

A Novel Regenerated Silk Fibroin-based Hydrogels with Magnetic and Catalytic Activities^{*}

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Abstract A simple and facile synthetic methodology for fabricating the regenerated silk fibroin (RSF)-based hydrogel which consisted of the *in situ* generated magnetic ferrihydrous oxide (Fe_3O_4) was developed. Using the co-precipitation of Fe^{2+} and Fe^{3+} within the RSF-based hydrogel with 90% RSF and 10% HPMC (hydroxypropyl methyl cellulose), the as-prepared RSF/ Fe_3O_4 hydrogel not only showed high strength of saturation magnetization, but also exhibited excellent catalytic activities. For example, with the assistant of 3,3',5,5'-tetramethylbenzidine (TMB), the RSF/ Fe_3O_4 hydrogel could detect H_2O_2 at a concentration as low as $1 \times 10^{-6} \text{ mol} \cdot \text{L}^{-1}$. In addition, the catalytic activities were able to be maintained for a long term under various conditions. These findings suggest that the RSF-based materials can be endowed with interesting properties, and have great potential for the applications in the fields of biotechnology and environmental chemistry.

Keywords Natural polymer; Silk; Hydrogel; Nanoparticle; Magnetic and catalytic activity

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INTRODUCTION

Hydrogels can retain a large amount of water or biological fluids while maintaining their structures^[1]. Besides a few of enzymes such as glucose oxidase or horseradish peroxidase^[2–4], many metallic or inorganic nanoparticles (*e.g.* Pt, Au, Ag and TiO_2)^[5–8] are also able to be immobilized within hydrogels to produce the robust composites with catalytic activities. In addition, hydrophilic networks of hydrogels can provide a specific micro-environment for the *in situ* synthesis of inorganic nanoparticles which address the main issues such as the aggregation and stability^[9], while the combination of hydrogels and inorganic nanoparticles can endow the materials with the desired properties and the advantages from them.

As one of the magnetite materials, Fe_3O_4 has attracted many researchers' attention in the past few decades due to its great potential in the biomedical and technological applications, such as magnetic resonance imaging (MRI)^[10], cancer therapy^[11], targeted drug delivery^[12], waste water treatment^[13] and so on. Besides, another important feature of Fe_3O_4 is the peroxidase-like activity^[14]. As a kind of artificial mimetic enzyme made of inorganic materials, Fe_3O_4 shows some advantages over the natural enzymes, including relatively high catalytic activities under a wide range of environmental conditions (pH = 2–7 or temperature at 70 °C), the cheaper fabricating process than that of the natural enzymes^[15]. Thus, Fe_3O_4 is suitable to act as the catalysts for a broad range of usage in biotechnology and environmental chemistry. The combination of Fe_3O_4 and hydrogels results

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in the obtained hydrogel as not only an ideal reactor but also a suitable carrier for the synthesis and immobilization of Fe_3O_4 ^[16]. Recently, a few of Fe_3O_4 loaded hydrogels have been successfully fabricated by various methods^[17–23]. However, multistep reactions for preparing hydrogels/ Fe_3O_4 composites and tedious purification process have limited their further application. Therefore, a facile approach for the fabrication and combination of Fe_3O_4 loaded hydrogels is highly desired.

Recently, natural polymer-based hydrogels become popular for achieving sustainable development. Nevertheless, the hydrogel loaded with functional inorganic nanoparticle is rarely reported mainly due to its uncertain sources and the poor mechanical performance of the natural polymer-based hydrogels. On the other hand, the silk proteins from various silkworms have a number of advantages, such as good mechanical properties, large amount of production and low cost, *etc.*, which has been approved to be a biomaterial by the U.S. Food and Drug Administration (FDA)^[24–26]. Thus, silk fibroin (SF), as a main component of the silk, is considered to be a new renewable biopolymer resource with the potential to replace current petrochemical-based materials from the view of economic and ecological impacts. An important feature of silk fibroin is its hydrophobic and hydrophilic moieties in which some functional groups ($-\text{OH}$, $-\text{COOH}$, $-\text{NH}_2$ or $-\text{CONH}_2$) are able to chelate metal ions^[27–30]. Usually, silk fibroin-based hydrogel consists of the protein networks with physical crosslinks of β -sheet region which can support and maintain their structures. Therefore, silk fibroin-based hydrogels are believed to be the ideal materials for adsorbing or chelating metal ions.

