibroblast cells were seeded on top of hydrogels at a cell density of 1.0×10^4 cells/well and maintained in a medium supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO_2. The cells following one day culture on hydrogels were observed by an inverted microscope (Axiovert 40 CFL).

RESULTS AND DISCUSSION

Synthesis of GC-SH conjugates and OAC-PEG-OAC triblock copolymers

Thiolated glycol chitosan (GC-SH, Scheme 1) derivatives were synthesized by reacting glycol chitosan (GC, $M_{w\ GC} =$ 80 kg/mol) with 3,3′-dithiodipropionic acid (DTP) in the presence of EDC and NHS coupling reagents at pH 5.0 followed by reductive cleavage of the disulfide bonds with DTT at pH 8.5. ^{1}H-NMR spectrum showed besides resonances characteristic of GC (δ 1.98, 2.97–3.16, 3.27–4.10, and 4.46) signals at δ 2.38–2.78 assignable to the methylene protons of DTP moieties (Fig. 1). The degree of substitution (DS), defined as the average number of DTP or free thiol groups per 100 disaccharide units, was estimated by comparing integrals of signals at δ 2.38–2.78 and 1.98. The results indicated that DS increased from 8.9 to 19.5 with increasing molar feed ratio of carboxyl groups of DTP to amino groups of GC from 0.56/1 to 1.33/1 (Table I). The amounts of thiol groups determined by Ellman's tests increased from 300 to 748 μmol/g GC-SH, corresponding to DS values of 6.9 to 17.8 (Table I), which were comparable to those calculated from ^{1}H-NMR within experimental errors. In the following studies, DS data determined by Ellman's tests were used.

OAC-PEG-OAC triblock copolymers were conveniently prepared by ring-opening polymerization of acryloyl cyclic carbonate (AC) using PEG ($M_{n\ PEG} = 6$ kg/mol) as an initiator and zinc bis [bis(trimethylsilyl) amide] as a catalyst (Scheme 2). We reported previously that AC can be readily synthesized and copolymerized with other cyclic ester monomers to afford acryloyl-functionalized biodegradable polymers that are amenable to post-polymerization modification with thiol-containing molecules via Michael-type addition reaction.[38] ^{1}H-NMR spectrum showed in addition to a peak at δ 3.64 due to the methylene protons of PEG also resonances at δ 5.81–6.48, 4.11, and 1.06 assignable to the acryloyl protons, methylene protons, and methyl protons of OAC, respectively [Fig. 2(A)]. The degree of polymerization (DP) of OAC blocks could be determined by comparing the intensities of signals at δ 5.81–6.48 and 3.64. To warrant water solubility, OAC-PEG-OAC copolymers were prepared with low average DP of 2 and 3 for OAC, which were denoted as OAC_2-PEG-OAC_2 and OAC_3-PEG-OAC_3, respectively. GPC revealed a unimodal distribution with low polydispersity indexes of 1.24 and 1.28 for AC_2-PEG-AC_2 and AC_3-PEG-AC_3, respectively (Table II). Gly-Arg-Gly-Asp-Cys (GRGDC) peptide was conjugated to OAC_3-PEG-OAC_3 copolymer at GRGDC/AC feeding molar ratio of 1/3 through Michael-type addition reaction in phosphate buffer at pH 8.0 at room temperature for 3 h (Scheme 2). The unconjugated GRGDC was removed by extensive dialysis. ^{1}H-NMR showed new signals attributable to GRGDC peptide and importantly signal intensity of acrylate protons decreased by approximately one-third relative to the methyl protons of OAC [Fig. 2(B)], indicating quantitative conjugation of GRGDC. Therefore, OAC-PEG-OAC copolymers allow versatile, efficient and

TABLE II. Synthesis of OAC-PEG-OAC Triblock Copolymers

Entry	Copolymer	$[AC]_0/[PEG]_0$ (mol/mol)	M_n (^{1}H-NMR)[a]	M_n (GPC)[b]	PDI (GPC)[b]	Yield (%)
1	OAC_2-PEG-OAC_2	10/1	400–6000–400	8800	1.24	80.0
2	OAC_3-PEG-OAC_3	12/1	600–6000–600	9300	1.28	75.2

[a] Molecular weights of OAC blocks determined from ^{1}H-NMR by comparing the intensities of signals at δ 5.81–6.48 and 3.64.
[b] Determined by GPC (eluant: THF, flow rate: 1.0 mL/min, standards: polystyrene).

clean (without need of a catalyst and organic solvent) conjugation of thiol-containing bioactive molecules.

