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Injectable biodegradable hybrid hydrogels based on thiolated collagen and oligo(acryloyl carbonate)–poly(ethylene glycol)–oligo(acryloyl carbonate) copolymer for functional cardiac regeneration

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ABSTRACT

Injectable biodegradable hybrid hydrogels were designed and developed based on thiolated collagen (Col-SH) and multiple acrylate containing oligo(acryloyl carbonate)-b-poly(ethylene glycol)-b-oligo(acryloyl carbonate) (OAC-PEG-OAC) copolymers for functional cardiac regeneration. Hydrogels were readily formed under physiological conditions (37 °C and pH 7.4) from Col-SH and OAC-PEG-OAC via a Michael-type addition reaction, with gelation times ranging from 0.4 to 8.1 min and storage moduli from 11.4 to 55.6 kPa, depending on the polymer concentrations, solution pH and degrees of substitution of Col-SH. The collagen component in the hybrid hydrogels retained its enzymatic degradability against collagenase, and the degradation time of the hydrogels increased with increasing polymer concentration. In vitro studies showed that bone marrow mesenchymal stem cells (BMSCs) exhibited rapid cell spreading and extensive cellular network formation on these hybrid hydrogels. In a rat infarction model, the infarcted left ventricle was injected with PBS, hybrid hydrogels, BMSCs or BMSC-encapsulating hybrid hydrogels. Echocardiography demonstrated that the hybrid hydrogels and BMSC-encapsulating hydrogels could increase the ejection fraction at 28 days compared to the PBS control group, resulting in improved cardiac function. Histology revealed that the injected hybrid hydrogels significantly reduced the infarct size and increased the wall thickness, and these were further improved with the BMSC-encapsulating hybrid hydrogel treatment, probably related to the enhanced engraftment and persistence of the BMSCs when delivered within the hybrid hydrogel. Thus, these injectable hybrid hydrogels combining intrinsic bioactivity of collagen, controlled mechanical properties and enhanced stability provide a versatile platform for functional cardiac regeneration.

1. Introduction

Myocardial infarction (MI) results in myocardial damage, scar formation, the death of cardiomyocytes and ventricular dysfunction [1]. The adult heart has limited self-regeneration capacity and the loss of cardiomyocytes in the infarcted heart leads to adverse ventricular remodeling, often leading to heart failure [2,3]. The gold standard treatment for those with end-stage heart failure remains cardiac transplantation, but donor heart availability remains a major limitation. Intra-myocardial hydrogel injection following MI provides an approach for reducing the stress experienced by the infarcted ventricular wall as elucidated by the law of Laplace, which states that wall stress is proportional to the pressure and radius and inversely proportional to the wall thickness [4,5]. Furthermore, injectable hydrogels have been demonstrated as a promising platform to enhance the engraftment and persistence of stem cells, thus improving the effects of the cell therapy on heart regeneration. It is interesting to note that several hydrogels based on alginate and collagen have already been approved for different phases of clinical trials (MAGNUM, PRESERVATION 1) addressing patients with myocardial infarction [6,7].

Natural materials that are intrinsically bioactive, such as fibrin, collagen, Matrigel and gelatin, can regulate the inflammation and remodeling process following MI, and have been widely used for

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cardiac regeneration [8–10]. Kofidis et al. [11] reported that Matrigel injection in a mouse model was capable of improving fractional shortening (FS) and wall thickening 4 weeks after treatment. Christman et al. [12] reported that local injection of fibrin hydrogels in the MI zone of a rat model could maintain FS and preserve the infarct scar thickness. In a later study, injectable hydrogels based on fibrin, collagen and Matrigel demonstrated the ability to significantly increase capillary formation compared to a saline control group [13]. Suuronen et al. [14] demonstrated that the incorporation of chitosan into a collagen hydrogel could stimulate greater vascular growth and recruit more endothelial/angiogenic cells than a collagen-only matrix in vivo. Wang et al. [15,16] reported that a chitosan hydrogel could improve the MI microenvironment, enhance the survival of engrafted adipose-derived stem cells (ADSCs) and significantly increase the differentiation rate of ADSCs into cardiomyocytes in vivo. Furthermore, ADSC-laden chitosan prevented adverse matrix remodeling, increased angiogenesis and preserved heart function. Cell–matrix interaction was shown to be required in the synergistic effect of cells and collagen matrix therapy in improving perfusion, viability and function of the infarcted mouse myocardium, highlighting the importance of bioactivity of the injectable hydrogel [17]. Although these natural hydrogels demonstrated great potency in treating MI, they may suffer from inferior mechanical properties and suboptimal durability, which have recently been recognized as critical factors for the improvement of heart function and neovascularization [18–20]. Several synthetic hydrogels with controllable degradation rate and tunable mechanical properties have been explored for cardiac repair. For example, Wagner et al. [21,22] developed a degradable hydrogel with a mechanical stiffness of 20 kPa and a degradation time of greater than 2 months in vivo based on a thermally responsive copolymer of *N*-isopropyl acrylamide, acrylic acid and hydroxyl ethyl methacrylate–poly(trimethylene carbonate). Injection of this hydrogel into a chronic rat infarct prevented the progression of cardiac dilation and remodeling. Li et al. [23] reported on a biodegradable synthetic hydrogel based on poly(δ-valerolactone)-b-poly(ethylene glycol)–poly(δ-valerolactone) that preserved ventricular function after MI by stabilizing the infarct and inducing angiogenesis. Wang et al. [24] demonstrated that injection of oligo(poly(ethylene glycol) fumarate) hydrogel with embryonic stem cells into a rat MI model could decrease the infarct size and improve heart function and revascularization. Jiang et al. [25,26] reported that injection of BMSCs with a synthetic hydrogel consisting of α-cyclodextrin and poly(ethylene glycol)–poly(caprolactone)–poly(ethylene glycol) (PEG-PCL-PEG) into the infarcted myocardium in rabbits could increase the survival and retention of transplanted cells and improve cardiac function. However, these synthetic hydrogels generally cannot provide optimal biological activity for cardiac repair. Hybrid hydrogels combining representative advantages of both natural and synthetic materials have thus been developed, providing biological properties together with well-controlled mechanical strength and degradation profiles. Previous studies have produced hybrid hydrogels mainly utilizing functional group-terminated PEG and natural polymers such as collagen [27,28], gelatin [29], fibrinogen [30], hyaluronic acid [31] and alginate [32].