Herein, we report the fabrication of magnetic silk fibroin-based hydrogels *via* the *in situ* formation of Fe_3O_4 , which are able to chelate the metal ions. The fabrication process is rather simple and facile, endowing the as-prepared magnetic silk hydrogels with magnetic and catalytic activities. Besides, the catalytic activities of such hydrogels can be maintained for a long time.

EXPERIMENTAL

Materials

Hydroxypropyl methyl cellulose (HPMC), Iron(II) chloride tetrahydrate ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), Iron(III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), hydrogen peroxide and ammonia were purchased from Aladdin Co. Ltd. (Shanghai, China). 3,3',5,5'-Tetramethylbenzidine (TMB) was purchased from Sinopharm (Shanghai, China). All the reagents were used as received without further purification.

Preparation of Regenerated Silk Fibroin (RSF) Aqueous Solutions

To produce the RSF, the silk fiber were degummed, dissolved and dialyzed according to the established procedures^[31]. The concentration of the original RSF aqueous solution was about 4 wt%. Then the original RSF solution was concentrated by dialyzing against PEG solution to reach 10 wt% as the stock solution for later use.

Preparation of RSF Hydrogels

RSF hydrogels were prepared according to our previous report^[32]. Briefly, different concentrations of RSF solution (6%, 8% and 10%) and hydroxypropyl methyl cellulose (HPMC) solution (6%, 8% and 10%) were mixed at room temperature to obtain the mixtures at the ratio (RSF:HPMC) of 9:1 (*V:V*) with the solid contents of 6%, 8% and 10%, respectively. Then the obtained mixtures were heated in a water bath at 70 °C for 2 h.

Preparation of RSF Hydrogels with Fe_3O_4 (RSF/ Fe_3O_4)

Fabrication of Fe_3O_4 in RSF hydrogels was carried out *via in situ* co-precipitation of FeCl_2 and FeCl_3 . Hydrogels with different solid contents (6%, 8% and 10%) were introduced into the solution containing FeCl_2 (0.2 mol·L⁻¹) and FeCl_3 (0.3 mol·L⁻¹), where they were left at 4 °C for 72 h. Afterwards, all these RSF-based hydrogels were washed using deionized water thoroughly, and then placed in ammonium hydroxide for another 2 h, resulting in the formation of Fe_3O_4 within the RSF hydrogels (named 6%-0.2/0.3, 8%-0.2/0.3 and 10%-0.2/0.3).

Characterization of RSF/ Fe_3O_4 Hydrogels in Dry State

All the RSF-based hydrogels were air-dried for X-ray diffraction (XRD), Raman spectroscopy, scanning electron microscopy (SEM), thermogravimetric analysis (TGA) and magnetic measurements, respectively. XRD patterns

were recorded on an X-ray diffractometer (D8 ADVANCE and DAVINCI design (Bruker, Germany)) with Cu $K\alpha$ radiation in the range of 10° – 70° . Raman spectra were detected by Renishaw inVia Reflex when a He-Ne laser was used to give 6 mW of energy at 785 nm, and the Raman shifts were chosen in the range of 200 cm^{-1} to 1000 cm^{-1} . For SEM observation, an S-4800 (Hitachi, Japan) operating in high vacuum was used, and an acceleration voltage of 2 kV was chosen for recording the images. Due to the good conductivity of the samples, there is no need to have the sample coated with gold except for the neat RSF one. The mineral contents of RSF/ Fe_3O_4 hydrogels were determined by means of TGA (Perkin-Elmer pyris1), and the tests were carried out from 50°C to 800°C at a heating rate of $20\text{ K}\cdot\text{min}^{-1}$ under a constant air flow. Magnetization loop measurement of the samples was performed in a Superconducting Quantum Interference Device (SQUID, Model 6000 (Quantum Design, USA)) magnetometer at 25°C . The magnetization of all the Fe_3O_4 was calculated according to the ratio between magnetic moment and sample mass.

