Injectable shear-thinning xanthan gum hydrogel reinforced by mussel-inspired secondary crosslinking

Zhijia Liu and Ping Yao*

Shear-thinning hydrogels fabricated by dynamic and weak interactions usually possess low mechanical strength, high swelling behavior and rapid corrosion/dissociation rate. A xanthan gum (XG) aqueous solution presented weak gel and shear-thinning properties due to XG double helical strand structure. In this study, we introduced a secondary chemical crosslinking network in the XG double helical strand network. Dopamine (Dopa) was conjugated to XG via an amidation reaction. The conjugated Dopa groups were oxidized by horseradish peroxidase (HRP) and H$_2$O$_2$ to form a crosslinked covalent network, or coordinated/oxidized by Fe$^{3+}$ ions to form a crosslinked coordination/covalent network. At the early stage of the secondary crosslinking, the hydrogels displayed excellent thixotropic property. After the formation of the secondary network, the thixotropic property disappeared, but the mechanical strength, swelling ratio and degradation rate of the hydrogels were greatly improved. Both in vitro and in vivo investigations revealed that the XG–Dopa/H$_2$O$_2$ hydrogel was an injectable, biocompatible and biodegradable hydrogel material. This study demonstrated that forming a Dopa-mediated covalent crosslinking network in a shear-thinning hydrogel network is a facile and effective strategy for maintaining the injectable shear-thinning property as well as improving the stability and mechanical strength of the hydrogel.

Introduction

Hydrogels are promising biomaterials for drug and cell delivery, medical device fabrication and regenerative medicine because of their remarkable characteristics, such as high water content, tunable physicochemical properties, good biocompatibility and similarity to native extracellular matrices. Injectable hydrogels can be administrated to the target sites in a minimally invasive manner and can easily fill arbitrary shaped defects in vivo. Shear-thinning hydrogels can flow under a shear stress and recover their mechanical properties after removal of the stress, therefore, they can avoid failure of the injection and also the leakage of the hydrogel precursor solutions into the neighboring tissues. On the other side, shear-thinning hydrogels fabricated by dynamic and weak interactions usually possess low mechanical strength, high swelling behavior and rapid corrosion/dissociation rate.

Xanthan gum (XG) is a commercially available polysaccharide produced by bacterial fermentation. XG is composed of a linear (1→4)-β-D-glucose backbone; every alternate glucose residue has a charged trisaccharide side chain consisting of β-D-mannose-(1,4)-β-D-glucuronic acid-(1,2)-α-D-mannose; the inner and terminal mannosides may be substituted by acetate and pyruvate groups, respectively. The ordered double helical strands of XG tend to form network structure, thus XG aqueous solution displays unique weak gel and shear-thinning properties. XG solution does not readily form hydrogel by usual gelation methods; the formed weak gel swells badly at physiological condition. To address this problem, Dyondi et al. utilized physical blend of XG and gellan gum to produce injectable hydrogel as a tissue engineering scaffold for multiple growth factor delivery for bone regeneration. Recently, we used physical blend of XG and methylcellulose to produce thermo-responsive hydrogel composed of XG and methylcellulose double networks with injectable shear-thinning property for drug delivery.

In this study, we introduced chemical crosslinking network in XG double helical strand network to reinforce XG weak gel but also maintain the injectable shear-thinning property. Inspired by marine mussel adhesion, various hydrogel materials with excellent adhesiveness, self-healing, biocompatibility and biodegradability have been produced by introducing 3,4-dihydroxy-phenylalanine (DOPA) or catechol analogues, such as dopamine (Dopa), to synthetic and natural polymers for oxidation crosslinking and coordination crosslinking. Herein, we synthesized XG–dopamine conjugate (XG–Dopa) by an amidation reaction between the carboxyl group of XG and amine group of dopamine...
group of Dopa. An injectable shear-thinning XG–Dopa weak gel was prepared by dissolving XG–Dopa in pH 7.4 phosphate buffered saline (PBS, 10 mM phosphate buffer containing 0.138 M NaCl) at room temperature. The XG–Dopa weak gel was reinforced by covalent crosslinking in the presence of horseradish peroxidase and H$_2$O$_2$ (HRP/H$_2$O$_2$) or by coordination crosslinking followed by covalent crosslinking using Fe$^{3+}$ ions. The physicochemical properties of HRP/H$_2$O$_2$- and Fe$^{3+}$-mediated XG–Dopa hydrogels, including crosslinking kinetics, thixotropic property, mechanical strength and swelling behavior, were characterized in detail. The biocompatibility and biodegradability of HRP/H$_2$O$_2$-mediated XG–Dopa hydrogel were investigated in vitro and in vivo.