In this paper, we report on the development of injectable bioactive hybrid hydrogels based on thiolated collagen (Col-SH) and multiple acrylate-containing oligo(acryloyl carbonate)-b-poly(ethylene glycol)–oligo(acryloyl carbonate) (OAC-PEG-OAC) copolymers, formed via a Michael-type addition reaction. These were tested for cardiac regeneration, by intramyocardial injection ± bone marrow mesenchymal stem cells (BMSCs) to the infarcted rat heart. These bioactive hybrid hydrogels were designed based on the following considerations: (i) collagen with intrinsic biocompatibility, biodegradation and cell-responsive characteristics has demonstrated great potential in regenerative medicine, including cardiac repair [33–35]; (ii) polycarbonate, with a slow hydrolytic degradation rate and non-acidic degradation products, provides a controllable degradation rate and improved mechanical properties [36–38]. In addition, the multiple acryloyl functional groups of polycarbonate allow facile adjustment of crosslinking density and physical properties; (iii) Michael-type addition hydrogels have been extensively explored for the in situ formation of biodegradable hydrogels in that they are formed quickly under physiological conditions without the aid of a catalyst and without generating any byproducts, fully circumventing potentially toxic contaminations [39–41]; and (iv) BMSCs have been widely investigated in preclinical animal studies and have shown a positive effect on cardiac function after MI, mainly through multiple paracrine effects [42–44]. In this study, the gelation times, mechanical properties and enzymatic degradation profiles of the hybrid hydrogels, and their use for in vitro BMSC culture and in vivo cardiac regeneration, were investigated.

2. Materials and methods

2.1. Materials

Toluene and dichloromethane were dried by refluxing over sodium wire and CaH_2, respectively, and distilled prior to use. PEG (M_n = 6 kDa, Alfa Aesar) was dried by azeotropic distillation from dry toluene. Type I porcine collagen was obtained from the skin of pig (M_w = 80 kDa, Sichuan Mingrang Bio-Tech Co., Ltd.). Succinic anhydride (99%, Alfa Aesar), 2-mercaptoethylamine hydrochloride (MEA, 98%, Alfa Aesar), collagenase (type I *Clostridium histolyticum*, 0.25–1.0 FALGPA U mg solid^{-1}, Sigma), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 98%, Alfa Aesar), *N*-hydroxysuccinimide (NHS, 98%, Sigma), 1,4-dithio-DL-threitol (DTT, 99%, Merck), zinc bis(bis(trimethylsilyl) amide) (97%, Aldrich), 2,4,6-trinitro-benzenesulfonic acid (TNBSA, 5 w/v% in water, Aladdin) and 3-carboxy-4-nitrophenyl disulfide (99%, Adamas Reagent Co., Ltd.) were used as received. Acryloyl cyclic carbonate (AC) and OAC-PEG-OAC were synthesized according to our previous report [45,46].

2.2. Preparation of Col-SH

Col-SH derivatives were prepared with different degrees of substitution (DSs) by reacting collagen with an excess of succinic anhydride to yield carboxylated collagen (Col-COOH), followed by amidation with varying amounts of MEA. In a typical example, a solution (100 ml) of succinic anhydride (15 g, 0.15 mol) in acetone at 4 °C was added dropwise to a solution of collagen (1 g, 0.0125 mmol) in deionized water (150 ml) adjusted to pH 10 with NaOH (4.0 M). The pH of the reaction mixture was maintained at pH 9.0 and the mixture was stirred for 4 h at 4 °C. The reaction solution was then dialyzed (MWCO 14000) for 24 h against deionized water and the Col-COOH product was collected by freeze-drying. The amount of remaining amine groups in the Col-COOH, determined by TNBSA assay, was found to be zero, indicating that the DS of the carboxyl groups in Col-COOH was close to 100%. Next, the aqueous solutions of EDC (0.5 g, 2.6 mmol), NHS (0.3 g, 2.6 mmol) and MEA (0.2 g, 1.76 mmol) were sequentially added to a solution of Col-COOH (0.5 g, 0.00625 mmol) in deionized water (50 ml). The pH of the reaction mixture was adjusted to pH 5.5 with HCl (1.0 M) and the mixture was stirred for 24 h at room temperature. The solution pH was then adjusted to pH 8.5 with NaOH (4.0 M) and treated with DTT (1.5 g, 9.7 mmol) for 24 h. The reaction mixture was purified by dialyzed (MWCO 14000) against salt solution (pH 3.5, 100 mM NaCl) for 24 h and deionized water for

5 h. Col-SH was obtained as a white solid after lyophilization. The amount of free thiol groups in Col-SH was determined by Ellman's test. The yield was determined to be 90.5–93.3%.

2.3. In situ forming collagen-polycarbonate hybrid hydrogels and rheological properties

Col-SH and OAC-PEG-OAC were separately dissolved in phosphate-buffered saline (PBS, pH 7.4, 100 mM) at concentrations ranging from 1 to 5 w/v%. An equal volume (0.25 ml) of Col-SH and OAC-PEG-OAC solutions were mixed well for 30 min. The resulting cylindrical hydrogels were 2 mm in height and 10 mm in diameter.

Rheological analysis was performed using a RS6000 rheometer (Thermo-Fisher, Germany) with parallel plates (20 mm diameter) configuration at 37 °C in oscillatory mode. The evolutions of the storage modulus (G') and of the loss modulus (G'') were recorded as functions 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. The elastic modulus was determined when the gel was in the linear viscoelastic regime for both the frequency and strain sweeps.

2.4. Gel content

To evaluate the crosslinking density of hybrid hydrogels, the gel content was measured. For this, 0.50 ml of the hybrid hydrogel samples was lyophilized and weighed (W_d). The samples were extensively extracted with deionized water for over 2 days to remove any unreacted monomers until they showed a constant weight. The remaining hydrogels were then lyophilized and weighed (W_s). The gel content was expressed as $W_s/W_d \times 100\%$.

