Electroactive nanoparticles loaded Silk protein/Chitosan macromolecular injectable hydrogel to improve therapeutic efficacy of mesenchymal stem cells in functional recovery after ischemic myocardial infarction

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Research Article

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Abstract

Background

Currently, cardiac regeneration by stem cell-based tissue engineering is considered an important strategy for overcoming myocardial infarction. Therefore, this study is designed to explore the potential for differentiation of gold nanoparticles loaded injectable Silk protein/Chitosan hydrogel along with mesenchymal stem cells towards a cardiomyogenic phenotype.

Methods

The incorporated gold nanoparticles into chitosan-silk fibroin hydrogel (Au@Ch-SF) was validated by various analysis including FT-IR, NMR, XRD and SEM analysis. The major properties of Au@Ch-SF hydrogel such as weight loss, mechanical test and drug releasing activities also investigated. Further, the mesenchymal stem cells (MS) were encapsulated into hydrogel by incubating the MS cells with 100 µg/mL of Au@Ch-SF hydrogel in a humidified incubator at 37°C for 3 days in the presence of 5% CO₂. In vitro toxicity effect of MS loaded Au@Ch-SF hydrogel was tested against cardiac myoblast H9C2 cells. Further, the tissue regenerative activities in myocardial infraction rats were examined by histology, apoptosis, and Cx43 cardiac-specific marker analysis.

Results

The gel formation time of Au@Ch-SF was comparatively lower than Ch and Ch-SF hydrogels which demonstrates the stronger intermolecular interactions between Ch and SF. The toxicity study showed that the prepared MS loaded Au@Ch-SF hydrogels did not possess toxicity against cardiac myoblast H9C2 cells. Further, the myocardial infarction rats were treated with MS loaded Au@Ch-SF hydrogel promotes the cardiac muscle fibers regeneration performance which was confirmed by β-MHC and Cx43 cardiac markers.

Conclusions

We demonstrate for the first time that encapsulation of MS with Au@Ch-SF hydrogels could promotes tissue regenerative activity in myocardial infraction tissues. The findings of this study suggest that MS encapsulated Au@Ch-SF hydrogels might be useful in the treatment of myocardial infarction.

1. Introduction

Cardiovascular diseases are the foremost causes of morbidity and mortality in the world [1]. Cardiac failure due to myocardial infarction (MI) is a serious issue accountable for almost 7.3 million mortality per year and has limited healing options worldwide [1, 2]. Although heart transplantation and the
ventricular assisted devices could act as a treatment option in the current scenario they have limited efficacy [3]. The failure in the survival rate of MI patients necessitates new therapeutic strategies such as regenerative medicine and tissue engineering approaches to rectify the injured cardiac tissues [4]. When blood flow stops to a part of cardiac tissue and subsequently the cardiac muscle gets injured due to lack of oxygen supply which is known as ischemic condition. Tissue regeneration therapies have gained great attention as a potential therapy for restoring heart functions [5, 6]. It is essential to prevent the condition but regenerative capacity of the heart muscle is very less when compared to other tissues [7]. The ultimate goal of cardiac tissue engineering is to create a constructive tissue microenvironment for cells in ischemic regions, in order to support the cells physically as well as direct them biochemically by similar to the native extracellular tissue [8, 9].

Modern research in regenerative medicine for cellular and tissue repair using mesenchymal stem cells (MS) have emerged as a promising strategy to treat various organ diseases due to their distinctive properties such as immuno-suppressive, self-renewal and ability to trans-differentiate [10]. The injectable hydrogel utilized as stem cells vehicle is considered as an alternative tool due to minimally invasive surgeries [11, 12]. The natural biopolymers Silk fibroin (SF) and Chitosan (Ch) have been used in tissue engineering as a drug carrier due to its excellent mechanical strength, biocompatibility and slow degradation [13–15]. In a dry state, SF is brittle in nature and unsuitable for practical uses. In order to overcome this limitation, silk fibroin has been reported to blend with other synthetic polymers. Ch has been used in many studies on the blends of chitosan with verities of polymers to obtain some improved properties. Hence the, blend of Ch-SF is interesting and prepared for this study. However, without electrical communications in infarcted myocardium, the success of the hydrogel-based stem cell therapy after MI is a challenging task [16].

In the recent years, incredible efforts have been made to construct electroactive injectable biomaterials for MI treatment using conductive carbon nanotubes [17, 18], metal particles [19], graphene [20], and conductive polymers [21]. Incorporation of gold nanoparticles into tissue scaffolds was already proved to provide electrical conductivity, mechanical stability and biocompatibility. In this way, nano-scaffolds combining Ch-SF and gold take advantages of both materials for biological applications [22]. The present study, an attempt has been made to construct an injectable electroactive gold loaded Ch-SF hydrogel scaffolds along with mesenchymal stem cells for the functional recovery behind ischemic myocardial infarction.

2. Materials And Methods

2.1. Chemical and reagents

Chitosan (95% deacetylation degree) was obtained from Zhejiang Aoxing Biotechnology Co., Ltd. (Kanmen, Zhejiang, China). Silkworm cocoons (Bombyx mori) were attained from Institute of Biotechnology of Southwest University, Chongqing, China. Hydrogen tetrachloroaurate (III) (HAuCl$_4$.3H$_2$O)
was purchased from AppliChem GmbH, Darmstadt, Germany. TUNEL-DAPI kit was purchased from Beyotime Institute of Biotechnology, Shanghai, China.