Catalytic Activities of RSF/ Fe_3O_4 Hydrogels and Their Application on the H_2O_2 Detection

The RSF/ Fe_3O_4 hydrogels were cut into the small cubes of $5\text{ mm} \times 5\text{ mm} \times 5\text{ mm}$, while the air-dried ones were in a certain shape for the catalytic activity measurements or the H_2O_2 detection. In the catalytic experiment, the hydrogel or the air-dried sample was immersed in the solution containing $100\text{ mmol}\cdot\text{L}^{-1}$ H_2O_2 and $816\text{ }\mu\text{mol}\cdot\text{L}^{-1}$ 3,3',5,5'-tetramethylbenzidine (TMB) at room temperature, and the test lasted for 30 min. For H_2O_2 detection, the concentration of H_2O_2 ranged from $1\text{ }\mu\text{mol}\cdot\text{L}^{-1}$ to $100\text{ mmol}\cdot\text{L}^{-1}$ while the concentration of TMB was fixed at $816\text{ }\mu\text{mol}\cdot\text{L}^{-1}$, and the 10%-0.2/0.3 RSF/ Fe_3O_4 hydrogel was chosen as the representative. The solution was examined by wavelength-scan mode and photometry mode of 652 nm, using a UV-Vis spectrophotometer (UV-2550 (SHIMAZU, JAPAN)).

Stability of RSF/ Fe_3O_4 Hydrogel

Two samples of 10%-0.2/0.3 RSF/ Fe_3O_4 hydrogels were prepared. Then, one was left outside exposing to the air for 1 month while the other one was kept in tap water for 1 month. After their structures being re-characterized by XRD and Raman spectroscopy, the catalytic experiments of both the RSF/ Fe_3O_4 hydrogel and dry samples were carried out as mentioned above.

RESULTS AND DISCUSSION

In Situ Fabrication of Fe_3O_4 within RSF Hydrogel

To fabricate the RSF hydrogel loaded with Fe^{2+} and Fe^{3+} , as an example, the prepared neat RSF hydrogel with solid content of 10 wt% was submerged into the aqueous solution of Fe^{2+} and Fe^{3+} for 72 h until reaching the diffusion equilibrium. Such a brown and translucent hydrogel was subsequently transferred into ammonia for another 2 h to *in situ* incubate the formation of Fe_3O_4 . Finally, a black RSF/ Fe_3O_4 hydrogel was obtained. The scheme of the preparation procedure is shown in Fig. 1.

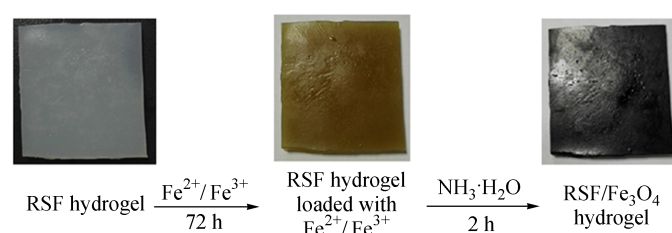


Fig. 1 Schematic processes for the fabrication of RSF/ Fe_3O_4 hydrogel

XRD was employed for characterizing the ferric oxide synthesized within the RSF hydrogel. As shown in Fig. 2(a), both XRD patterns of neat RSF hydrogel and RSF hydrogel with ferric oxide display a broad peak around $2\theta = 23^\circ$, which can be attributed to the amorphous structure of silk fibroin^[33]. Nevertheless, the pattern of the one with ferric oxide showed a few characterized peaks of either Fe_3O_4 or $\gamma\text{-Fe}_2\text{O}_3$ ^[16, 34]. Due to the

similarity of XRD patterns of $\gamma\text{-Fe}_2\text{O}_3$ and Fe_3O_4 , Raman spectroscopy was employed for further identifying the crystal structure of the ferric oxide synthesized within the RSF hydrogel matrix. As expected, a single and strong peak at 670 cm^{-1} , as the typical feature of Fe_3O_4 can be found in the Raman spectrum of the RSF hydrogel with ferric oxide. However, there are no peaks around 350 , 500 and 700 cm^{-1} , relating to the $\gamma\text{-Fe}_2\text{O}_3$ ^[34, 35], observed in Fig. 2(b). Thus, the substance synthesized within the RSF hydrogel was unambiguously determined to be Fe_3O_4 rather than $\gamma\text{-Fe}_2\text{O}_3$ or other structures of ferric oxide.