2.5. Swelling and enzymatic degradation of hydrogels

To determine the swelling ratio, hydrogel samples (0.50 ml) were prepared. The hydrogels were incubated with 3 ml of PBS at 37 °C. At regular time intervals, the medium was removed carefully and the hydrogels were gently blotted dry and weighed (W_s). After W_s had reached its equilibrium value ($W_{s,eq}$), the equilibrium mass swelling ratio was determined by the equation $(W_{s,eq} - W_d)/W_d$, where W_d is the initial solid weight of the sample. The medium was replaced once a day. The experiments were performed in triplicate.

For enzymatic degradation studies, hydrogel samples (0.50 ml) of different Col-SH concentrations were prepared as described above and accurately weighed (W_0). Then 3 ml of 0.1 M Tris–HCl buffer (pH 7.4, 5 mM $CaCl_2$) containing 10, 30 or 100 μg ml^{-1} of collagenase was added on the top of the hydrogels, followed by incubation at 37 °C. The solution was replaced every 24 h to maintain enzyme activity. The hydrogels were weighed at different time intervals, after the surface water had been gently blotted off. Three samples were tested for each hydrogel formulation. The percent residual mass of hydrogels was calculated according to the following equation: $W_t/W_0 \times 100\%$, where W_0 is the initial weight of the hydrogel and W_t is the weight of the hydrogel at each time point.

2.6. Cell adhesion and proliferation on hybrid hydrogels

Female Sprague–Dawley (SD) rat BMSCs, supplied by the Institute for Cardiovascular Science of Soochow University, China, were grown in SD rat mesenchymal stem cell growth medium (Cyagen Biosciences Inc.) supplemented with 10 v/v% fetal bovine serum, 1% glutamine and 1% penicillin–streptomycin. All cell culturing was performed at 37 °C in 5% humidified CO_2.

For cell adhesion studies, hybrid hydrogels formed from 4 w/v% Col-SH (DS 34.7) were cast in 24-well plates using 200 μl of solution per well. After gelation, hydrogels were sterilized with 75% ethanol, then washed several times with PBS and growth medium. BMSCs were then seeded on the top surface of the gels (cell seeding density: 1×10^5 cells ml^{-1}) and were cultured in complete medium for 2 days before being stained. The medium was removed and gels were washed twice in PBS prior to fixing in 4 wt.% paraformaldehyde for 15 min at room temperature. After washing three times with PBS, the gels were stained with fluorescein isothiocyanate (FITC)–phalloidin (100 μl, 10 μg ml^{-1}) and 4′,6-diamidino-2-phenylindole (DAPI, 100 μl, 0.2 μmol l^{-1}) to visualize F-actin filaments and cell nuclei, respectively.

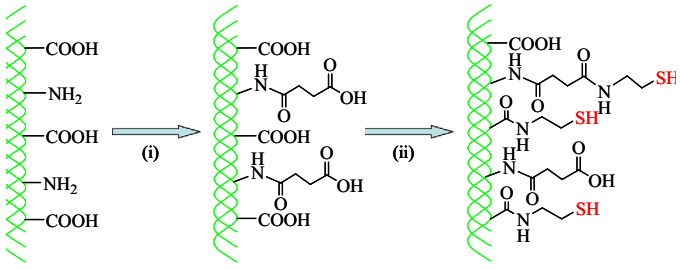
2.7. Rat myocardial infarct model and surgical procedure

A rat model of infarction and left ventricle (LV) remodeling was used to assess the therapeutic potential of the injected hybrid hydrogels and BMSC-laden hydrogel composites. Rats were handled under protocols approved by Soochow University Laboratory Animal Center. Female SD rats (150–200 g) were anesthetized with chloral hydrate (10 w/v%, i.p.). The animals were then incubated and ventilated by a volume-regulated respirator during surgery. The heart was exposed by a left thoracotomy and the proximal left anterior descending coronary artery was ligated. Successful ligation was confirmed by the typical MI waves (ST segment elevation and regional cyanosis) observed by limb-lead electrocardiography (Nihon Kohden, cardiofax ECG-6951E) [22,47].

Animals were randomly assigned to the following four groups ($n = 5$ per group): PBS, BMSCs, hybrid hydrogels and BMSC-encapsulating hybrid hydrogels. A 120 μl aliquot of PBS, BMSCs, hybrid hydrogels or BMSC-encapsulating hybrid hydrogels was injected 30 min after the onset of ischemia into four sites around the infarct with a 28-gauge insulin syringe (30 μl per injection) and the incision was closed. The number of cells injected was 3×10^6 cells per animal in the BMSC-containing groups. Animals were individually housed in cages (accessible to water and food) at a room temperature of 24 ± 2 °C (a normal day/night cycle).

2.8. Cardiac functional assessment and histological analysis

Cardiac performance was evaluated using echocardiography immediately before MI, and at 7 and 28 days after injection. Under the left lateral decubitus position, M-mode tracing of the LV was obtained close to the papillary muscle level using the short-axis imaging plane. The left-ventricular end-diastolic diameter (LVEDD), left-ventricular end-systolic diameter (LVESD) and heart rate (beats per minute) were measured. The LV ejection fraction (EF) was calculated as follows: $EF(\%) = ((EDD^3 - ESD^3)/EDD^3) \times 100$. All procedures and analyses were performed by an experienced researcher blinded to the treatment groups.



Scheme 1. Synthesis of Col-SH. Conditions: (i) succinic anhydride, 0 °C, pH 9, 4 h; and (ii) 2-mercaptoethylamine hydrochloride, EDC, NHS, N_2, 24 h.

Table 1
Synthesis of Col-SH.

Entry	Molar feed ratio of amino groups of MEA to COOH groups of Col-COOH	DS[a] (Ellman's test)	SH (μmol g^{-1}) (Ellman's test)	Yield (%)
1	0.3/1	14.1	205	90.5
2	0.5/1	20.5	297	91.9
3	2/1	34.7	503	93.3

[a] Degree of substitution, defined as the average number of free thiol groups per 100 carboxyl groups of Col-COOH, determined by Ellman's test.