2.2. Preparation of SF solution

SF solutions were prepared by the method of Raia et al.[23]. Briefly, cocoons were degummed in boiling 0.02 M sodium carbonate solution for 30 min and washed with distilled water (DW) to remove wax and sericin. The procedure was repeated once and the degummed SF was dried and then dissolved in 9.3 M lithium bromide at 60 °C for 2 h. Then the solution was dialyzed against DW for 3 days. Every 6 h, the water was changed to remove solvent and salts. Then, the solution was centrifuged to take away the insoluble particulates and concentrated to dryness by freeze drying with a Dry winner DW8 Freeze-Dryer (Heto, Denmark) at −40 °C overnight.

2.3. Preparation of gold nanoparticles loaded injectable chitosan/silk fibroin (Ch-SF) hydrogels

The chitosan was dissolved in 1% of acetic acid and mixed with SF solution at 1:1 ratio (v/v). Simultaneously, β-glycerophosphate solution (50 wt.%) was prepared using DW. The prepared β-glycerophosphate solution (BGP) was added into pre-chilled Ch-SF mixture drop by drop with a 1:1 ratio (v/v) under stirring in an ice-cold water bath for 20 min. The reaction mixture was stored at 4 °C. The prepared Ch-SF was treated with 10 mM HAuCl₄·3H₂O solution followed by 0.4 ml of 100 mM NaBH₄ (reducing agent) under stirring condition. The colour variation (Rapid color changed into yellow to wine-red) was observed and the stirring continued for another 2 h to get Au loaded Ch-SF hydrogels. The functional groups of hydrogels were characterized by FT-IR analysis using KBr pellet method with a Nexus 670 FT-IR spectrometer (Nicolet, USA) in the 500-4000 cm⁻¹ spectral region at a resolution of 8 cm⁻¹. Also, ¹H NMR spectra were recorded on a Bruker AV400 MHz spectrometer.

2.4. Gelation property analysis

The gel formation temperature measurements of the injectable thermo gelling Ch, Ch-SF and Au@Ch-SF hydrogels were determined from rheological analysis of the viscoelastic properties using a Bohlin Gemini 200HR Nano Rotational Rheometer (Malvern, UK). The gelation temperature of the hydrogels was determined by the procedure described earlier [15]. The gelation time of the Ch, Ch-SF and Au@Ch-SF hydrogels was investigated by a vial inverting method [24] in water bath at 37 °C by observing the fluidity every 1 min.

2.5 Stability and morphology analysis

The UV-Vis spectra (UV-1800, Shimadzu) was used to confirm the gold nanoparticle (530 nm) and evaluate photo stability of samples up to 30 days. The crystallinity and gel structure was characterized by Powder X-ray diffraction (XRD) analysis using Bruker D8 Advance diffractometer (Karlsruhe, Germany) with Cu K(α) radiation (λ = 1.54060 Å) at an accelerating voltage of 40 kV and a current of 40 mA. The
spectra were collected from 2θ in the range of 20°–80°. The morphological analysis was carried out by Scanning Electron Microscope (Carl Zeiss, Germany).

2.6 Weight loss measurement

The weight loss performance of Ch-SF and Au@Ch-SF hydrogels were analyzed by two types of method. Thermogravimetric analysis (TGA) was performed by using TA Instruments SDT-Q600 equipment. 1 mL of each thermo gelling hydrogel was formed and were pre-weighed (Wi) about 10–15 mg. Each hydrogel was loaded onto an alumina pan and heated in a nitrogen atmosphere from 25 to 800 °C at a heating rate of 5 °C/min. Each thermal point was monitored for decomposition of hydrogels and weighed (Wt). The physiological conditions were analyzed in phosphate buffer solution (PBS) using immersion method for weight loss measurements at 37 °C for 15th day and 30th day. At predetermined time intervals, each hydrogel was collected and weighed. The weight loss was calculated by the following equation.

\[ \text{Weight loss (W\%)} = \frac{W_i - W_t}{W_i} \times 100 \]

2.7 Mechanical tests

The mechanical properties of hydrogels were evaluated by stretch test with Lloyd LS500 Material Testing Machine (Lloyd, England). About, 30 mm × 5 mm (length × width) size of samples were cut from scaffolds and the results were obtained as load versus deflection which was then transformed into stress-strain data. Tensile strength (TS), modulus of elasticity (E) and percent elongation at break (EAB) were calculated from the stress-strain curves. Mechanical properties of the hydrogels were determined by Instron 5969 testing frame (Instron Instruments, USA) [25].

2.8 In vitro drug releasing activity

The release of Au from Au@Ch-SF hydrogel was determined by immersing method using PBS at different pH condition (5.7 and 7.4). Over certain time intervals, the amount of Au released was examined by reading the absorbance at 485 nm using UV-visible spectrophotometer. The Au release from Au@Ch-SF hydrogel was compared with the quantity of free Au released standard calibration curve. The experiment was carried out in triplicate and the average value was consider applying the following equation.

\[ \% \text{ of Au release} = \left[ \frac{\text{Concentration} \times \text{dissolution bath volume} \times \text{dilution factor}}{1000} \right] \]

2.9 Static contact angle and electroactivity test

Static contact angle (SCA) measurements were performed using manual goniometer (Kernco Instruments Co. Inc.) In this study, deionized water was used as a test liquid. The electroactivity of the hydrogels Ch-
SF and Au@Ch-SF was evaluated by Cyclic voltammetry (CV) using an electrochemical workstation (CHI660D, China) through a conventional three-electrode system [16].