**Experimental section**

**Materials**

Xanthan gum (XG, viscosity 800–1200 cps, from *Xanthomonas campestris*), dopamine hydrochloride (Dopa), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS) and horseradish peroxidase (HRP) were purchased from Sigma–Aldrich. Hydrogen peroxide (H$_2$O$_2$) was from Jiangsu Tongsheng Chemical Reagent Co., Ltd. Other chemicals were from Sinopharm Chemical Reagent Co., Ltd. KB and HEK 293 cell lines were from American Type Culture Collection. DMEM cell culture medium and fetal bovine serum were from Gibco BRL Life Technologies Inc. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was from Promega Co.

**Synthesis and characterization of XG–Dopa conjugate**

Dopa was conjugated to XG via an amidation reaction. XG was dissolved in deionized water and then was dialyzed (cutoff molecular weight 3.5 kDa) against pH 3 HCl solution to remove inorganic salts and protonate the carboxylic groups of XG. After dilution with deionized water, the XG solution with 1.5 mg mL$^{-1}$ XG concentration of 500 mL was adjusted to pH 4.5 with NaOH, and 615 mg EDC and 370 mg NHS were added. The mixed solution was kept at room temperature for 2 h to activate the carboxylic groups of XG. Subsequently, 600 mg Dopa was added. The solution was adjusted to pH 5–5.5 then reacted at room temperature for 24 h. During the reaction, the pH was maintained at 5–5.5. After the reaction, the produced XG–Dopa was purified by dialysis against pH 4 HCl solution. During the dialysis, the free Dopa in the dialysate was monitored by UV absorption to ensure that the free Dopa was removed completely. After the dialysis, the XG–Dopa solution was centrifuged at 6000 rpm for 10 min. The supernatant was freeze-dried and the purified XG–Dopa was obtained. The yield was about 80%.

FTIR spectra were obtained on a Nicolet 6700 spectrometer (Thermo Fisher) with a resolution of 4 cm$^{-1}$ and accumulation of 128. FTIR samples were prepared by pressing XG or XG–Dopa with KBr. $^1$H-NMR spectra were acquired on an AVANCE III 500 MHz NMR spectrometer (Bruker BioSpin International) at 80 °C. $^1$H-NMR samples were prepared by dissolving XG or XG–Dopa in D$_2$O with a concentration of 8 mg mL$^{-1}$. The conjugation degree of Dopa in XG–Dopa was determined by the maximum absorption of Dopa around 280 nm$^{23}$ measured on a Shimadzu UV-2550 spectrometer based on Dopa standard curve.

**Preparation of XG and XG–Dopa weak gel**

XG or XG–Dopa of 30 mg was dissolved in 2 mL PBS followed by 12 h stir to obtain XG or XG–Dopa weak gel.

**Preparation of HRP/H$_2$O$_2$-mediated XG–Dopa (XG–Dopa/HRP/H$_2$O$_2$) hydrogel**

XG–Dopa of 30 mg was dissolved in 1.7 mL PBS followed by 12 h stir to obtain XG–Dopa weak gel. HRP and H$_2$O$_2$ solutions were separately added after the weak gel was adjusted to desired pH value. The mixture was immediately stirred to ensure homogeneous mixing. The final concentrations of XG–Dopa and HRP in the mixture were 15 mg mL$^{-1}$ and 10 U mL$^{-1}$, respectively; the final H$_2$O$_2$ concentration was 1, 3, or 5 mM.

**Preparation of Fe$^{3+}$-mediated XG–Dopa (XG–Dopa/Fe$^{3+}$) hydrogel**

XG–Dopa of 30 mg was dissolved in 1.7 mL PBS followed by 12 h stir. After FeCl$_3$ solution was added into XG–Dopa weak gel, the mixture was adjusted to desired pH value. The final concentrations of XG–Dopa and Fe$^{3+}$ in the mixture were 15 mg mL$^{-1}$ and 0.72 mM, respectively; the molar ratio of Dopa to Fe$^{3+}$ was 3 : 1.