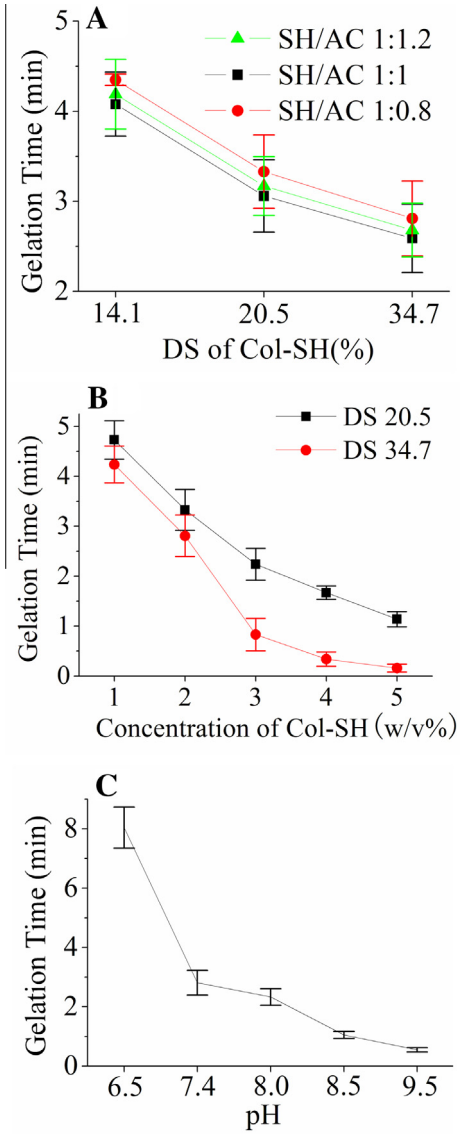


Fig. 1. Hybrid hydrogels formed in situ from Col-SH and OAC-PEG-OAC in PBS (100 mM) at 37 °C. (A) Gelation times of hybrid hydrogels as a function of DS at 2 w/v% Col-SH and pH 7.4; (B) gelation times of hybrid hydrogels as a function of Col-SH concentration at pH 7.4 and an SH/AC molar ratio of 1/1; and (C) gelation times of hybrid hydrogels as a function of pH at 2 w/v% Col-SH, DS 34.7 and an SH/AC molar ratio of 1/1. Data are presented as mean ± SD (n = 3).

Four weeks after treatment, the animals were euthanized and their hearts were explanted. Frozen sections 3 μm thick were cut from the apex to the level just below the ligation site MI zone of the rat hearts. The specimens used for light microscopy were fixed in 4% paraformaldehyde solution and embedded in paraffin and stained with Masson's trichrome (MT) for immunohistological analysis. MT staining was used to measure and calculate the thickness values of the peri-infarct (border zone) and infarct areas (the central portion) with a computer-based image analysis system (Image-Pro® Plus, Media Cybernetics, Silver Spring, MD, USA) at ×10 magnification and converted to millimeters. The infarct size was expressed as a percentage of the total LV circumference, and the scar thickness was quantified as the average of three equidistant measurements on each section [48,49].

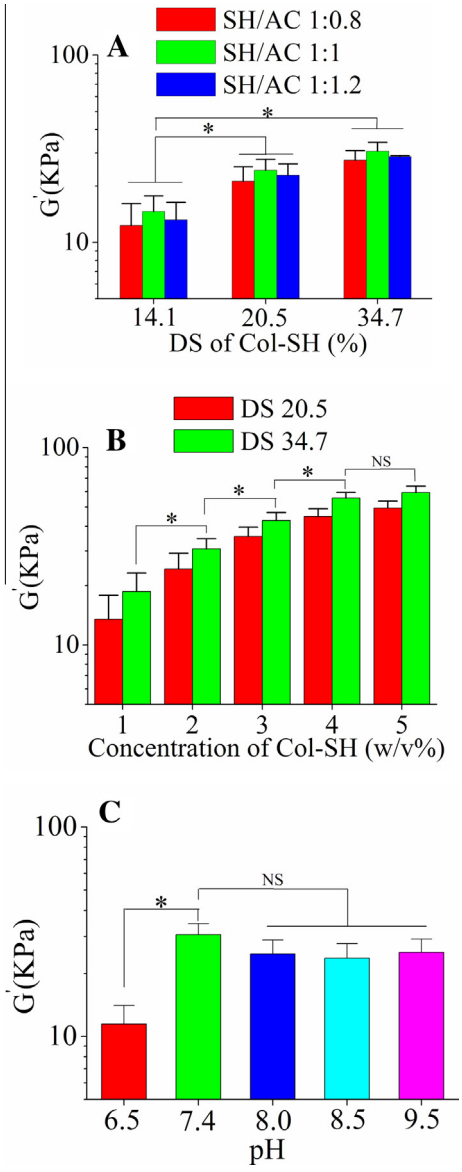


Fig. 2. Storage moduli of hybrid hydrogels in PBS (100 mM) at 37 °C determined by rheology: (A) as a function of DS at 2 w/v% Col-SH and pH 7.4; (B) as a function of Col-SH concentration at pH 7.4 and an SH/AC molar ratio of 1/1; (C) as a function of pH at 2 w/v% Col-SH, DS 34.7 and an SH/AC molar ratio of 1/1. Data are presented as mean ± SD (n = 3; $^*p < 0.05$). NS indicates no significant difference.

The arteriole density in the infarct and border zones was quantified by staining myocardial sections with α-smooth muscle actin (α-SMA) antibody (Boster). Images were taken from five randomly selected areas using inverted fluorescence microscopy (Nikon, ECLIPSE, Ti-S), and the vascular densities were counted manually within each section.