2.10 Cell culture and maintenance

The rat myocardial cells (H9c2) were purchased from ATCC, USA. The cells were cultured using Dulbecco’s modified Eagle’s medium (DMEM) supplement with 15% fetal bovine serum (FBS) under humidified (37 °C, 5% CO₂) condition. Subsequently, the human bone marrow derived mesenchymal stem cells were cultured in α-MEM supplemented with 20% FBS and specific antibiotics at 37 °C in the presence of 5% of CO₂.

2.11 Encapsulation of mesenchymal stem cells into hydrogel

Mesenchymal stem cells (1×10⁵ cells) were collected in culture media and mixed with 10× PBS and 100 µg/mL of Au@Ch-SF hydrogel liquid in a ratio of 1:1:2 and then incubated for 37 °C for the gelation. The culture was maintained in DMEM containing 20% FBS for 3 days.

2.12 Preliminary analysis of cell viability in presence of Au@Ch-SF

The cytotoxic effect of Au@Ch-SF on MS cells were examined by MTT assay. The experiment was performed with three groups namely, MS cells with medium (Negative control), MS cells treated with 30% DMSO (Positive control) and MS cell treated with supernatant of Au@Ch-SF hydrogel (100 µl). The control and a treated group of cells were incubated at 37 °C with 95% of humidity and 5% of CO₂ for 24 h. A freshly prepared MTT solution (20 µl) was added into the cells and continue the incubation period for 4 h. The formation of crystals was dissolved by the addition of DMSO and the absorbance was read at 570 nm.

2.13 Cell viability assay

MTT assay was perform to determine the cell viability. The fabricated hydrogels were sterilized with UV light and ethanol and they had been immersed in PBS for 24 hrs. Briefly, H9c2 cells (8 × 10³ cells/well) were seeded in 96-well plates for 24 h. After, the growth medium was replaced with fresh medium and Ch, Ch-SF, Au@Ch-SF, MS and MS along with Au@Ch-SF hydrogel polymer was added into the cell plates. The different time intervals (1st, 5th and 15th day), relative cell proliferation was measured by addition of 20 µl of MTT. The formation of crystals was dissolving by the addition of dimethyl sulfoxide (DMSO). The absorbance was read at 570 nm. Independently, H9c2 cells treated with DMSO and MS served as a control for this experiment. The results are presented as mean values ± SD calculated from at least three samples per condition.

In order to find the differentiation of bone marrow-derived mesenchymal stem cells into cardiomyocytes along with Au@Ch-SF hydrogel transplantation to H9c2 cells, DAPI (4′,6-diamidino-2-phenylindole) staining and immunofluorescence staining of β-MHC which is known to expressed in cardiac hypertrophy
and heart failure. The dishes with H9c2 cells were re-suspended the culture medium were washed with PBS, fixed by 4% formaldehyde, and permeabilized with 0.1% Triton X-100. The plates were blocked with blocking buffer containing 5% non-fat dry milk for an hour. The H9c2 cells were then incubated with PBS containing primary antibody NOQ7.5.4D mouse anti-β-MHC (Beta-myosin heavy chain) conjugated to Alexa 647 (red) and incubated at 4 °C dark for overnight. Then, they were washed with PBS at 37 °C for 1 hour and the fluorescence images were taken by Confocal Laser Scanning Microscopy (CLSM).

2.14 MI establishment and transplantation of injectable hydrogel

Adult male Sprague-Dawley (SD) rats (250 ± 20 g) were randomly divided into the five groups namely control group, saline administrated group, MS cells treated group, MS cells loaded with Ch-SF hydrogels and MS cells loaded with Au@Ch-SF hydrogels group. The animal studies were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of “Capital Medical University” and all experiments were conducted with the approval of the Animal Ethical Committee at the Capital Medical University, China. All the experiments were carried out by triplicates. Rats were sedated with minimum amount of diethyl ether and the left lateral thoracotomy and pericardiectomy was tenderly ligated with a 6 prolene suture for few minutes. The infarction in heart was observed as a pale region. Then, 200 µL of PBS or hydrogels was injected with a 28-gauge needle along peri-infarct zone at the targeted tissue. After injection is over, the muscle and the skin were closed with double 10 prolene suture and the treated rats received penicillin anti-microbial therapy intramuscular twice daily up to 3 days.

2.15 Histology evaluation

All the groups of rats were anesthetized and sacrificed at four weeks after surgery and the hearts were carefully harvested and fixed with 4% paraformaldehyde at 4 °C and processed and stained using Masson's-trichrome staining as described earlier [15]. Microscopic images from trichrome stain were also used to estimate the fibrosis area and infarct size with ImageJ software.

2.16 TUNEL-DAPI staining

Apoptosis in MI induced cardiac tissues were analyzed by TUNEL-DAPI staining. Briefly, treated and processed cardiac tissue sections were incubated with TUNEL solution for 1 h at 37 °C in darkness. The cells were stained with DAPI for 10 min and washed with PBS thrice. The apoptotic cells were detected by CLSM.