**Rheological measurements**

Rheological measurements were performed on a stress-controlled HAAKE MARS III rheometer (Thermo Fisher) with parallel plate geometry (25 mm diameter and 1.5 mm gap). The parallel plate was carefully sealed with silicone oil to minimize water evaporation during measurement. The mechanical properties of the samples were determined within the linear viscoelastic region at a stress of 2 Pa and a frequency of 1 Hz. Oscillation amplitude sweep between low shear rate (0.1 s$^{-1}$) and high shear rate (10 s$^{-1}$) at 25 °C were carried out to investigate the rheological behavior of XG–Dopa. Oscillation frequency sweep and time sweep were performed to measure the storage modulus (G$'$) and loss modulus (G$''$) changes of the samples at 25 and 37 °C. The mechanical strength recovery properties were investigated at high stress (50 Pa) and low stress (0.5 Pa) for XG–Dopa/HRP/H$_2$O$_2$ hydrogel and at high stress (300 Pa) and low stress (3 Pa) for XG–Dopa/Fe$^{3+}$ hydrogel at 25 °C. The mechanical strength recovery behavior of XG–Dopa/Fe$^{3+}$ hydrogel was also monitored when linearly increasing the stress from 0.1 to 1000 Pa and then immediately reducing the stress to 1 Pa at 25 °C.

**In vitro swelling measurements**

Cylindrical XG–Dopa/HRP/H$_2$O$_2$ and XG–Dopa/Fe$^{3+}$ hydrogel samples with 10 mm diameter and 5 mm thickness were...
In vitro cytotoxicity

The sample was fractured.

\[
\text{Swelling ratio (\%)} = \left( \frac{W_t - W_i}{W_i} \right) \times 100\%,
\]

where \(W_i\) was the initial weight of the unswollen sample and \(W_t\) was the wet weight of the sample after \(t\) days of the immersion.

Compression tests

The compressive strength of the hydrogels was measured on a universal testing machine (CMT4104) at room temperature. Cylindrical XG–Dopa/HRP/H\_2O\_2 and XG–Dopa/Fe\^{3+} hydrogel samples with 10 mm diameter and 5 mm thickness were immersed in PBS at 37 \(^\circ\text{C}\) for 24 h to reach swelling equilibrium. Subsequently, the sample was put on the lower plate and compressed by the upper plate at a rate of 0.5 mm min\(^{-1}\) until the sample was fractured.

In vitro biodegradation and biocompatibility

The animal experiments of this study were performed at Experimental Animal Center of School of Pharmacy of Fudan University in full compliance with the guidelines approved by Experimental Animal Center of School of Pharmacy of Fudan University. The animal experiments of this study were performed at Experimental Animal Center of School of Pharmacy of Fudan University in full compliance with the guidelines approved by Experimental Animal Center of School of Pharmacy of Fudan University in full compliance with the guidelines approved by Experimental Animal Center of School of Pharmacy of Fudan University in full compliance with the guidelines approved by Experimental Animal Center of School of Pharmacy of Fudan University in full compliance with the guidelines approved by Experimental Animal Center of School of Pharmacy of Fudan University. Male SD rats (about 200 g) were subcutaneously injected in the backside with 0.5 mL XG–Dopa/HRP/H\_2O\_2 hydrogel using a syringe with 23G needle. At a predetermined interval, the rats were sacrificed, the injection sites were carefully opened, and photos were taken. The implanted hydrogels were taken out and lyophilized.

In vivo degradation of the hydrogel was calculated using the following equation:

\[
\text{Remnant dry weight (\%)} = \frac{W_f}{W_i} \times 100\%,
\]

where \(W_i\) and \(W_f\) were the dry weights of the hydrogel samples after one day and \(t\) days of the implantation, respectively. The tissues of the rats around the implanted hydrogels were surgically taken out, fixed, dehydrated and embedded in paraffin in succession. The inflammatory cells in the specimens were assayed as described previously.

Results and discussion

Synthesis and characterization of XG–Dopa conjugate

The synthetic route of XG–Dopa is illustrated in Scheme 1. Firstly, the commercial XG was purified and protonated by dialysis against pH 3 HCl solution. Subsequently, Dopa was conjugated to the carboxylic groups of XG via an amidation reaction with EDC and NHS as activator and coupling reagent.