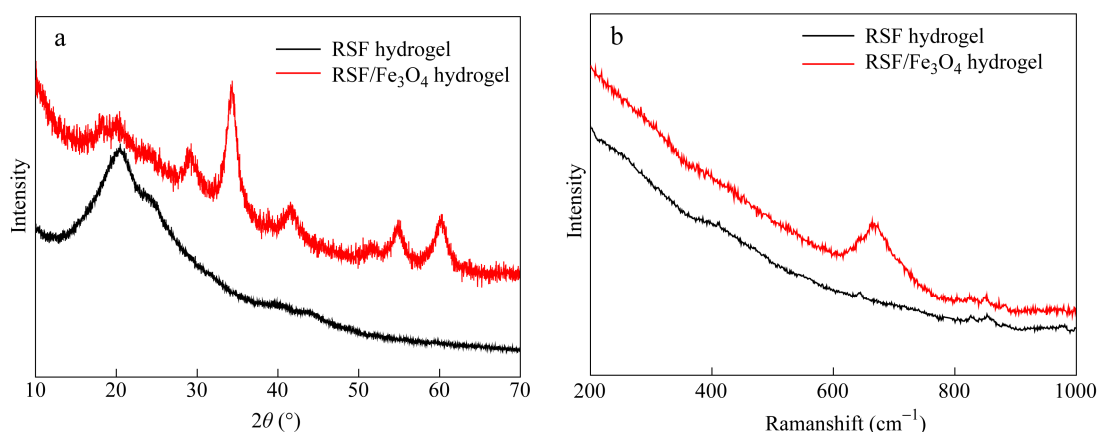


Fig. 2 (a) XRD patterns and (b) Raman spectra of the neat RSF hydrogel and RSF/ Fe_3O_4 hydrogel (10%-0.2/0.3) (Solid content of the both hydrogels was 10 wt%.)

Combining the results of those RSF hydrogels with the solid contents of ferric oxide in 6 wt% and 8 wt%, whose XRD patterns and Raman spectra are shown in Fig. S1 (in supporting information, SI), we could conclude that Fe_3O_4 was successfully fabricated within RSF hydrogels *via* the *in situ* co-precipitation method.

Magnetic Properties of RSF/ Fe_3O_4 Hydrogels

The loading capacity of Fe_3O_4 in the RSF hydrogel and its magnetic properties were determined by thermogravimetric analysis (TGA) and superconducting quantum interference device (SQUID).

As a control, the neat RSF hydrogel demonstrated that almost all of the silk fibroin was burned under the constant air flow, due to the fact that its weight remained only 1.8% in the TGA test (Fig. 3a). Indeed, the weight loss of the RSF sample divided into three stages, *i.e.* the temperatures were lower than $200\text{ }^\circ\text{C}$, between $200\text{ }^\circ\text{C}$ and $500\text{ }^\circ\text{C}$, and higher than $500\text{ }^\circ\text{C}$, corresponding to the evaporation of the residual water, the decomposition of side groups of silk fibroin chain, and the oxidative decomposition of organic skeleton and C—C backbone of silk fibroin, respectively^[36]. On the other hand, although the TGA curves of RSF hydrogel loaded with Fe_3O_4 were in the same fashion, there were significant residues in the samples even at the temperature over $800\text{ }^\circ\text{C}$. Besides, the amount of these confessed inorganic residues was around 18%, 22% and 29% for the samples of 10%-0.2/0.3, 8%-0.2/0.3 and 6%-0.2/0.3, respectively. The result could be easily understood that more Fe^{2+} and Fe^{3+} could diffuse or transfer into the RSF matrix to produce Fe_3O_4 with the decrease of the solid content of the hydrogel.

Subsequently, the magnetic measurement for the RSF/ Fe_3O_4 hydrogels demonstrated that all the samples not only had ferromagnetic properties, but also exhibited the saturation magnetizations with the as-prepared Fe_3O_4 around $40\text{ emu}\cdot\text{g}^{-1}$ (Fig. 3b). These saturation magnetization data of the Fe_3O_4 were much higher than those synthesized in other hydrogels in a similar way, which were presented as 14, 26.3 or $32.7\text{ emu}\cdot\text{g}^{-1}$, respectively^[16, 33, 37]. The expanded magnetization curves in the range from -100 Oe to 100 Oe are shown in Fig. S2 (in SI), and the Fe_3O_4 content and magnetic data of these RSF/ Fe_3O_4 hydrogels are summarized in Table S1 (in SI).