2.17 Immunofluorescent staining of cardiac marker Cx-43

Connexin – 43 (Cx-43) immunostaining was evaluated as cardiac specific-marker. About, 4 mm frozen sections were dewaxed in series of xylene and rehydrated using anhydrous ethanol and DW. Tissue sections were treated with EDTA (Ethylenediaminetetraacetic acid) antigen repair buffer, washed with PBS, dried and blocked with 3% bovine serum albumin (BSA) for 30 min at room temperature. The primary antibody anti-Connexin 43 Monoclonal (CX-1B1) was applied to the section and incubated at 4 °C in a wet box overnight. Then, the sample was washed with PBS and treated with secondary antibody at
room temperature for 60 min. The sections were stained using DAPI by dropwise for 10 min and the film was sealed with anti-fluorescent. The images were observed under a CLSM. The fixed and processed sections were incubated with TUNEL staining as described earlier to find the apoptotic area.

2.18 Evaluation of biocompatibility

In this study, the biocompatibility of the prepared different hydrogels was tested on rats using hematological parameters, liver enzymes and histomorphological analysis. The treatment group of rats were individually treated by 200 µL of Ch-SF, MS cells loaded with Ch-SF hydrogels and MS cells loaded with Au@Ch-SF hydrogels, respectively. At the same time, the control group received an equal amount of PBS (200 µL). Over the 20 days of treatment, the changes in body weight were recorded. The blood was collected from rats for haematological parameters, liver enzymes analysis such as haemoglobin (HGB), red blood cells (RBC), aspartate aminotransferase (AST), and alanine transaminase (ALT). The analysis was carried out by following the instruction provided in the commercial kits (Beijing Leadman Biochemistry Co., Ltd. (Beijing, China). Finally, the rats were dissected out and the organs including heart, liver, spleen, lung and kidney were subjected to histomorphological analysis.

2.19 Statistical analysis.

All experimental data was presented as means ± SD. The statistical significance differences between the groups were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett multiple-comparisons as a post hoc test. The probability value less than 0.05 (P<0.05) considered as a statistically significant.

3. Results And Discussion

3.1. Characterization of Ch-SF hydrogel

In order to overcome the long-term cytotoxicity of metal nanoparticles in tissue engineering applications, hydrogels were incorporated with metal nanoparticles which are efficient with electrical and biological characteristics of biological tissues that require the conductivity [26,27]. In recent years, the blends of natural polymers have been attractive considerably significant due to their potential in replacing synthetic polymers in various applications in addition of being renewable resources, non-toxic, inexpensive and leave biodegradable waste [28,29]. Among natural polymers, chitosan and its blends have received special interest due to its versatility and suitability for a numerous number of applications. Further, the chitosan properties have been improved by blending with synthetic and naturally occurring macromolecules [30–34]. In this aspect, the hydrogel polymer was prepared by gold loaded with chitosan-silk fibroin complex which are well known as the conductive hydrogel.

FT-IR spectroscopy was used to find out the functional groups present in Chitosan (Ch) and Chitosan/Silk fibroin (Ch-SF) hydrogel (Figure 1). The IR spectrum of the Ch displayed main absorption peaks at 3481, 2930, 1745, 1702, 1212 and 693 cm⁻¹ respectively. Whereas, the IR spectrum of Ch-SF had shown major
absorption peaks at 3468, 3042, 1842, 1740, 1496 and 1164 cm\(^{-1}\) respectively. The observed broad absorption peaks at 3400 to 3500 cm\(^{-1}\) was due to the stretching vibration of \(-\text{NH}_2\) and \(-\text{OH}\) groups. The band at 3042 cm\(^{-1}\) found out the vibrational stretches of methylene present in the Ch. The band at 1700 to 1900 cm\(^{-1}\) was due to the absorption of the peptide backbones of N–H bending vibration of amides I, II, and III, respectively present in Ch and Ch-SF hydrogels [35]. The peak differences in the 1900 to 1200 cm\(^{-1}\) have confirmed the differentiation pattern of two hydrogels. The overlapped absorption bands, intensity and differences indicated the intermolecular H bond interaction and conjugation of Ch with SF [36]. Further, the structures of the hydrogel Ch and Ch-SF conjugates were confirmed by \(^1\text{H} \text{NMR}\) (Figure 1). The spectrum of Ch-SF exhibited new signals at \(\delta 4.14, \delta 4.21\) and \(\delta 3.31\) when compared with Ch spectrum which indicated the existence of the conjugated protons of Ch-SF.

3.2 Development of Gold loaded Ch-SF hydrogel

Gold nanoparticle formation was evidently observed with the pink-red color formation in the reaction mixture containing HAuCl\(_4\)∙3H\(_2\text{O}\) with the Ch-SF. UV-Vis spectral analysis has confirmed the presence of AuNPs by the absorbance peak at 530 nm. The comparative analysis of newly prepared Au@Ch-SF hydrogel and the same after 30 days were depicted in Figure 2. Both the spectra were found to be almost same which confirms the stability of the synthesized hydrogel. Varieties of metal nanoparticles have been used in the preparation of nano-scaffold hydrogels in the field of biomaterials including gold [37] and silver [38]. Since metal nanoparticles possess the desired electrical conductivity, magnetic properties, and antibacterial properties. The nano-scaffold hydrogels along with metal nanoparticles are widely used in conductive scaffolds [39–41].