Fig. 1A shows the absorption spectra of XG and XG–Dopa. The only absorption peak at about 280 nm, which represents the aromatic ring structure of Dopa groups, indicates that Dopa groups were successfully conjugated to XG and the conjugated Dopa groups were not oxidized. Fig. 1B shows the FTIR spectra of XG and XG–Dopa. XG displays a broad band at 3444 cm\(^{-1}\) due to the O–H stretching; the characteristic peaks at 1730 and 1618 cm\(^{-1}\) represent the stretching of carbonyl (C=O) of the acetyl groups and asymmetrical stretching of C=O of the carboxylic groups, respectively. The new peaks at 1530 cm\(^{-1}\) (amide bond, N–H bending) and 1068 cm\(^{-1}\) (amide bond, C–N stretching), and the weakened peak at 1618 cm\(^{-1}\) in XG–Dopa spectrum indicate the formation of amide bond between the amine group of Dopa and the carboxylic group of XG. Fig. 1C shows the \(^1\)H-NMR spectra of XG, Dopa and XG–Dopa. The spectra were acquired at 80 \(^\circ\text{C}\) to decrease the viscosity of XG and XG–Dopa solutions.

The signal at 3.4 ppm in XG–Dopa spectrum corresponds to the protons of methylene adjacent to the aromatic ring, and the signals around 7.3 ppm correspond to the aromatic ring protons of the Dopa groups, confirming the conjugation of Dopa groups onto XG. The conjugation degree of Dopa in XG–Dopa was 2.2 ± 0.2 mol% determined by the UV absorption, which means that about 2.2 Dopa groups were conjugated in per 100 alternate glucose residues on average.

Rheological behaviors of XG and XG–Dopa

In aqueous solution, XG possesses an ordered and rigid double helical strand structure at low temperature, whereas a disordered and flexible coil structure at high temperature. The midpoint transition temperature (\(T_m\)) is about 40–50 \(^\circ\text{C}\) depending on ionic strength. When the temperature is below its \(T_m\), the ordered double helical strands form a three-dimensional network, therefore, XG solution exhibits weak gel-like, good shear-thinning and rapid recovery behaviors. The carboxylic groups, which involve in the hydrogen bonding, play an important role in the helical strand structure of XG. Fig. 2A shows that XG–Dopa aqueous solution retained the weak gel property of XG and the linear viscoelastic region of XG–Dopa was slightly broader than the region of XG. At 25 \(^\circ\text{C}\) and a shear rate of 10 s\(^{-1}\), XG–Dopa exhibited a shear-thinning behavior, once the shear rate was changed to 0.1 s\(^{-1}\), XG–Dopa instantaneously recovered its viscosity (Fig. 2B). The same thixotropic property of XG–Dopa and XG samples confirm that the conjugation of Dopa on the carboxylic groups of XG does not influence the double helical strand structure, which is consistent with the result of the conjugation of octyl groups on the carboxylic groups of XG reported by Roy et al.
higher $G'$ values compared with XG weak gel, which possibly result from the hydrogen bonding and $\pi-\pi$ stacking of the conjugated Dopa groups. The results in Fig. 2 indicate that XG–Dopa weak gel can be delivered by injection under a shear stress and can rapidly recover its viscosity after injection.

Preparation and characterization of XG–Dopa/HRP/H$_2$O$_2$ hydrogel

It was reported that the conjugated Dopa groups are readily oxidized to form highly reactive quinone at alkaline conditions in the presence of oxygen, which can covalently crosslink the polymer. Fig. 3 shows time-dependent $G'$ and $G''$ changes of XG and XG–Dopa weak gels. XG–Dopa weak gel displayed higher $G'$ values than XG weak gel at both pH 8 and 9, confirming the self-crosslinking of XG–Dopa. Besides, changing the temperature from 25 to 37 °C at pH 9 and changing the pH from 8 to 9 at 37 °C caused the $G'$ and $G''$ to increase. However, after 90 min of the measurement, all the $G'$ values of the XG–Dopa samples were smaller than 10$^3$ Pa. This result indicates that without additional oxidant, the covalent crosslinking rate of the

Fig. 1 (A) UV-vis absorption spectra, (B) FTIR spectra and (C) $^1$H-NMR spectra of XG and XG–Dopa. The $^1$H-NMR spectra were acquired at 80 °C.