The magnetization measurement and TGA test indicated that a large number of $\text{Fe}^{2+}/\text{Fe}^{3+}$ ions could be

chelated by the RSF and sufficient Fe_3O_4 could be synthesized within the RSF hydrogels in an *in situ* precipitation method due to the strong interaction between $\text{Fe}^{2+}/\text{Fe}^{3+}$ and the functional groups of RSF molecular chain^[38]. Moreover, the presence of RSF hydrogels has a less impact on the magnetic properties of the Fe_3O_4 which is around $50 \text{ emu}\cdot\text{g}^{-1}$ to $60 \text{ emu}\cdot\text{g}^{-1}$. According to a previous report^[37], the samples had relatively high saturation magnetization over $40 \text{ emu}\cdot\text{g}^{-1}$.

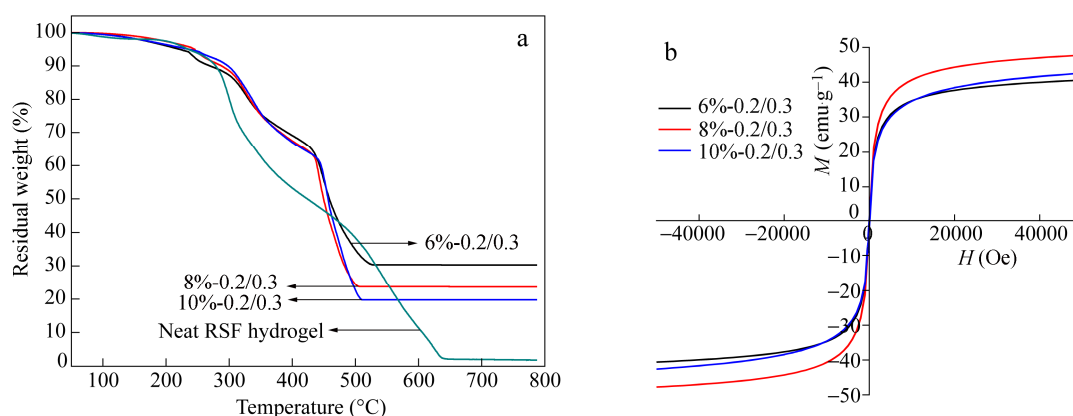


Fig. 3 (a) TGA curves of dried neat RSF hydrogel and the RSF/ Fe_3O_4 hydrogels with various solid contents and (b) magnetization curves of dried RSF/ Fe_3O_4 hydrogels at 20°C

Morphologies of RSF/ Fe_3O_4 Hydrogels

The cross-sections of these air-dried hydrogels based on RSF imaged by SEM are shown in Fig. 4. It could be seen that the neat RSF presented a relatively smooth and featureless cross-section (Fig. 4a), while the ones loaded with Fe_3O_4 displayed the fractural roughness surfaces (Figs. 4b, 4c and 4d), indicating that lots of Fe_3O_4 particles were successfully fabricated within the hydrogel. Additionally, it also could be found that the Fe_3O_4 particles in the RSF hydrogel with the diameter around 30–40 nm were distributed evenly in the samples, which hardly were affected by the solid content in the RSF hydrogel.

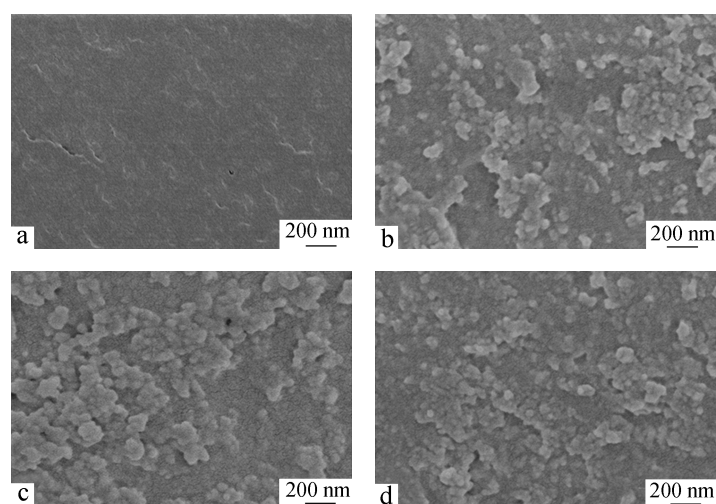


Fig. 4 The cross sections of the air-dried (a) neat RSF hydrogel, (b) 10%-0.2/0.3 hydrogel, (c) 8%-0.2/0.3 hydrogel and (d) 6%-0.2/0.3 hydrogel imaged by SEM