3.3 Mechanical properties of Au@Ch-SF hydrogel

Preparation of injectable hydrogel is a challenging task due to immediate gelation properties of hydrogels. The slow gelation rate leads to the formation intermediate reactions before the gelling which is the desired property [42]. The gelation property of Ch, Ch-SF and Au@Ch-SF has shown in Figure 2. The gelation time of Au@Ch-SF was comparatively less than the time taken for Ch and Ch-SF hydrogels. The gelation time of Au@Ch-SF hydrogel was 4 ± 0.33 min and the time of Ch and Ch-SF hydrogels were 9 ± 0.33 and 7 ± 0.33 min, respectively. This gelation time of gold incorporated hydrogel Au@Ch-SF shows the stronger intermolecular interactions between Ch and SF hydrogels. Recent study also documented that Chitosan conjugated SF hydrogels forms gelling property approximately 4 min [15]. Moreover, temperature played a significant role in gelation property. At initial testing temperature (4 °C) 4 min, the gelation was not observed. Beyond 24 °C, the viscosity was increased and temperature increment favored to the gelation after 28 °C. The clear gel was formed at 37 °C and 4 min (Figure 2). The rheological measurement was conducted by a method of dynamic viscoelastic as a function of temperature. The temperature dependence of the elastic (storage) modulus (\(G'\)) and viscous (loss) modulus (\(G''\)) were recorded between 20 - 50 °C and the results were displayed in Fig. 2. The \(G'\) represents the measure of the deformation of stored energy during a shear process or elastic response of materials. \(G''\) denoted the measure of the dissipated energy during the shear process as heat or the viscous response of the
While the G\(_e\) was lower than G\(_i\) at the initiating stage of gelation, it was a viscous liquid. Meanwhile, G\(_e\) was higher than G\(_i\), it’s become an elastic solid [43]. Our study results showed that the elastic modulus (G\(_e\)) value of the Ch was 60 Pa at 50 °C while G\(_e\) value of the Ch-SF and Au@Ch-SF were 20 and 12.5 Pa at 50 °C, respectively. Hence, the Ch hydrogels had a stronger elastic gel than the Ch-SF and Au@Ch-SF hydrogels. This incidence due to the electrostatic interactions between the hydroxyl/amine groups of SF and Ch molecules resulting in the charge density of the Ch molecules was decreased. Additionally, the incorporation of SF into the hydrogels might be decrease interaction between the ammonium group of Ch and phosphate group of BGP. Therefore, the addition of SF into the Ch hydrogels did not promote their mechanical properties. Moreover, the critical gel transition temperature (T\(_{gel}\)) was tested. The T\(_{gel}\) of ch hydrogels was 40 °C while T\(_{gel}\) of the Ch-SF and Au@Ch-SF hydrogels were 36 and 32-34 °C, respectively. These results confirmed that the incorporation of SF enhances the gelation through increasing entanglements and intermolecular interactions. The mechanical properties of Ch, Ch-SF and Au@Ch-SF hydrogel such as compression force, compression strength, tensile force, tensile strength and elongation was reported in Table 1. These mechanical properties possessed sufficient strength to retain its structure and shape during gel formation.

3.4 Structural analysis

XRD analysis of Ch-SF and Au@Ch-SF hydrogels demonstrated that the diffractions from (111), (200), (220) and (311) were due to the formation of nanocrystalline gold. As shown in Figure 3A, the absence of peaks in Ch-SF and the presence of peaks in Au@Ch-SF confirmed the incorporation of gold in Ch-SF hydrogel. FT-IR analysis was carried out to investigate the changes of functional groups in the Au@Ch-SF. Figure 3B shows the FT-IR spectrum of Ch-SF and Au@Ch-SF hydrogels. The Au@Ch-SF shows the bands at 3461, 2947, 1612, 1560, 1309, 1047 and 645 cm\(^{-1}\) respectively. On the contrary, Ch-SF showed absorption band peaks at 3367, 2961, 1621, 1321, 1284, 1257, 1128, 1049 and 649 cm\(^{-1}\) respectively. The differences in peaks in the region 1300 to 1100 cm\(^{-1}\) was due to the formation of intermolecular hydrogen bond interaction cross links which confirmed the gelling property.

3.5 Morphology of Au@Ch-SF hydrogel

The micro morphologies of Ch, SF, Ch-SF and Au@Ch-SF hydrogels were analyzed using scanning electron microscope (Figure 4). The cross-sectional hydrogels were prepared and observed which has a pore sizes ranging from 50–300 \(\mu\)m with inter connected structures. In the previous studies, it has been shown that silk fibroin hydrogel with 90–250 \(\mu\)m pore sizes had provided the better atmosphere for adhesion and proliferation of chondrocytes [44]. In addition, the porous structure all hydrogels Ch, SF, Ch-SF and Au@Ch-SF could be promote the passage of water or biomolecules into the hydrogel which is helpful for any drug molecules to diffuse through hydrogels [45]. The EDAX spectrum of Au@Ch-SF hydrogel had illustrated the characteristic Au peaks (Figure 4). The elements Au, C, N and O were documented with different composition in Ch-SF and Au@Ch-SF hydrogels.