Scheme 1 Synthetic route of XG–Dopa.
conjugated Dopa groups is slow at pH 8 and 9, and the mechanical strength of XG−Dopa hydrogel is still weak. HRP and H2O2 were added into XG−Dopa weak gel to accelerate the oxidation and covalent crosslinking. HRP concentration was fixed at 10 U mL⁻¹, and H2O2 concentration was 1, 3, or 5 mM. Fig. 4A shows that at pH 6.5, XG−Dopa weak gel did not change its \( G' \) and \( G'' \) values significantly from 0 to 90 min. After mixing XG−Dopa weak gel with HRP and H2O2, the samples gradually increased their \( G' \) and \( G'' \) values. At 2 h post-mixing, XG−Dopa/HRP/H₂O₂ hydrogels displayed at least two orders of magnitudes higher \( G' \) values than XG−Dopa weak gel, and the \( G' \) values of XG−Dopa/HRP/H₂O₂ hydrogels increased with H₂O₂ concentration (Fig. 4B). These results confirm that HRP and H₂O₂ can accelerate the covalent crosslinking of XG−Dopa and can greatly improve the mechanical strength of the hydrogels.

Fig. 4 shows that XG−Dopa/HRP/H₂O₂ samples were in weak gel state during the first 10 min after mixing XG−Dopa weak gel with HRP and H₂O₂. Fig. 5A shows that XG−Dopa/HRP/H₂O₂ samples with pH 6.5 and 8 displayed faster crosslinking rates at 37 °C compared with the rates at 25 °C, and at the same temperature, the crosslinking rates were faster at pH 8 than the rates at pH 6.5. All the samples did not increase their \( G' \) values significantly during the first 10 min of the oxidation crosslinking reaction, which implies that the covalent crosslinking network had not formed at the early stage of the reaction. The shear-thinning property of XG−Dopa/HRP/H₂O₂ sample at 10 min post-mixing was investigated. The similar \( G' \) and \( G'' \) changes of XG−Dopa/HRP/H₂O₂ and XG samples under alternating shear stresses of 0.5 and 50 Pa shown in Fig. 5B confirm that XG−Dopa/HRP/H₂O₂ hydrogel still possesses thixotropic property at the early stage of the oxidation crosslinking reaction. The results in Fig. 5 imply that XG−Dopa/HRP/H₂O₂ hydrogel can be delivered by injection at the early stage, its

**Figure 2** (A) Stress-dependent \( G' \) and \( G'' \) changes of XG and XG−Dopa weak gels measured at a frequency of 1 Hz and 25 °C; (B) viscosity changes of XG and XG−Dopa weak gels monitored between low shear rate (0.1 s⁻¹) and high shear rate (10 s⁻¹) at 25 °C.

**Figure 3** Time-dependent \( G' \) and \( G'' \) changes of XG and XG−Dopa weak gels with different pH values measured at 25 and 37 °C, 2 Pa stress and 1 Hz frequency.

**Figure 4** (A) Time-dependent \( G' \) and \( G'' \) changes of XG−Dopa/HRP/H₂O₂ samples containing 15 mg mL⁻¹ XG, 10 U mL⁻¹ HRP and various concentrations of H₂O₂ with pH 6.5 at 25 °C; (B) frequency-dependent \( G' \) and \( G'' \) changes of the XG−Dopa/HRP/H₂O₂ samples at 2 h post-mixing XG−Dopa with HRP and H₂O₂ at 25 °C.
premature gelation in syringe can be avoided, its viscosity can be rapidly recovered after injection, and its mechanical strength can be reinforced at body temperature.