Catalytic Activities of the RSF/ Fe_3O_4 Hydrogels

It is well known that the nanoparticles of Fe_3O_4 present peroxidase-like activity^[16]. To confirm such catalytic

property of the Fe_3O_4 in RSF hydrogel, TMB was chosen as the substrate while H_2O_2 was employed as an oxidant. In this case, TMB is able to be oxidized to generate the substance with the maximum absorbance around 652 nm^[14].

As expected, the mixed solution of TMB and H_2O_2 produced a blue color in the presence of the RSF/ Fe_3O_4 hydrogel (Fig. 5a). It could be found that the maximum absorbance of the mixed solution linearly increased to around 0.7 to 0.9 within 30 min by the catalysis of RSF/ Fe_3O_4 composites, no matter what the solid content of the RSF/ Fe_3O_4 hydrogel was (Fig. 5b and Fig. S3 in SI). In addition, in the case that the RSF/ Fe_3O_4 composite dried from the hydrogels was employed as the catalyst, it showed a slightly lower catalytic activity than those of the hydrogel counterparts (Fig. 5b). It is reasonable because the high density of dried RSF/ Fe_3O_4 composite may retard the interaction among the Fe_3O_4 , TMB and H_2O_2 .

The experiment results mentioned above indicated that these Fe_3O_4 nanoparticles produced in RSF hydrogel *in situ* not only kept their inherent magnetic properties, but also maintained the peroxidase-like activity.

The release of H_2O_2 by cells or glucose oxidation usually relates to human health and disease^[38, 39], hence detecting trace amount of H_2O_2 is of great significance in biotechnology. The 10%-0.2/0.3 RSF/ Fe_3O_4 hydrogel was selected for the H_2O_2 detection experiment. As shown in Fig. S4 (in SI), all the solution with different H_2O_2 concentrations (in the range between 1 $\mu\text{mol}\cdot\text{L}^{-1}$ and 100 $\text{mmol}\cdot\text{L}^{-1}$ while the concentration of TMB was fixed at 816 $\mu\text{mol}\cdot\text{L}^{-1}$) turned blue in the presence of the RSF/ Fe_3O_4 hydrogel. Besides, the UV-Vis test showed that the RSF/ Fe_3O_4 hydrogel had a sensitive and rapid response towards H_2O_2 concentration, which was of great potential in detection of minute amount of H_2O_2 (Fig. 5c).