3.6 Weight loss of hydrogel
The conformational changes of Ch-SF and Au@Ch-SF hydrogels were analyzed by TGA thermograms (Figure 5A). TGA was carried out to find the function of percentage weight loss against temperature. 10% of decomposition was observed in the temperature range from 175 to 185 °C and after increasing temperatures favored decomposition of both samples. 50% weight loss was observed for Ch-SF at 238 °C and Au@Ch-SF at 392 °C. The initial decomposition temperature was found to be higher for Au@Ch-SF (232°C) than Ch-SF (181 °C) hydrogel. It was reported that, the decomposition initiation was due to the dehydration and loss of volatile molecules and further stage decomposition was due to depolymerization reactions [46]. The final decomposition temperature of Au@Ch-SF at 505 °C showed better thermal stability of the crosslinked hydrogel.

The weight loss of hydrogel was evaluated in PBS medium (pH 7.4) up to 30 days (Figure 6A). The results showed that the weight loss of all hydrogels favored with increasing submersion period. Comparatively Au@Ch-SF is stable than Ch-SF hydrogel and the submersion analysis confirmed that more than 65% of hydrogel was stable at 30 days.

3.7 Mechanical properties

The elasticity of the nanocomposite hydrogel has been recognized as a dominating factor of cell fate in TE [47]. The compression test was performed on fully-formed hydrogels to verify the elastic properties of the Ch-SF and Au@Ch-SF hydrogels. The stress-strain curve results showed that incorporation of gold nanoparticle into the Ch-SF hydrogel improved compressive modulus from 14.2 MPa to 26.3 MPa (Fig. 5B). Au@Ch-SF hydrogel could withstand compressive forces without breakage than the Ch-SF hydrogel.

3.8 SCA measurements

SCA were used to determine the surface hydrophilicity of Ch-SF and Au@Ch-SF hydrogels. The contact angles of of Ch-SF and Au@Ch-SF hydrogels are reported in Figure 5D. The contact angle value of 61.8 ± 0.8° was observed for Ch hydrogel and the Ch-SF and Au@Ch-SF hydrogels were found to be 87.8 ± 0.8° and 58.8 ± 0.8°. A recent study shows that the electro-synthesized hydrogel resulted in the contact angle value of 67 ± 4° [48].

3.9 In vitro drug release studies

The percentage of Au release from Au@Ch-SF hydrogels was plotted against time. As shown in Fig. the hydrogel exhibited a pH-responsive releasing behavior which displayed 78.76% Au release after 24 h at pH 5.7. In contrast, the lower level of Au release (33.6%) was recorded at pH 7.4 it may be due to the greater hydrolytic stability of Schiff base linkages. Moreover, the higher Au releasing activity at pH 5.7 attributed to the greater swelling capacity of Au@Ch-SF hydrogels at lower pH.

3.10 Electrical conductivity property

The conductivity property of the Ch-SF and Au@Ch-SF hydrogels were measured by Cyclic Voltammetry (CV) measurement. The closed CV curves exhibited the capacitive capacity of tested hydrogels and
shown in Figure 5C. The capacitive capacity of Ch-SF hydrogel is relatively weak due to the smallest enclosed area in CV curve and the Au@Ch-SF hydrogel showed enlarged circles which confirm the stronger capacitance. As shown in Figure 6B, Au@Ch-SF hydrogel displayed a conductivity of 0.164 S/m which is higher than the hydrogel without gold nanoparticles. Recently Baei et al. [49] synthesized a thermosensitive conductive hydrogel by combining AuNPs and chitosan with the conductivity of 0.13 S/m which is reported as closer to native myocardium. This conductivity of 0.164 S/m from Au@Ch-SF hydrogel could support the metabolism, viability, migration, and proliferation of myocardial cells.

3.11 *In vitro* studies

3.11.1 Cytotoxicity analysis

*In vitro* biocompatibility of implant material is one of the key factors in biological systems to avoid toxic effects. An increasing body of evidence has proved that the incorporation of Ch-SF hydrogels might enhance the biocompatibility of the Au nanoparticle-based hydrogels to cells by promoting cell attachment and proliferation. The biocompatibility of Au@Ch-SF on MS cells were presented in Fig. S1 (supplementary data). MS cells treated with the supernatant of Au@Ch-SF showed 101.4±2% cell viability over the positive control (3.7±1%) which indicates high biocompatibility of Au@Ch-SF hydrogel. Also, there are no significant differences were found between MS cells cultured using normal media and media containing Au@Ch-SF hydrogel supernatant. Further, the cytotoxicity assay was carried out by H9c2 cells. The cells were treated with Ch, Ch-SF, Au@Ch-SF and mesenchymal stem cells along with Au@Ch-SF hydrogel polymer coatings. On day one, the viability of H9c2 cells cultured with all the above hydrogels ranged between ~52 and ~64% (Figure 7A) and the viability were increased on 15th day ranged between ~72 and ~93%. This activity was higher than treatment of MS alone that showed the activity ~83% at 15 days. The cytotoxicity results demonstrated that the hydrogels loaded gold nanoparticles and mesenchymal stem cells exhibited no toxicity against H9c2 cells. These results confirmed that these hydrogels had the potential for use in biomedical applications.

3.11.2 Immunofluorescent staining method

Immunofluorescent staining revealed that H9c2 cells induced by mesenchymal stem cells along with Au@Ch-SF hydrogel was strongly positive for beta-myosin heavy chain (Figure 7ii). DAPI stain showed nuclear region and the red stained β-MHC expression confirmed the cardiac differentiation of mesenchymal stem cells. The H9c2 cells were incubated with anti-β-MHC conjugated to Alexa 647 resulting the cells treated with MS cells + Au@Ch-SF hydrogel showed more red-stained β-MHC expression which confirm the cardiac differentiation of mesenchymal stem cells. These findings were well matched with the previous studies by Shi et al., [50].