In situ hydrogel formation and gelation time

Hydrogels were formed *in situ* via Michael-type addition between GC-SH and OAC-PEG-OAC in PBS at pH 7.4 and 37°C. The aqueous solutions of GC-SH and OAC-PEG-OAC were quickly mixed and applied to the rheometer. The kinetics of hydrogel formation was followed by monitoring the storage modulus (G') and loss modulus (G'') in time. The gelation time was defined as the time point where G' equals to G''. Figure 3 shows typical gelation kinetics of GC-SH (DS 10.0 or 14.1) and OAC_2-PEG-OAC_2 in phosphate buffer (pH 7.4, 100 m*M*) at 37°C at a GC-SH concentration of 1.5 wt % and an SH/AC molar ratio of 1/1. Notably, the storage modulus increased rapidly upon mixing GC-SH and OAC-PEG-OAC solutions, in which gelation times of 1.8 min and 1.1 min were observed for GC-SH DS 10.0 and 14.1, respectively. The storage modulus (G') leveled off within 30 min, indicating completion of crosslinking process.

The influences of DS, polymer concentration, SH/AC molar ratio, and pH on the gelation time were systematically investigated. Figure 4(A) shows that at a low GC-SH concentration of 1 wt % using OAC_2-PEG-OAC_2 as a crosslinker at a SH/AC molar ratio of 1/1, gelation time decreased from ca. 12.9 min to 2.3 min with increasing DS from 6.9 to 14.1, as a result of increased crosslinking density. However, further increasing DS to 17.8 resulted in longer gelation time (ca. 4 min), which is most likely due to that the crosslinking reaction becomes sterically hindered at such a high DS. Notably, variation of SH/AC molar ratios from 1/0.83 to 1/1.2 did not much influence the gelation time [Fig. 4(A)]. As expected, the gelation time decreased with increasing solution pH from 6.5 to 8.0 [Fig. 4(B)]. For example, GC-SH 14.1 had gelation times of 15.8 min, 5.7 min, 2.3 min, and 10 s at pH 6.5, 7.0, 7.4, and 8.0, respectively, at 1 wt % concentration and SH/AC molar ratio of 1/1. The faster gelation at higher pH is due to the presence of higher concentration of thiolate anions that are the actual reactive species for the Michael-type addition reaction.[39] The gelation time decreased dramatically, from 2.5 min to 10 s for GC-SH 14.1 and from 4.3 min to 0.9 min for GC-SH 10.0, when increasing GC-SH concentrations from 1.0 wt % to 2.0 wt % at pH 7.4 [Fig. 4(C)], which could be attributed to an increase in the number of functional groups per volume of gel precursors. It is evident, therefore, that the gelation times of GC-SH/OAC-PEG-OAC hydrogels can be nicely controlled from about 10 s to several minutes by DS of GC-SH, solution pH, and polymer concentration.

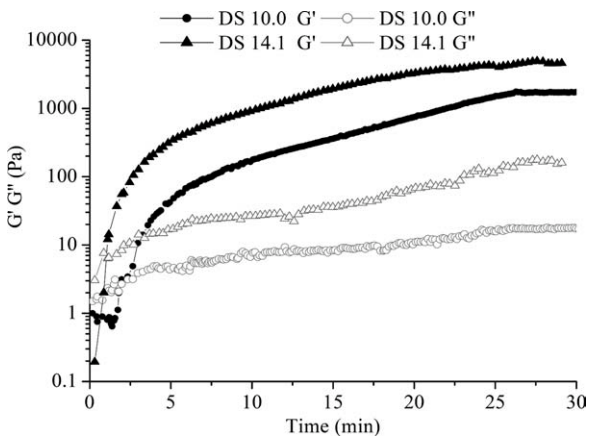


FIGURE 3. Evolution of storage modulus (G') and loss modulus (G'') upon mixing GC-SH (DS 10.0 or 14.1, 1.5 wt %) and OAC_2-PEG-OAC_2 in phosphate buffer (pH 7.4, 100 m*M*) at 37°C. SH/AC molar ratio was fixed at 1/1.