2.9. Statistical analysis

All data are presented as mean ± SD. For hydrogel characterization experiments, differences between groups were assessed using

Student's *t*-test. The effects of treatment (PBS, hybrid hydrogels, BMSCs, BMSC-encapsulating hydrogels) on cardiac function, infarct size and wall thickness, and vascular density were compared using a one-way analysis of variance. For all comparisons, $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Synthesis of Col-SH

Col-SH derivatives were prepared by reacting collagen with an excess of succinic anhydride to yield Col-COOH, followed by amidation with varying amounts of MEA in the presence of EDC and NHS coupling agents at pH 5.5 (Scheme 1). ^{13}C-NMR displayed signals at δ 29.68 owing to the methylene carbons of succinic acid (Fig. S1). The amount of remaining amine groups in Col-COOH was determined by a TNBSA assay in borate saline buffer (0.1 M, pH 9.13). The zero absorbance at 420 nm observed from the UV spectrum suggested that the DS of the carboxyl groups in Col-COOH was close to 100% (Fig. S2). Col-SH derivatives with different DSs were obtained by coupling Col-COOH with varying amounts of MEA at pH 5.5, followed by reductive cleavage of possible disulfide bonds with DTT at pH 8.5. The DS, defined as the average number of free thiol groups per 100 carboxyl group of Col-COOH, was determined by Ellman's test. The amount of thiol groups increased from 205 to 503 μmol g^{-1} Col-SH, corresponding to DS values of 14.1 to 34.7, with increasing molar feed ratios of amine groups of MEA to carboxyl groups of Col-COOH from 0.3/1 to 2/1 (Table 1).

3.2. Hydrogels formed in situ and their rheological properties

Hybrid hydrogels were formed in situ via Michael-type addition between Col-SH and OAC-PEG-OAC in PBS (pH 7.4, 100 mM) at 37 °C without any catalyst. The ^{1}H-NMR spectrum of the Col/OAC-PEG-OAC hybrid hydrogel showed resonance characteristic of collagen and OAC-PEG-OAC (δ 3.64, 4.11 and 1.06), but peak resonances at δ 5.81–6.48, assignable to the acryloyl protons of OAC, were absent (Fig. S3), indicating that the Michael-type addition reaction between Col-SH and OAC-PEG-OAC was almost completed. The rheology measurement showed that a mixture of Col-SH (DS 34.7) and OAC-PEG-OAC in PBS at 37 °C at a Col-SH concentration of 2 w/v% and an SH/AC molar ratio of 1/1 formed hydrogels rapidly (Fig. S4).

The influence of DS, polymer concentration, pH and SH/AC molar ratio on the gelation time were systematically investigated (Fig. 1A–C). The results showed that, at a low Col-SH concentration of 2 w/v%, using OAC-PEG-OAC as a crosslinker and at a fixed SH/AC molar ratio of 1/1, the gelation time decreased from 4.1 to 2.6 min with increasing DS from 14.1 to 34.7, as a result of increased crosslinking density (Fig. 1A). Notably, variation of the SH/AC molar ratio from 1/0.8 to 1/1.2 had little influence on the gelation time (Fig. 1A). The gelation time decreased dramatically from 4.2 min to 9 s for Col-SH with a DS of 34.7, and from 4.7 to 1.1 min for Col-SH with a DS of 20.5, when increasing Col-SH concentrations from 1.0 to 5.0 w/v% at pH 7.4 (Fig. 1B), which could be attributed to an increase in the number of functional groups per volume of gel precursor. The influence of the buffer solution pH was also investigated, since the Michael-type reaction is known to be base-catalyzed. As expected, the gelation time decreased from 8.0 min to 30 s with an increase in pH from 6.5 to 9.5 at 2 w/v% Col-SH (Fig. 1C). It is evident, therefore, that the gelation times of Col-SH/OAC-PEG-OAC hybrid hydrogels can be nicely controlled from about 20 s to several minutes by the DS of Col-SH, the polymer concentration and its pH. Hydrogels with fast gelation rate have the capacity to prevent blocking blood flow and subsequently tissue necrosis, thus are often desired for cardiac implantation [50,51].

The mechanical properties of Col-SH/OAC-PEG-OAC hybrid hydrogels were also studied as a function of DS, polymer concen-

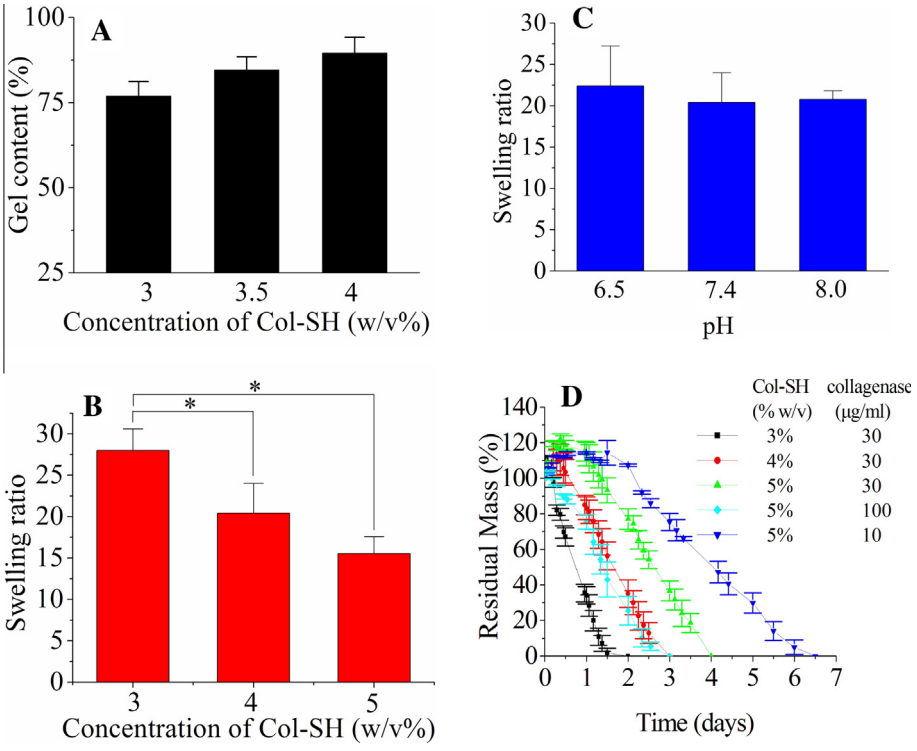


Fig. 3. Properties of hybrid hydrogels formed from Col-SH (DS 34.7%) and OAC-PEG-OAC solution in PBS at 37 °C with an SH/AC molar ratio of 1/1. (A) Gel content as a function of Col-SH concentration; (B) swelling ratios of hybrid hydrogels as a function of polymer concentration; (C) swelling ratios of hybrid hydrogels as a function of pH; (D) enzymatic degradation of hybrid hydrogels with type I collagenase (30 μg ml^{-1}) in Tris–HCl buffer (pH 7.4, 5 mM $CaCl_2$) at 37 °C.