Preparation and characterization of XG–Dopa/Fe3+ hydrogel

Self-healing hydrogel materials constructed by catechol–Fe3+ coordination crosslinking have been reported.17,18 The stoichiometry of catechol-Fe3+ coordination complexes depends on the medium pH: the mono-complex is dominant at pH < 6.5, the bis-complex at 6.8 < pH < 8, and the tris-complex at pH > 8.31 Fig. 6 shows that after addition of Fe3+ ions and pH adjustment to 9 at 25 °C, the G’ values of XG–Dopa/Fe3+ hydrogel is about 5-fold higher than the G’ values of XG–Dopa weak gel shown in Fig. 3. The G’ and G” values of XG–Dopa/Fe3+ hydrogel did not change significantly during the first 105 min of the coordination crosslinking, subsequently, the G’ and G” values increased gradually (Fig. 6). It was reported that Fe3+ ions lead to catechol oxidation and subsequently covalent crosslinking.32 In order to verify that the increased G’ and G” values were caused by the oxidation crosslinking of the conjugated Dopa groups, we measured the absorption spectra of XG–Dopa/Fe3+ solutions containing 1 mg mL−1 XG–Dopa and a molar ratio of Dopa to Fe3+ 3 : 1 with pH 8.5 and 10.5. Fig. 7 shows that the absorbance of the pH 8.5 and 10.5 solutions in 250–700 nm range increased with time, confirming the oxidation of the Dopa groups.25,31 The absorbance of the pH 10.5 solution at around 290 nm increased more significantly compared with the absorbance of the pH 8.5 solution, which is consistent with the report that the formation of dicatechol is favored at elevated pH levels.19 The results in Fig. 6 and 7 demonstrate that XG–Dopa/Fe3+ hydrogel can improve its mechanical strength via both coordination crosslinking and oxidation crosslinking.
As mentioned above, the medium pH has important influence on the stoichiometry of catechol–Fe³⁺ coordination complexes. We studied the mechanical strength of XG–Dopa/Fe³⁺ hydrogels with different pH values by measuring the frequency-dependent $G'$ and $G''$ changes of the hydrogels at 25 °C. In order to minimize the impact of oxidation crosslinking, the measurements were performed from high frequency (10 Hz) to low frequency (0.01 Hz) immediately after the addition of Fe³⁺ ions and pH adjustment. Fig. 8 shows that the pH 6 and 8.2 hydrogels presented almost the same mechanical strength, and the pH 9 hydrogel displayed the maximum mechanical strength; above pH 9, the mechanical strength decreased with the increase of hydrogel pH. As we know, the formation of the tris-complex is favored at elevated pH levels and at pH around 9 the tris-complex is dominated. On the other side, the hydrolysis of Fe³⁺ ions at pH above 9 can reduce the coordination interaction between catechol and Fe³⁺. That is, the stronger mechanical strength of the pH 9 hydrogel is due to the formation of the tris-complex in the hydrogel.

Fig. 6 indicates that the oxidation crosslinking of XG–Dopa is not serious during the first 105 min post-addition of Fe³⁺ ions, after that, the formation of oxidation crosslinking network causes the increase of mechanical strength. The mechanical strength recovery properties of XG–Dopa/Fe³⁺ hydrogel with pH 9 at 30 and 180 min post-addition of Fe³⁺ ions were investigated at 25 °C, and the results are shown in Fig. 9. For the hydrogel measured at 30 min post-addition of Fe³⁺ ions, the $G'$ values were larger than $G''$ values at lower stress but smaller than $G''$ values at higher stress, and the $G'$ and $G''$ immediately recovered to almost 100% of the original levels when the stress was reduced to lower one. This property implies that at the early stage of the coordination crosslinking, XG–Dopa/Fe³⁺ hydrogel can be delivered by injection and can immediately recover its mechanical strength after injection. For the hydrogel measured at 180 min post-addition of Fe³⁺, the $G'$ values slightly decreased with the increase of shear stress and could not recover when the stress was changed to lower one. These results indicate that at the later stage, the XG–Dopa/Fe³⁺ network cannot be broken completely under the stress applied and the network cannot recover once being broken. Fig. 9 reveals that XG–Dopa/Fe³⁺ hydrogel possesses thixotropic property at the early stage and the thixotropic property disappears on the formation of the oxidation crosslinking network in the hydrogel. The photo in Fig. 10A confirms that XG–Dopa/Fe³⁺ hydrogel is injectable at the early stage using a syringe with 23G needle. Fig. 10B shows the self-healing property of the hydrogel, which is the same as DOPA–polyallylamine/Fe³⁺ hydrogel reported by Krogsgaard et al.

**In vitro swelling and compressive properties**

The swelling ratio of XG–Dopa/HRP/H₂O₂ hydrogel with pH 6.5 and 1 mM H₂O₂ was 6.8% and the ratio of XG–Dopa/Fe³⁺ hydrogel at pH 6.5 and 1 mM H₂O₂ was 5.6%.