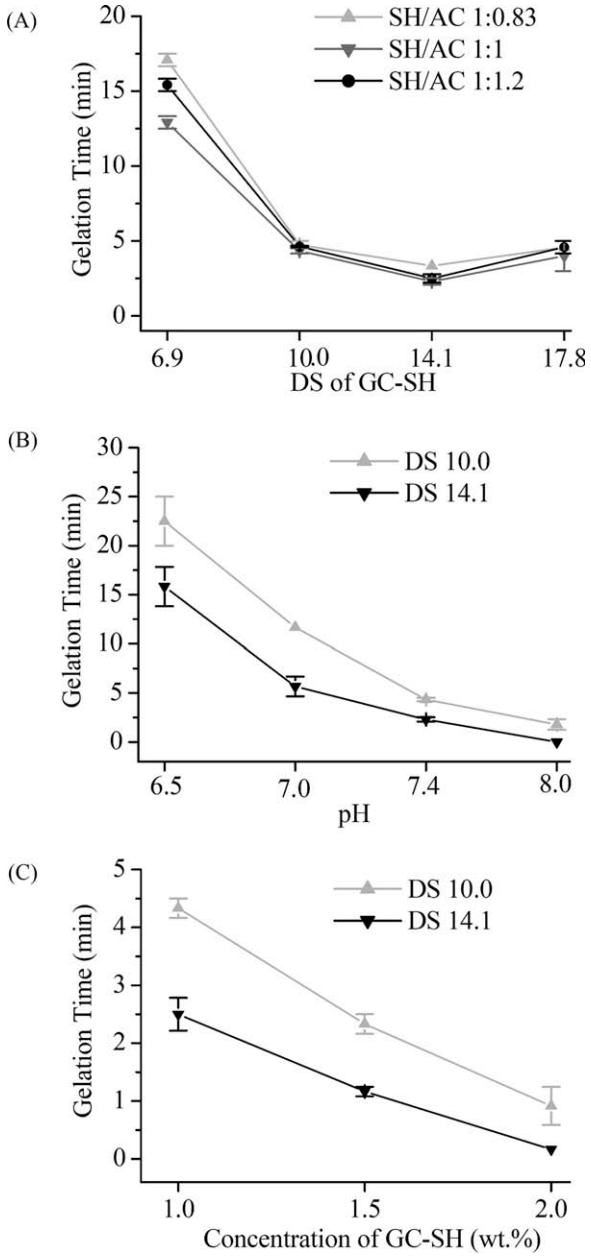


FIGURE 4. Gelation times of GC-SH/OAC_2-PEG-OAC_2 in phosphate buffer (100 m*M*) at 37°C determined by rheology (A) as a function of DS at 1 wt % GC-SH and pH 7.4; (B) as a function of pH at 1 wt % GC-SH and SH/AC molar ratio of 1/1; and (C) as a function of GC-SH concentration at pH 7.4 and SH/AC molar ratio of 1/1.