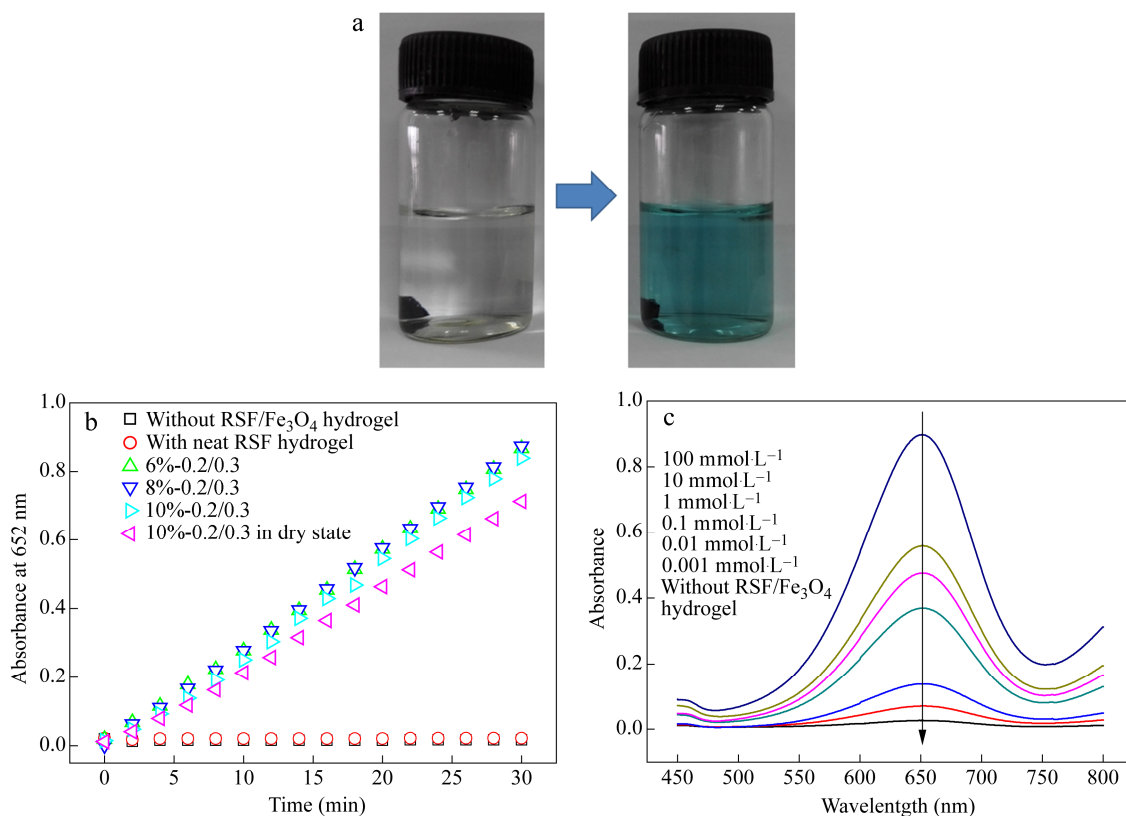


Fig. 5 (a) In the presence of RSF/ Fe_3O_4 hydrogel, the original colorless mixed solution of TMB and H_2O_2 turns into blue within a couple of minutes; (b) The absorbance of solution containing 100 $\text{mmol}\cdot\text{L}^{-1}$ H_2O_2 and 816 $\mu\text{mol}\cdot\text{L}^{-1}$ TMB at 652 nm, in the presence of different samples as noted; (c) UV-Vis spectra of the TMB solution (concentration of TMB was fixed at 816 $\mu\text{mol}\cdot\text{L}^{-1}$) with different H_2O_2 concentrations (10%-0.2/0.3 RSF/ Fe_3O_4 hydrogel is used as the catalyst, and H_2O_2 concentration is in a range from 1 $\mu\text{mol}\cdot\text{L}^{-1}$ to 100 $\text{mmol}\cdot\text{L}^{-1}$.)

Stability of the RSF/Fe₃O₄ Hydrogel

It is a common knowledge that Fe₃O₄ usually tends to be oxidized readily in various conditions, and then may lose its inherent catalytic activities. Therefore, in the purpose of the long time application, it is necessary to prevent Fe₃O₄ from being oxidized. Indeed, the nanoparticles of Fe₃O₄ loaded in the RSF hydrogel presented significant stability, as the RSF might act as a protective material to Fe₃O₄ in the oxygen containing environments such as air and tap water.

The as-prepared 10%-0.2/0.3 RSF/Fe₃O₄ hydrogels were either left in air (actually it turned into dried state within 2 days) or kept in the water for 1 month. The XRD pattern and Raman spectrum of the dried sample (Figs. 6a and 6b) were nearly identical with those presented in Figs. 2(a) and 2(b), demonstrating that the Fe₃O₄ loaded in RSF matrix maintained its crystal structure even being exposed in air for 1 month. More importantly, either the xerogel of RSF/Fe₃O₄ or the one kept in water for 1 month still had sufficient catalytic activity (Figs. 6c and 6d). These results indicated that both the structure and catalytic activity of Fe₃O₄ produced in RSF hydrogel *in situ* could be successfully maintained for a long time and the RSF matrix could act as a protector for keeping the catalytic activities of Fe₃O₄.