---

**Fig. 8** Frequency-dependent $G'$ and $G''$ changes of XG–Dopa/Fe³⁺ hydrogels with various pH values at 25 °C. The measurements were performed from high frequency to low frequency immediately after the addition of Fe³⁺ ions and pH adjustment.

**Fig. 9** $G'$ and $G''$ changes of XG–Dopa/Fe³⁺ hydrogel with pH 9 measured at 30 and 180 min post-addition of Fe³⁺ ions: (A) applying a stress from 0.1 to 1000 Pa then immediately changing the stress to 1 Pa; (B) applying alternating stresses of 3 and 300 Pa.

**Fig. 10** Photos of (A) injection of XG–Dopa/Fe³⁺ hydrogel with pH 9 at the early stage post-addition of Fe³⁺ ions and (B) self-healing of the hydrogel after being cut into two pieces.
The hydrogel with pH 9 was 6.3% after swelling equilibrium in 37 °C PBS (Fig. 11A), which are much smaller than the swelling ratio of XG weak gel reported previously. It is obvious that the covalent crosslinking network in XG–Dopa/HRP/H₂O₂ hydrogel and also in XG–Dopa/Fe³⁺ hydrogel restricts the swelling of XG. The compressive strength of the XG–Dopa/HRP/H₂O₂ hydrogel decreased from 47.7 to 39.4 kPa after 24 h swelling, while the compressive strength of the XG–Dopa/Fe³⁺ hydrogel decreased from 59.6 to 47.6 kPa. XG–Dopa/Fe³⁺ hydrogel presented higher compressive strength and smaller swelling ratio compared with XG–Dopa/HRP/H₂O₂ hydrogel, suggesting stronger crosslinking network in XG–Dopa/Fe³⁺ hydrogel.

### In vitro Cytotoxicity

Fig. 12 shows KB and HEK 293 cell viabilities after 48 h incubation in the culture media containing XG–Dopa solution, XG–Dopa/HRP/H₂O₂ hydrogel and XG–Dopa/Fe³⁺ hydrogel. The cell viabilities were almost 100% when the medium containing 0.5 mg mL⁻¹ XG–Dopa or less (Fig. 12A), demonstrating that XG–Dopa does not have significant cytotoxicity. It was reported that free Dopa is highly cytotoxic. The excellent biocompatibility of XG–Dopa sample indicates that the free Dopa had been completely removed during the purifying process. For XG–Dopa/HRP/H₂O₂ hydrogel, both KB and HEK 293 cells

![Fig. 11](image1.png)

(A) Swelling ratios of XG–Dopa/HRP/H₂O₂ hydrogel with pH 6.5 and 1 mM H₂O₂ and XG–Dopa/Fe³⁺ hydrogel with pH 9 after immersion in 37 °C PBS; (B) compressive strength of the hydrogels (a) before and (b) after 24 h swelling in 37 °C PBS. The insets show the photos of the hydrogels.

![Fig. 12](image2.png)

KB and HEK 293 cell viabilities after 48 h incubation in the culture media containing (A) XG–Dopa solutions with different XG–Dopa concentrations, (B) XG–Dopa/HRP/H₂O₂ hydrogel samples with pH 6.5 and different H₂O₂ concentrations and (C) XG–Dopa/Fe³⁺ hydrogel samples with different pH values.
presented full viabilities when H2O2 concentration was 0.5 or 1 mM in the hydrogel, but the viabilities reduced when the H2O2 concentration was 3 mM or higher (Fig. 12B). For XG–Dopa/Fe3+ hydrogel, the cell viabilities were higher than 90% when the hydrogel pH was 8.2 or 9, but the cell cytotoxicity increased when the hydrogel pH was higher than 9 (Fig. 12C). These results indicate that the XG–Dopa/HRP/H2O2 hydrogel containing 1 mM H2O2 and XG–Dopa/Fe3+ hydrogel with pH 9 are biocompatible.