3.12 *In vivo* studies

3.12.1 Evaluation of apoptosis by TUNEL-DAPI staining
The histochemical analysis was performed with Masson's-trichrome staining to view nuclear, collagenous and cytoplasmic region of infarcted and treated heart sections. Figure 8 shows the micrographs of the infarcted area of blue collagen which showed a severe infarcted myocardium. The MI hearts treated with MS cells showed little improved heart tissue which showed myofibers with central nuclei indicated that the cardiac muscle fibers are regenerating. Similarly, the treatment of MS cells loaded Ch-SF showed the cardiac muscle fibers regeneration characteristics. However, the MS cells + Au@Ch-SF hydrogel treated sections showed much improves fibers and the collagens were stained less than the other treated group. These findings were confirmed the regenerated tissues. The quantitative image data in Figure 8 showed 26 ± 3% fibrosis area of infarcted tissue. The infarcted tissue treated with MS cells + Ch-SF hydrogel showed 7 ± 2% fibrosis. However, the lowest fibrosis area was recorded in MS cells + Au@Ch-SF hydrogel (9.3 ± 3.5%) which is the protective effect of electroactive nanoparticles loaded Ch-SF hydrogel along with MS cells. In order to find the apoptotic cells in the hearts after MI, TUNEL-DAPI co-staining was used to evaluated the tissue sections. The infarcted tissues showed significant apoptosis as noted by increased TUNEL-DAPI positive cells. However, treatment with MS cells + Au@Ch-SF hydrogel effectively prevented induced apoptosis than the cells treated with MS cells alone and the combination of MS cell and Ch-SF (Figure 8). The quantitative image data showed 41 ± 3% of apoptotic cells in the saline-treated infarcted tissues and the same was improved upon treatment with MS cells (37.6 ± 3%) and MS+Ch-SF (33.3 ± 2%). However, the superior activity was noted in MS cells+ Au@Ch-SF hydrogel treated tissue which showed 23 ± 2 % of apoptotic cells.

3.12.2 Improvement and restoration of myocardial damage by β-MHC and Cx43

To evaluate the process of cardiomyogenesis, immunohistochemical staining of Cx43 was carried out which served as cardiac-specific marker. As shown in Fig. 8, the infarcted tissues treated with saline showed the least amount of Cx43 expression than the untreated tissue (Normal). In contrast, the infarcted tissue treated with MS cells alone showed positive Cx43 cells expression. Among the treatments, the highest expression of Cx43 positive cells were observed in MS cells + Au@Ch-SF hydrogel treatment group, suggesting a large restoration of the myocardial damage after MI. This gold incorporated Ch-SF hydrogel might have provided electromechanical signals in the infarcted myocardial tissue. The quantitative image data of Cx43-DAPI ratio showed the expression pattern of Cx43 in MS cells + Au@Ch-SF hydrogel treated group was higher than the other treatments. This confirmed that injectable hydrogels, conductivity and mesenchymal stem cells had a constructive effect on the improvement and restoration of cardiac function after MI. Although the native cardiac cells do not have proper regeneration capacity following the myocardial infarction, the implanted MS cells transferred via hydrogels were found to secrete new tissues which are confirmed through β-MHC and Cx43 cardiac markers. In parallel with the above results, a relationship between expressions of cardiac-specific markers and electro-conductive elements such as gold within the hydrogel scaffolds has been previously reported [51].

Myocardium engineering studies has been reported 4–6 weeks as a proper time frame for degradation of hydrogels and in our study 4 weeks treated SD rats are analysed for the protective role of MI [52]. In addition, the prepared Au@Ch-SF hydrogel resembled native myocardium mechanics in terms of
providing electro conductivity and biocompatible tissue microenvironment [53,54] which could have increased the propensity of MS cells to become cardiomyocytes [55].

3.12.2 Biocompatibility analysis

Further, in vivo biocompatibility test was carried out by examining the hematological parameters (HGB and RBC), liver enzymes (AST and ALT), and histomorphological changes after Ch-SF, MS cells loaded with Ch-SF hydrogels and MS cells loaded with Au@Ch-SF hydrogels treatment. At the time of the experiment, it was demonstrated that there were no significant differences in weight between the control and treated groups, i.e., the prepared hydrogels did not cause any changes in the weight of the rat (Fig. 9A). Both AST and ALT are liver enzymes used to evaluate liver function. Increased levels of AST and ALT are indicators of liver damage [56]. In this study, it was found that the levels of AST and ALT in the hydrogel-injected groups were similar to those in control, meaning that the hydrogels treatment did not cause liver damage in rats (Fig. 9B and C). Similarly, hematological parameters such as HGP and RBC were found at a similar level to the control group (Fig. 9D and E). Furthermore, histomorphological examination did not show any internal injury or infectious lesions, showing that it was normal (Fig. 9F). From the biocompatibility results of this study, it is demonstrated that the prepared hydrogels did not show any side effects during the treatment period.