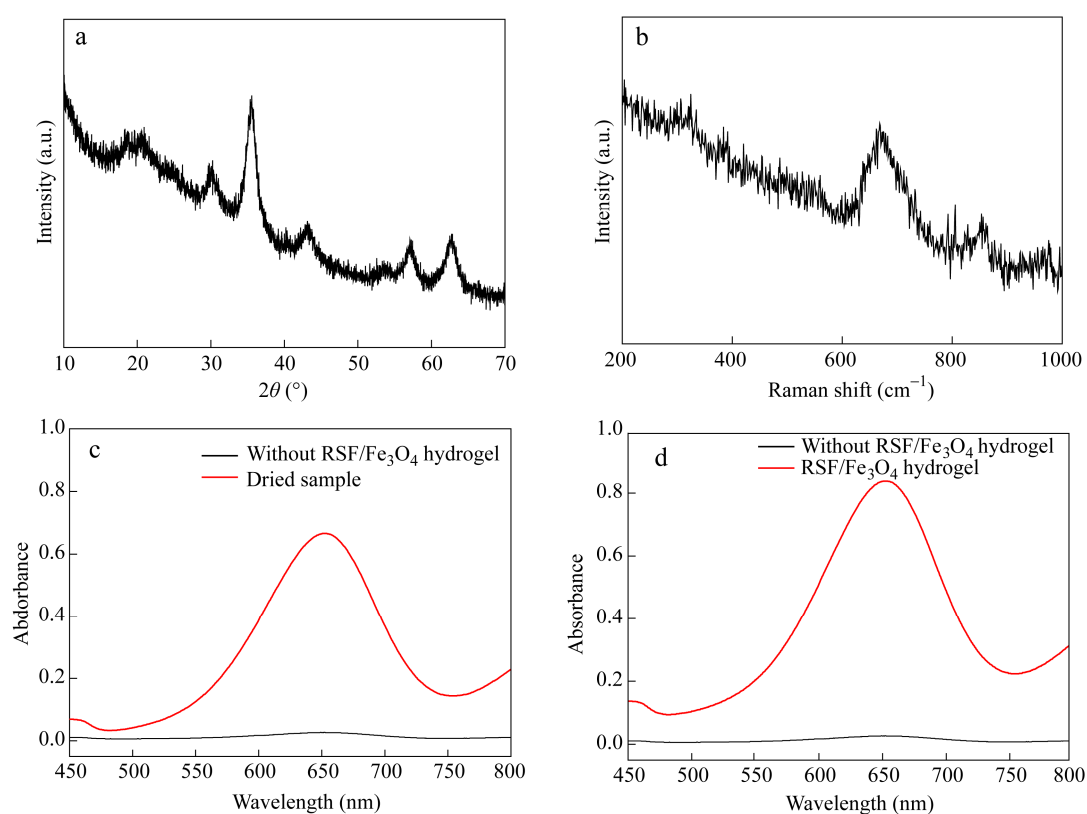


Fig. 6 XRD pattern (a) and Raman spectrum (b) of the RSF/Fe₃O₄ hydrogel preserved in the air for 1 month; UV-Vis spectra of the solution containing H₂O₂ and TMB with the dried RSF/Fe₃O₄ hydrogel exposed in air for 1 month or without the RSF/Fe₃O₄ hydrogel (c) and with the RSF/Fe₃O₄ hydrogel kept in water for 1 month or without the RSF/Fe₃O₄ hydrogel (d)

Reaction time: 30 min. Samples used were 10%-0.2/0.3 hydrogels.

CONCLUSIONS

By utilizing the chelating interaction between RSF molecules and Fe²⁺/Fe³⁺, the RSF hydrogel loaded with Fe₃O₄ particles is successfully fabricated *via* an *in situ* precipitation method which is rather simple and low-cost. Such prepared Fe₃O₄ within RSF hydrogels have relatively high saturation magnetization, compared with that of

Fe₃O₄ synthesized in other hydrogel by the same method. Besides, the inherent peroxidase-like activity of Fe₃O₄ with the sensitivity to H₂O₂ is preserved at a concentration as low as 1×10^{-6} mol·L⁻¹ after the fabrication of Fe₃O₄ within RSF hydrogels. Furthermore, the catalytic activity of the RSF/Fe₃O₄ hydrogel can be maintained for a long time. Thus, combining the well-known biocompatibility of RSF, the RSF/Fe₃O₄ hydrogel holds great promise in the applications of environmental chemistry and biotechnology.

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