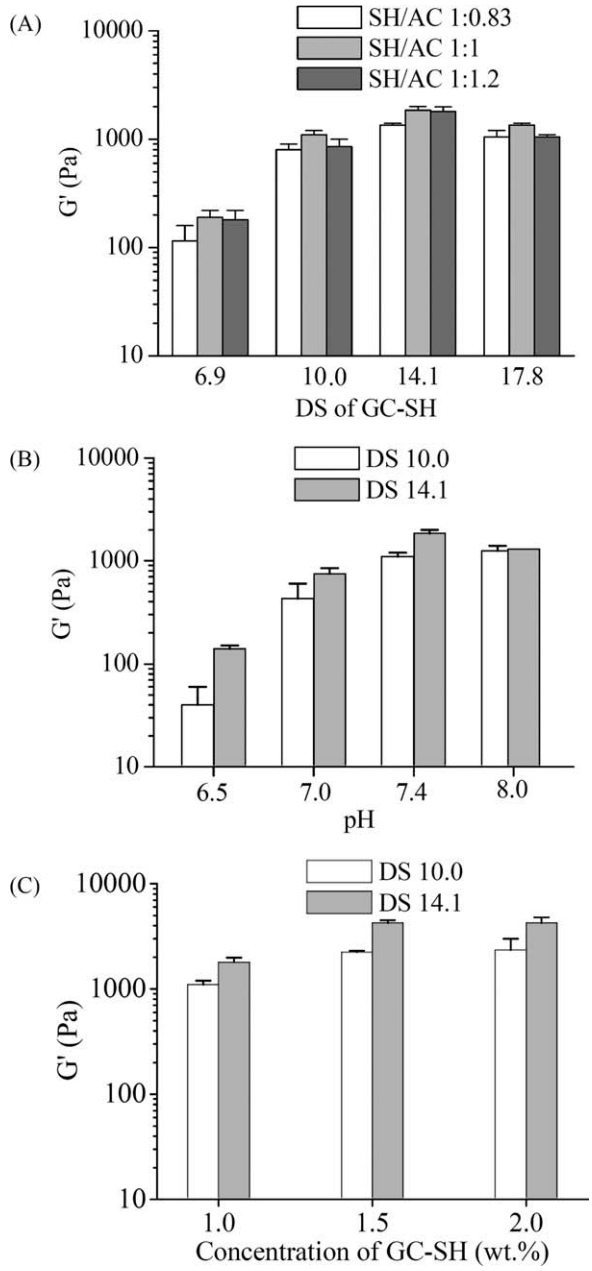


FIGURE 5. Storage moduli of GC-SH/OAC_2-PEG-OAC_2 hydrogels in phosphate buffer (100 m*M*) at 37°C determined by rheology (A) as a function of DS at 1 wt % GC-SH and pH 7.4; (B) as a function of pH at 1 wt % GC-SH and SH/AC molar ratio of 1/1; and (C) as a function of GC-SH concentration at pH 7.4 and SH/AC molar ratio of 1/1.

Hydrogel mechanical properties and morphologies

The mechanical properties of GC-SH/OAC_2-PEG-OAC_2 hydrogels were studied as a function of DS, polymer concentration, SH/AC molar ratio, and pH. The results showed that at a low GC-SH concentration of 1 wt % and SH/AC molar ratio of 1/1, the storage modulus (G') increased from 190 to 1850 Pa with increasing DS from 6.9 to 14.1 due to increased crosslinking density [Fig. 5(A)]. The storage modulus slightly decreased to 1350 Pa when further increasing DS to 17.8, probably because of the steric hindrance at such

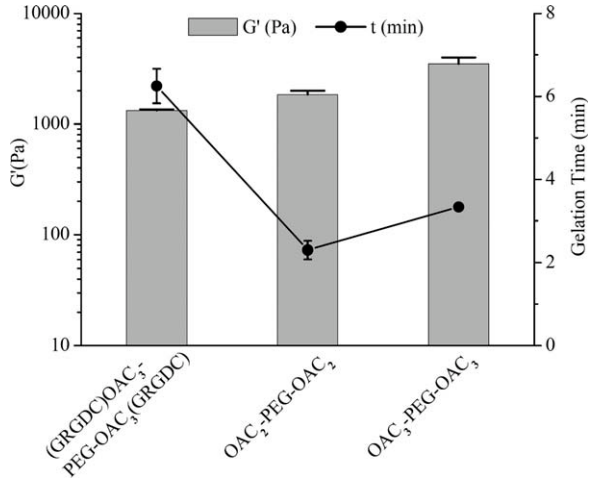


FIGURE 6. Storage moduli and gelation times of GC-SH (DS 14.1, 1 wt %) hydrogels crosslinked with (GRGDC)OAC_3-PEG-OAC_3(GRGDC), OAC_3-PEG-OAC_3, and OAC_2-PEG-OAC_2 in phosphate buffer (pH 7.4, 100 m*M*) at 37°C and SH/AC molar ratio of 1/1.

a high DS yielding less perfect hydrogel networks. SH/AC molar ratios had little influence on the storage modulus [Fig. 5(A)]. The storage modulus increased with increasing solution pH from 6.5 to 7.4 and did not change or even decreased when further increasing pH to 8.0 [Fig. 5(B)], indicating that pH 7.4 is an optimal condition for Michael-type addition reaction to accomplish high conversion. The reduced storage modulus for GC-SH 14.1 hydrogels at pH 8.0 is most likely due to its too fast and thereby inhomogeneous and incomplete gelation. The storage modulus first increased and then leveled off with increasing GC-SH concentrations from 1.0 wt % to 2.0 wt % [Fig. 5(C)]. For example, GC-SH 14.1 hydrogels had storage moduli of 1800, 4300, and 4300 Pa at 1.0 wt %, 1.5 wt %, and 2.0 wt %, respectively. Hence, the storage moduli of GC-SH/OAC-PEG-OAC hydrogels could be broadly tuned from 100 to 4300 Pa. It should be noted that these hydrogels had remarkably low solid concentrations of 1.5–4.5 wt %, which