In vivo biocompatibility and biodegradation

In vivo biocompatibility and biodegradation of XG–Dopa/HRP/H2O2 hydrogel with pH 6.5 and 1 mM H2O2 were evaluated by implantation of the hydrogel into SD rats. After mixing XG–Dopa with HRP and H2O2, the hydrogel was immediately implanted into the backside subcutaneous tissue by injection using a syringe with 23G needle. The photos in Fig. 13A show the implanted XG–Dopa/HRP/H2O2 hydrogels, in which leakage was not observed. The hydrogel volume decreased with the implantation time. The remnant dry weight of the hydrogel was 47% after 8 weeks of the implantation as shown in Fig. 13B. The degradation/erosion rate of XG–Dopa/HRP/H2O2 hydrogel in vivo is much slower than the rate of XG/methylcellulose blend hydrogel, which disappeared completely after 36 days of the implantation. This result confirms that the covalent crosslinking network can reduce the degradation/erosion rate of the shear-thinning injectable hydrogel. The degradation/erosion mechanism of XG–Dopa/HRP/H2O2 hydrogel is not clear at this stage.

The implanted XG–Dopa/HRP/H2O2 hydrogel did not induce extensive inflammatory response because there was neither any observable abnormality on the skin surfaces, nor any sign of edema, redness or tissue necrosis in the surrounding tissues. Fig. 14 shows the hematoxylin–eosin strained histological images of the tissues around the implanted hydrogel. The inflammatory cells in the tissues decreased with the implantation time and disappeared completely after 16 days of the implantation. The results in Fig. 13 and 14 demonstrate that XG–Dopa/HRP/H2O2 is an injectable, biocompatible and biodegradable hydrogel material.

Complementary properties of XG weak gel and Dopa-mediated chemical crosslinking network

As we know, hydrogel implantation by injection highly depends on gelation kinetics of hydrogel precursor solution. The gelation rate of Dopa modified polymers can usually be tuned by varying Dopa conjugation degree, polymer concentration, oxidant or complexant concentration, and/or precursor solution pH. In this study, Fig. 12 shows that increasing polymer or H2O2 concentration or hydrogel pH, the facile ways to accelerate the gelation rate, result in cytotoxicity. Therefore, it is not easy to control the gelation time for Dopa modified polymers to prevent the leakage of the precursor solutions and also to avoid the cytotoxicity. Gelation rate is not a critical factor for shear-thinning hydrogels because they can flow under a shear stress and recover their mechanical properties on removal of the stress. However, shear-thinning hydrogels usually possess low mechanical strength, high swelling ratio and rapid corrosion/dissociation rate in vivo. Herein, we combined shear-thinning hydrogel properties with covalent chemical crosslinking.
thinning hydrogel and Dopa-mediated hydrogel with complementary properties together. By introducing Dopa-mediated chemical crosslinking network in XG double helical strand network, we successfully fabricated Dopa-mediated XG hydrogels which possess injectable shear-thinning property, good biocompatibility and biodegradation, excellent stability and mechanical strength.

Conclusions
XG–Dopa conjugate was synthesized by an amidation reaction between the carboxyl group of XG and the amine group of Dopa. XG–Dopa aqueous solution displayed the same shear-thinning and rapid recovery weak gel properties as XG solution. The conjugated Dopa groups were oxidized by HRP and H$_2$O$_2$ to form crosslinked covalent network or coordinated/oxidized by Fe$_{3+}$ ions to form crosslinked coordination/covalent network. At the early stage of the crosslinking reactions at which the secondary network had not formed, XG–Dopa/HRP/H$_2$O$_2$ and XG–Dopa/Fe$_{3+}$ hydrogels displayed excellent thixotropic property. After the formation of the secondary network, the thixotropic property disappeared, but the mechanical strength, swelling ratio and degradation/erosion rate of the hydrogels were greatly improved. Both in vitro and in vivo investigations demonstrated that XG–Dopa/HRP/H$_2$O$_2$ hydrogel containing 15 mg mL$^{-1}$ XG, 10 U mL$^{-1}$ HRP and 1 mM H$_2$O$_2$ was biocompatible and biodegradable. This study demonstrated that forming Dopa-mediated secondary covalent crosslinking network in shear-thinning hydrogel network is a facile and effective strategy for maintaining the injectable shear-thinning property and also improving the stability and mechanical strength of the hydrogel.

Acknowledgements
Financial supports of National Natural Science Foundation of China (NSFC Project 21474018 and 21274026) are gratefully acknowledged.

Notes and references
18 M. Krogsgaard, M. A. Behrens, J. S. Pedersen and H. Birkedal, Biomacromolecules, 2013, 14, 297–301.