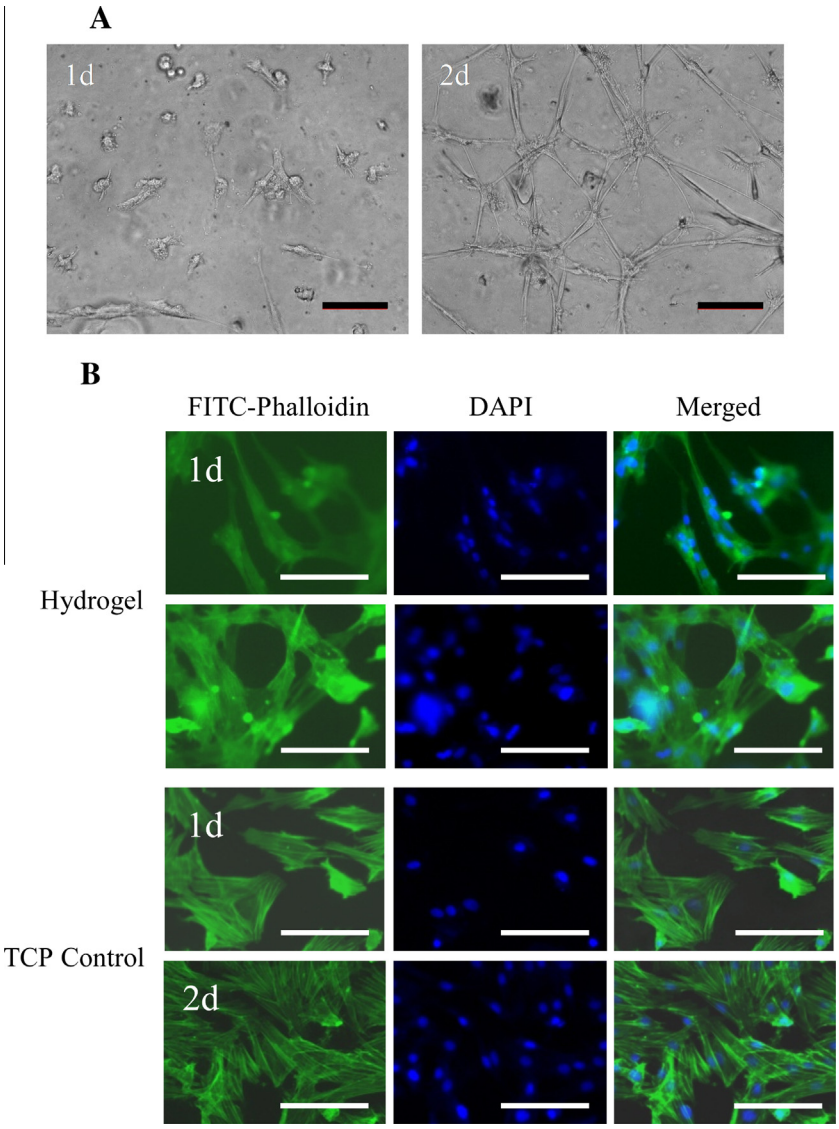


Fig. 4. Cell adhesion and growth on hybrid hydrogels. (A) Cell morphology of BMSCs on hybrid hydrogels after 1 and 2 days of culture; (B) cytoskeletal F-actin (FITC–phalloidin) and cell nuclei (DAPI) staining of BMSCs cultured on hybrid hydrogels for 1 and 2 days, respectively. The scale bar is 100 μm. Cell seeding density: 1×10^5 cells ml^{-1}.

tration, pH and SH/AC molar ratio. The results showed that, at a low Col-SH concentration of 2 w/v% and an SH/AC molar ratio of 1/1, the storage modulus (G') increased from 14.6 ± 3.1 to 30.7 ± 3.53 kPa with increasing DS from 14.1 to 34.7 due to increased crosslinking density (Fig. 2A). SH/AC molar ratios changing from 1/1.2 to 1/0.8 were observed to have little influence on the storage modulus (Fig. 2A). The storage moduli of hybrid hydrogels increased from 18.6 ± 4.5 to 55.9 ± 3.7 kPa with increasing Col-SH concentrations from 1.0 to 4.0 w/v% (Fig. 2B). However, further increasing the Col-SH concentration to 5.0 w/v% resulted in an insignificant increase in storage modulus. The storage modulus increased with increasing solution pH from 6.5 to 7.4 but did not change or even decreased when further increasing it to 9.5 (Fig. 2C), indicating that pH 7.4 is the optimal condition for the formation of hybrid hydrogels. The reduced storage modulus for hybrid hydrogels at higher pH (8.0–9.5) is most likely due to gelation that is too fast (<2.5 min), non-homogeneous and incomplete. Notably, the mechanical strength of these hybrid hydrogels is much higher than that of hydrogels based on collagen alone (generally lower than 100 Pa) [52,53], and is in the range of the rat and human myocardium (1–140 kPa for rat myocardium and

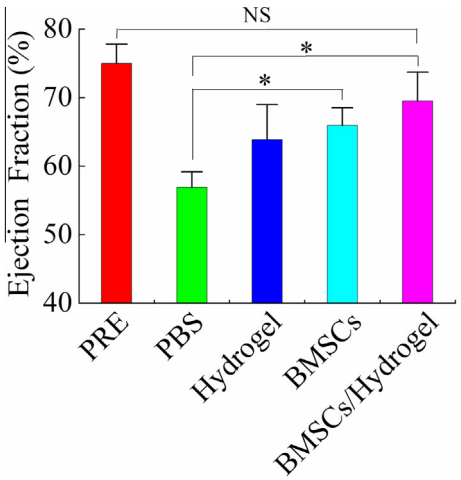


Fig. 5. Cardiac function (LVEF) examined by echocardiography at 28 days post-MI injection of BMSCs, hybrid hydrogels and BMSC-encapsulating hybrid hydrogels. Data are presented as mean ± SD ($n = 5$; $^*p < 0.05$). PRE indicates rats on the day of surgery prior to MI surgery. NS indicates no significant difference.