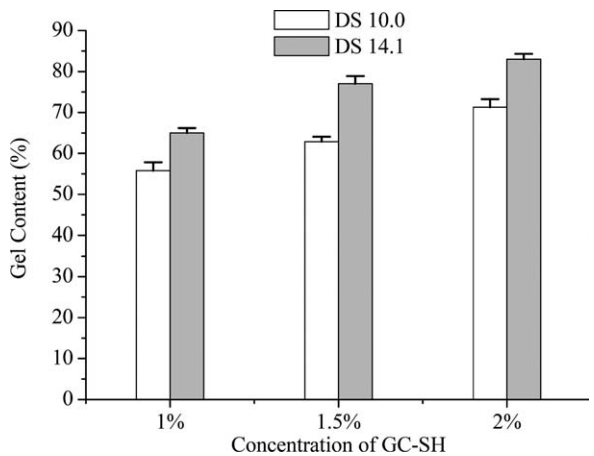


FIGURE 7. Gel content of GC-SH/OAC_2-PEG-OAC_2 hydrogels as a function of GC-SH concentration in phosphate buffer (pH 7.4, 100 m*M*) at 37°C.

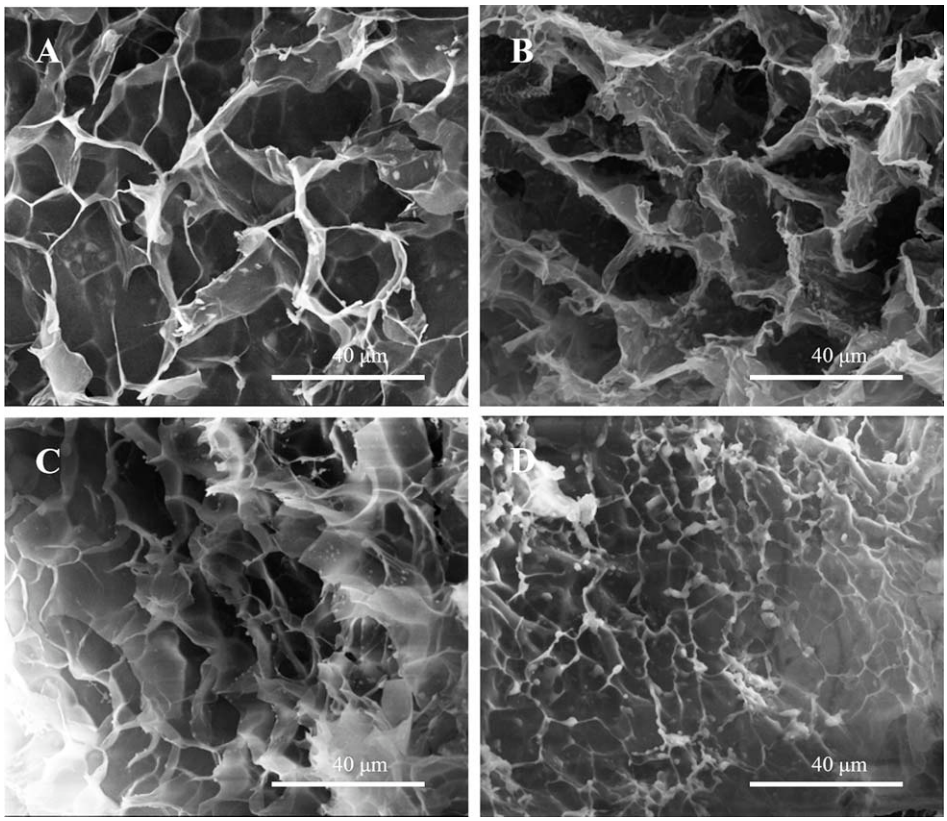


FIGURE 8. SEM photographs of lyophilized GC-SH/OAC_2-PEG-OAC_2 hydrogel samples. The hydrogels were prepared in phosphate buffer (pH 7.4, 100 m*M*) at 37°C with GC-SH at two different DS (10.0 or 14.1) and concentrations (1 or 2 wt %). (A) DS 10.0, 1 wt %, (B) DS 10.0, 2 wt %, (C) DS 14.1, 1 wt %, and (D) DS 14.1, 2 wt %.

corresponded to hydrogels with high water contents of 95.5–98.5 wt %.