Conclusion

Overall, we constructed an injectable electroactive gold nanoparticle loaded silk fibroin chitosan composite hydrogel to improve therapeutic significance of mesenchymal stem cells in functional improvement after ischemic myocardial infarction. Our data recognized that the Au loaded Ch-SF hydrogel scaffolds with good conductivity, stability and biocompatibility properties provided an appropriate material for cardiac tissue engineering studies.

Abbreviations
| Abbreviation | Description |
|--------------|-------------|
| ALT          | Alanine transaminase |
| ANOVA        | Analysis of variance |
| AST          | Aspartate aminotransferase |
| Au@Ch-SF     | Gold nanoparticles incorporated chitosan-silk fibroin hydrogel |
| α-MEM        | Alpha minimum essential medium |
| BGP          | Beta-glycerophosphate solution |
| BSA          | Bovine serum albumin |
| β-MHC        | Beta-myosin heavy chain |
| Ch           | Chitosan |
| CLSM         | Confocal laser scanning microscopy |
| CV           | Cyclic voltammetry |
| Cx-43        | Connexin – 43 |
| DAPI         | 4',6-Diamidino-2-phenylindole dihydrochloride |
| DMEM         | Dulbecco's modified eagle's medium |
| DMSO         | Dimethyl sulfoxide |
| DW           | Distilled water |
| EAB          | Elongation at break |
| EDAX         | Energy dispersive spectroscopy |
| EDTA         | Ethylenediaminetetraacetic acid |
| FBS          | Fetal bovine serum |
| FT-IR        | Fourier-transform infrared spectroscopy |
| HGB          | Haemoglobin |
| MI           | Myocardial infarction |
| MS           | Mesenchymal stem cells |
| MTT          | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NMR          | Nuclear magnetic resonance |
| PBS          | Phosphate buffer solution |
| RBC          | Red blood cells |
| SCA          | Static contact angle |
SD rats : Sprague-dawley rats
SEM : Scanning electron microscope
SF : Silk fibroin
TGA : Thermogravimetric analysis
TS : Tensile strength
TUNEL : Terminal deoxynucleotidyl transferase dUTP nick end labeling
XRD : X-ray powder diffraction

Declarations

Ethics approval and consent to participate

In this study, all animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of “Capital Medical University” and all experiments were conducted with the approval of the Animal Ethical Committee at the Capital Medical University, China.

Consent for publication

Not applicable

Availability of data and material

Not applicable

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Authors’ contributions

ZW contributed to the conception and coordination of the study, the assessment of the quality of trials, and drafting the manuscript. SC and SW contributed to the extracting and screening of all references for eligibility, the assessment of the quality of trials, and drafting the manuscript. WL consolidated the data and statistical analysis. JL contributed to the conception and coordination of the study, the assessment of the quality of trials, and drafting the manuscript.

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Competing Interests
The authors declare that they have no competing interests.

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Scheme

Scheme 1 not provided with this version. You can leave an HTML note for this.

Figures

Figure 1

(A) FT-IR and 1H NMR analysis of chitosan (Ch) and a mixture of chitosan and silk fibroin (Ch-SF).
Figure 2

(A) UV spectrum of Au@Ch-SF, (B) Gelation time determination; insert figure shows temperature change of hydrogel formation and Rheological properties of hydrogels: (C) Ch, (D) Ch-SF and (E) Au@Ch-SF.
Figure 3

(A) XRD and (B) FT-IR analysis of Ch-SF and Au@Ch-SF.

Figure 4

Examination of morphological analysis using scanning electron microscope; (A) Chitosan, (B) Silk fibroin, (C) Chitosan and silk fibroin (D) Gold nanoparticles loaded Chitosan and silk fibroin. The insert spectrum
represents an EDX analysis of Ch-SF and Au@Ch-SF.

**Figure 5**

(A) TGA thermograms, (B) mechanical properties, (C) contact-angle values and (D) electrochemical cyclic voltammetry analysis of Ch-SF and Au@Ch-SF hydrogels.
Figure 7

In vitro analysis of cardiac myoblast H9C2 cells treated by Au@Ch-SF hydrogels and co-culture of mesenchymal stem cells with Au@Ch-SF hydrogels; (i) Cell viability assay (ii) Confocal scanning electron microscopic assay; (A) DAPI stained cells (B) DAPI+B-MHC Merged cell image. The probability value less than 0.05 (P<0.05) considered as a statistically significant. The letters a, b and c indicates statistically difference between the time periods and x, y and z denotes significantly difference between the groups.
Figure 8

In vivo analysis of the cardiac arrangement of infarcted hearts after treatment with Au@Ch-SF hydrogels. The histology and immunohistochemistry analysis of hearts for the control group (A), saline administrated group (B), mesenchymal stem cells treated group (C), mesenchymal stem cells and Ch-SF (D) and the combination treatment of mesenchymal stem cells and Au@Ch-SF hydrogels group (E). Quantitative analysis of Fibrosis area percentage, Apoptotic cell density and Cx43/DAPI pixel ration in
control and experimental groups. The statistical difference (*P < 0.05) represents the comparison between the saline and mesenchymal stem cells treated group with animal treated by combine treatment of mesenchymal stem cells and Au@Ch-SF hydrogels.

Figure 9

Biocompatibility of prepared hydrogels. (A) Bodyweight of the rat after various treatment. (B-C) Determination of liver enzymes such as AST and ALT. (D-E) Determination of hematological parameters
such as HGB and RBC. (F). Histomorphology of control and experimental groups.

**Supplementary Files**

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