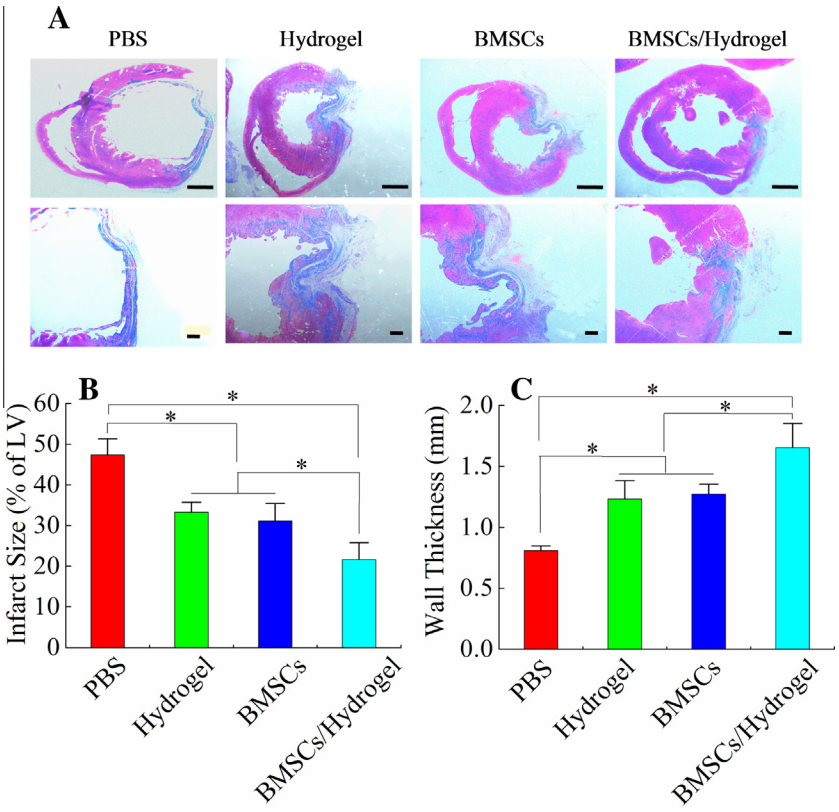


Fig. 6. Infarct size and infarct wall thickness. (A) Representative MT-stained sections of hearts treated with PBS, hybrid hydrogels, BMSCs and BMSC-encapsulating hydrogels. Scale bar = 2 mm. (B) Infarct size and (C) infarct wall thickness were statistically compared between different groups. Data are presented as mean ± SD ($n = 5$; $^{*}p < 0.05$).

20–500 kPa for human myocardium) [54]. Thus, hybrid hydrogels with a storage modulus matching that of the myocardium are promising for decreasing wall stress and attenuating myocardial remodeling [5,51].

3.3. Physical properties of hybrid hydrogels

With Col-SH concentrations increasing from 3 to 4 w/v%, the gel content of hybrid hydrogels increased from 77 to 89% (Fig. 3A), indicating that a higher concentration of Col-SH provided more functional groups and resulted in higher crosslinking density. The gel content was higher than 77%, suggesting that the Michael-type addition reaction between Col-SH and OAC-PEG-OAC was efficient. The results of swelling studies indicated that the swelling ratio decreased as the Col-SH concentration increased (Fig. 3B). For example, hybrid hydrogels formed from 3 w/v% Col-SH had a statistically higher swelling ratio (28.0 ± 2.6) than hydrogels formed from either 4 w/v% Col-SH (20.4 ± 3.6) or 5 w/v% Col-SH (15.5 ± 2.1). Hybrid hydrogels based on 4 w/v% Col-SH formed at different pHs had similar swelling ratios of 20, indicating that pH had little influence on the swelling ratio (Fig. 3C). It has been shown that hydrogels with swelling ratios higher than 15 possess adequate mesh sizes to facilitate nutrient and growth factor diffusion, promoting efficient differentiation of encapsulated marrow mesenchymal stem cells [55]. Enzymatic degradation of hybrid hydrogels was investigated in the presence of 30 μg ml^{-1} collagenase (Fig. 3D). The degradation time of hybrid hydrogels increased with increasing concentrations of Col-SH. For example, complete degradation was discerned on days 3 and 4 for hydrogels formed from 4 and 5 w/v% Col-SH, respectively. In addition, the degradation time for hydrogels formed from 5 w/v% Col-SH decreased from 6.5 to 2.5 days as the collagenase increased from 10 to 100 μg ml^{-1}. It is most likely that the degradation rate of hydrogels formed from 3 and 4 w/v% Col-SH against different collagenase concentrations would follow the same trend. In the following, hybrid hydrogels formed from 4 w/v% Col-SH (DS 34.7) with appropriate gelation time, swelling ratio and myocardium-matching mechanical properties were employed for further in vitro and in vivo experiments.

3.4. Cell adhesion and growth on hybrid hydrogels

Collagen is the most widely used tissue-derived natural polymer, which could be introduced into synthetic materials to support cell adhesion and promote proteolytic degradation [51]. BMSCs cultured on hybrid hydrogels were observed to elongate and adopt a spindle-shaped morphology after 1 day, and formed branched and interconnected multicellular networks by day 2 (Fig. 4A). Phalloidin staining of the cytoskeletal protein F-actin showed that BMSCs spread out and assembled stress fibers (Fig. 4B), which are critical to numerous physical cellular process, including cell adhesion, migration and division [56]. The excellent cell growth and network formation indicates that these hybrid hydrogels are promising scaffolds for cardiovascular tissue engineering.