The storage moduli and gelation times of GC-SH (DS 14.1) hydrogels crosslinked with (GRGDC)OAC_3-PEG-OAC_3(GRGDC), OAC_3-PEG-OAC_3, and OAC_2-PEG-OAC_2 in phosphate buffer (pH 7.4, 100 m*M*) at 1 wt % GC-SH and SH/AC molar ratio of 1/1 at 37°C were compared (Fig. 6). The results showed that hydrogels crosslinked with OAC_3-PEG-OAC_3 had slightly longer gelation time but much higher storage modulus than those with OAC_2-PEG-OAC_2. Notably, (GRGDC)OAC_3-PEG-OAC_3(GRGDC) also resulted in sufficiently fast gelation to afford hydrogels with storage

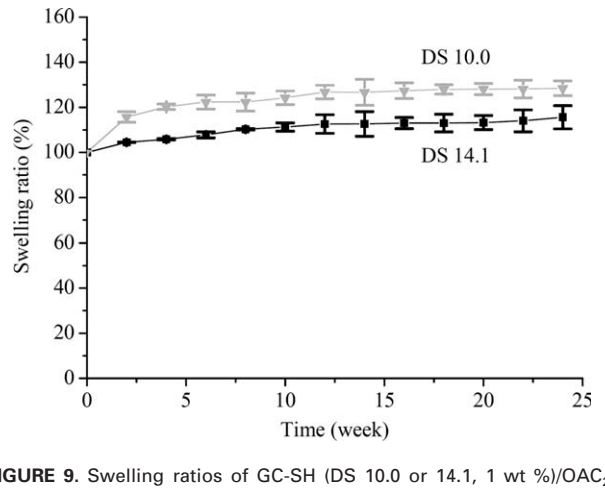


FIGURE 9. Swelling ratios of GC-SH (DS 10.0 or 14.1, 1 wt %)/OAC_2-PEG-OAC_2 hydrogels in phosphate buffered saline (pH 7.4, 150 m*M* NaCl) at 37°C.

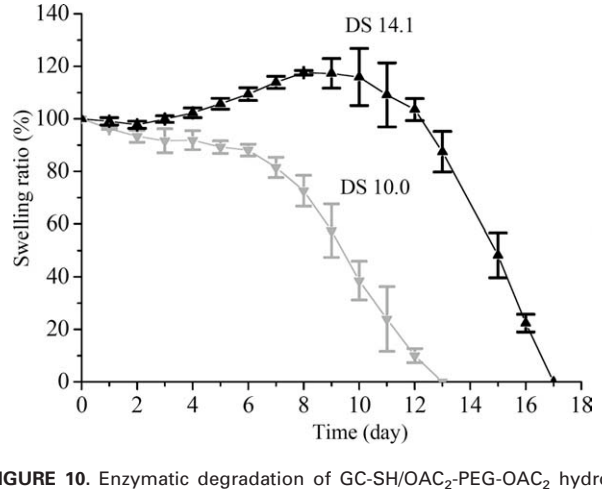


FIGURE 10. Enzymatic degradation of GC-SH/OAC_2-PEG-OAC_2 hydrogels with lysozyme (2 mg/mL) in phosphate buffered saline (pH 7.4, 150 m*M* NaCl) at 37°C. The hydrogels were prepared with GC-SH DS 10.0 or 14.1 at 1 wt % GC-SH concentration.

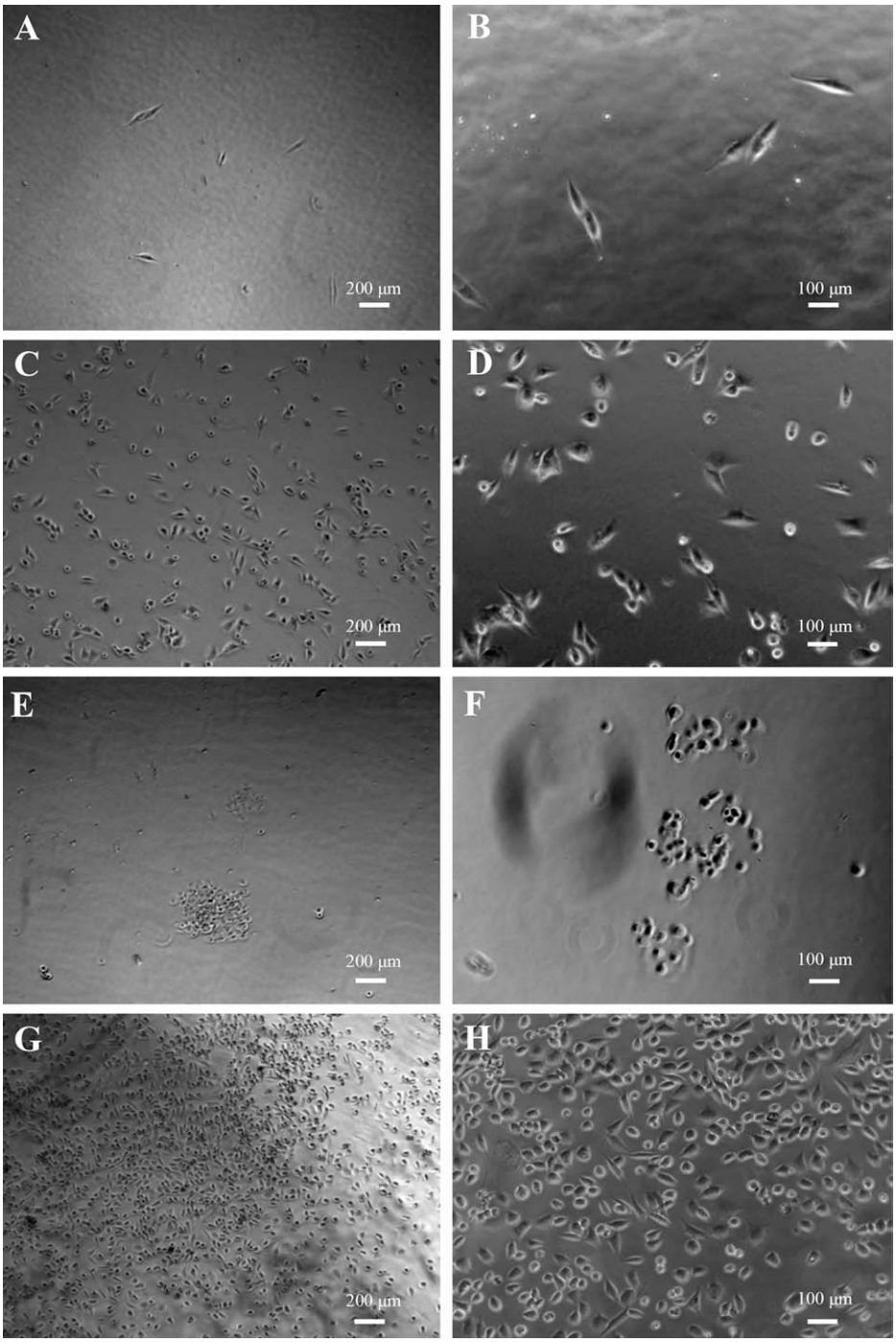


FIGURE 11. Images of MG63 and L929 cells cultured on GC-SH (DS 14.1) hydrogels crosslinked with OAC_2-PEG-OAC_2 (A,B; E,F) and (GRGDC)OAC_3-PEG-OAC_3(GRGDC) (C,D; G,H). The cells were cultured on top of hydrogels for one day and the images were taken at ×50 (A,C; E,G) and ×100 (B,D; F,H) magnifications.

modulus close to those formed with OAC_2-PEG-OAC_2. Therefore, injectable bioactive hydrogels could be readily obtained from GC-SH and OAC-PEG-OAC.

The gel contents of GC-SH hydrogels increased with increasing GC-SH concentrations and DS (Fig. 7). High gel contents of 77–83% were obtained at 1.5 and 2.0 wt % concentrations, respectively, for GC-SH (DS 14.1) hydrogels. To investigate hydrogel morphology and pore sizes, lyophilized GC-SH/OAC-PEG-OAC hydrogel samples were characterized by scanning electron microscopy (SEM). The results unveiled microporous hydrogel structures with average pore sizes ranging from several micrometers to several tens of micrometers depending on DS and concentration of GC-SH (Fig. 8). The higher the DS and polymer concentration, the smaller

the pore sizes. It is important for hydrogels to have microporous features which enable efficient transportation of nutrients and wastes throughout the hydrogel scaffolds.