3.5. Cardiac function of MI rat heart after treatment

Infarcted LVs were injected with PBS, hybrid hydrogels, BMSCs or BMSC-encapsulating hybrid hydrogels 30 min after infarction. Echocardiography was used to assess the cardiac function before and at 7 and 28 days after injection (Fig. 5). In the PBS group, the EF continued to decrease over time, indicating left ventricular dilatation and loss of contractility. In contrast, the EF for hearts treated with hybrid hydrogels, BMSCs and BMSC-encapsulating hybrid hydrogels was decreased at 7 days after implantation, but was then

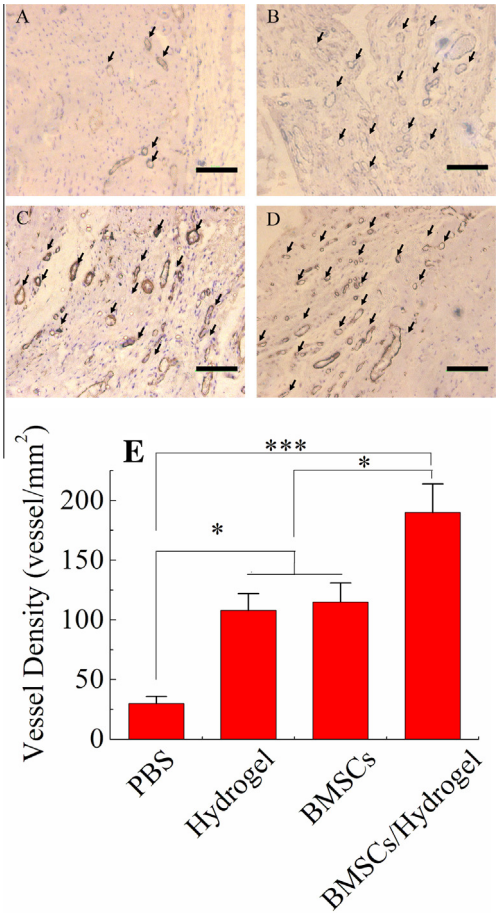


Fig. 7. Analysis of vascular density by α-SMA staining for arterioles. Representative immunohistological images of arterioles at 28 days post-MI injection of PBS (A), hybrid hydrogels (B), BMSCs (C) and BMSC-encapsulating hybrid hydrogels (D); (E) the vessel density in hearts at 28 days post-treatment, Scale bar = 100 μm. Data are presented as mean ± SD ($n = 5$; $^{*}p < 0.05$, $^{***}p < 0.001$). NS indicates no significant difference.

increased at 28 days, resulting in improved cardiac function (Fig. 5). For example, the EF for hybrid hydrogels, BMSCs and BMSC-encapsulating hybrid hydrogels at 28 days post-injection had increased to 63.86 ± 5.13, 65.91 ± 2.55 and 69.51 ± 4.20, respectively. Moreover, no significant difference was observed in the EF of the BMSC-encapsulating hybrid hydrogel group between day 28 and pre-treatment (prior to MI), indicating that BMSC-encapsulating hybrid hydrogels greatly preserved cardiac function.

3.6. Histology

A macroscopic study of the implanted hydrogels revealed that no residual hydrogel material was present in mice at day 28. MT staining shows collagen scar deposition (appears blue), while viable myocardium appears red (Fig. 6A). Computerized planimetry showed that the infarct size was significantly reduced by the injection of hybrid hydrogels (33.28 ± 2.49%), BMSCs (31.17 ± 4.30%) and BMSC-encapsulating hybrid hydrogels (21.61 ± 4.02%) compared to the injection of PBS (47.35 ± 4.04%) (Fig. 6B). The ventricular wall thickness in the central area of the MI was 810 ± 35, 1232 ± 140, 1272 ± 81 and 1655 ± 198 μm for rats treated with PBS, hybrid hydrogels, BMSCs and BMSC-encapsulating hybrid hydrogels, respectively (Fig. 6C). It is noteworthy that rats treated with BMSC-encapsulating hybrid hydrogels had the greatest wall thickness, possibly owing to enhanced engraftment and survival of BMSCs [57]. These findings suggest that hybrid hydrogels, both with and without stem cells, have the capacity to stabilize the infarct and prevent scar expansion and ventricular dilatation.

Collagen is a natural ECM protein that has been used for cardiac regeneration and has exhibited the ability to improve angiogenesis, resulting in increased cardiac function [13]. In this study, the capacity of hybrid hydrogels to improve angiogenesis in the post-MI heart was evaluated using α-SMA staining. The results showed that the densities of arterioles in the infarct and border zones in the hybrid hydrogel, BMSCs and BMSC-encapsulating hybrid hydrogel groups were significantly higher than that in the PBS group, with the BMSC-encapsulating hybrid hydrogel treatment group tending to have the highest arteriole density (Fig. 7). This finding suggests that transplantation of either hybrid hydrogels or BMSC-encapsulating hybrid hydrogels could promote neovascularization and concomitant cardiac functional benefits. It should be noted that this adaptable platform could be readily extended to the delivery of other bioactive molecules and cells of interest for heart repair [58,59].

4. Conclusions

We have demonstrated that injectable biodegradable hybrid hydrogels based on Col-SH and water-soluble OAC-PEG-OAC copolymers are an interesting platform for functional cardiac regeneration. The gelation time and mechanical properties of hybrid hydrogels can be nicely tuned by the DS of Col-SH, the solution pH and the polymer concentration. These injectable hybrid hydrogels, while rather stable under physiological conditions, are subject to enzymatic degradation by collagenase. Cell culture studies showed that BMSCs exhibit rapid cell spreading and extensive cellular network formation on the hybrid hydrogels. In a rat infarction model, echocardiography demonstrated that both hybrid hydrogel and BMSC-encapsulating hybrid hydrogel treatments could increase the ejection fraction at 28 days post-MI injection. Histological analysis demonstrated that the hybrid hydrogel-injected group had a significantly reduced infarct size and increased wall thickness, while there was a trend for greater infarct size reduction and the greatest wall thickness in the BMSC-encapsulating hydrogel group. Thus, injectable hybrid hydrogels, which combine the intrinsic bioactivity of collagen with controlled mechanical properties and enhanced stability, have tremendous potential for functional cardiac regeneration.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1–7 and Scheme 1, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2014.12.016.

Appendix B. Supplementary data

^{1}H NMR spectra of Col-SH, OAC-PEG-OAC and hybrid hydrogel, ^{13}C NMR spectra of collagen and Col-COOH, and UV spectra of the

complex of collagen and Col-COOH with TNBSA. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2014.12.016.

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