Swelling and degradation of hydrogels by lysozyme

The swelling/degradation behaviors of GC-SH/OAC_2-PEG-OAC_2 hydrogels formed at 1 wt.% GC-SH concentration were studied in phosphate buffered saline (PBS, pH 7.4, 150 m*M* NaCl) at 37°C. Interestingly, the results showed that these GC hydrogels had particularly low swelling, in which swelling ratios smaller than 120% and 130% were observed for hydrogels of GC-SH DS 14.1 and 10.0, respectively, after 24 weeks incubation in PBS (Fig. 9), indicating that these GC-SH/OAC_2-PEG-OAC_2 hydrogels had excellent stability and slow hydrolytic degradation under physiological conditions. This is in line with our expectation since oligocarbonate is less prone to hydrolytic degradation than oligoesters such as oligolactide. In contrast, fast degradation of GC-SH hydrogels was observed in the presence of 2 mg/mL lysozyme under otherwise the same conditions (Fig. 10). The degradation time increased with increasing DS of GC-SH. For example, complete degradation was discerned on day 13 and 17 for hydrogels formed with GC-SH DS 10.0 and 14.1, respectively. It is known that lysozyme is the primary enzyme responsible for the *in vivo* degradation of chitosan yielding biocompatible chitosan oligosaccharides of variable lengths as degradation products.[31] It should be noted, however, that physiological lysozyme concentration is usually low (much lower than 2 mg/mL). The *in vivo* degradation of GC-SH/OAC-PEG-OAC hydrogels would likely require several weeks to several months. The remarkable hydrolytic stability combined with enzymatic biodegradability of GC-SH/OAC-PEG-OAC hydrogels render them particularly interesting to be used as tissue scaffolds in which degradation of matrices and tissue remodeling is mainly dictated by local lysozyme concentrations.

Cell culture on hydrogels

The preliminary cell adhesion and growth behaviors of MG63 osteoblast and L929 fibroblast cells on GC hydrogels were investigated. The results showed that GC-SH/OAC_2-PEG-OAC_2 hydrogels were practically cell non-adhesive [Fig. 11 (A,B,E,F)], likely due to their highly hydrophilic and non-fouling nature. In contrast, GRGDC-functionalized GC hydrogels prepared from GC-SH and (GRGDC)OAC_3-PEG- OAC_3 (GRGDC) were found to well support adhesion and growth of both MG63 and L929 cells under otherwise the same culture conditions [Fig. 11 (C,D,G,H)]. Importantly, both cells retained their native morphologies. RGD peptide, a conserved adhesion motif in extracellular matrix (ECM) proteins such as fibronectin and fibrin, has been widely used for the fabrication of bioactive scaffolds for specific cell (including osteoblasts and fibroblasts) attachment and proliferation.[40,41] The cell non-adhesive feature together with facile functionalization with thiol-containing biomolecules under physiological conditions makes GC-SH/OAC-PEG-OAC hydrogels particularly interesting for the development of injectable bioactive biodegradable extracellular matrices for tissue engineering applications.

CONCLUSIONS

We have demonstrated for the first time that glycol chitosan-based hydrogels are rapidly formed upon mixing thiolated glycol chitosan and water soluble oligo(acryloyl carbonate)-*b*-poly(ethylene glycol)-*b*-oligo(acryloyl carbonate) triblock copolymers at remarkably low solid concentrations of 1.5–4.5 wt % under physiological conditions. The gelation time and mechanical properties of hydrogels can be nicely tuned by DS of GC-SH, solution pH, and polymer concentration. These microporous glycol chitosan-based hydrogels while rather stable under physiological conditions are subject to enzymatic degradation by lysozyme. Interestingly, these hydrogels are cell non-adhesive, and can be readily made into bioactive extracellular matrix with specific cell attachment and proliferation functions through immobilization with thiol-containing bioactive molecules such as GRGDC peptide via Michael-type addition reaction under physiological conditions. Glycol chitosan is enzymatically degradable *in vivo*, which offers a significant advantage over PEG and dextran. We are convinced that these rapidly *in situ* forming glycol chitosan hydrogels have great potential for the development of injectable bioactive biodegradable extracellular matrices for tissue engineering.

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