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Cite this: DOI: 10.1039/c0xx00000x

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COMMUNICATION

HRP-mediated polymerization forms tough nanocomposite hydrogels with high biocatalytic performance†

Teng Su, Da Zhang, Zhou Tang, Qing Wu and Qigang Wang*

Received (in XXX, XXX) Xth XXXXXXXXX 200X, Accepted Xth XXXXXXXXX 200X

DOI: 10.1039/b000000x

This communication describes a mild and quick construction of tough nanocomposite hydrogels via a horseradish peroxidase-mediated radical polymerization, which offers an effective platform for immobilizing enzyme to attain high catalytic performance in various solvents.

Hydrogels, formed from three-dimensional hydrophilic networks, can facilitate bio-related applications, including biocatalytic reactions, drug delivery, biosensor fabrication and tissue engineering.[1] Having manifested good efficiency in manipulating the local microenvironment of immobilized enzymes, hydrogels, such as polymeric hydrogels and supramolecular hydrogels, have been successfully designed to serve as competitive candidates for the immobilization of enzymes.[2] However, most hydrogels, covalently or non-covalently cross-linked, are mechanically weak, which limits the scope of their practical utilization. To solve this issue, many innovative strategies have been developed for fabricating tough hydrogels, such as nanocomposite hydrogels,[3] double network hydrogels,[4] macromolecular microsphere composite hydrogels,[5] and tetra-poly(ethylene glycol) hydrogels.[6] Nevertheless, to form a tough hydrogel usually requires complicated molecule designs or particular fabrication skills, which may damage the structures of enzymes and result in an adverse effect on their catalytic activities. Therefore, an enzymatically triggered hydrogelation that immobilizes the enzyme into a hydrogel *in situ* in a benign way would be particularly attractive.

To date, enzymatic reactions have been successfully utilized to regulate the self-assembly of small molecules into supramolecular hydrogels.[7] Increasing interest has also been devoted to the injectable enzymatically cross-linked hydrogels.[8] Horseradish peroxidase (HRP) has been well studied for cross-linking tyramine-conjugated macromolecules into hydrogels for protein delivery and cell culture. Inspired by these pioneering works, we choose a HRP mediated radical polymerization approach to prepare mechanically strong nanocomposite hydrogels, which has rarely been found in the literature. It is well known that oxidoreductase can catalyze electron transfer reactions and generate products capable of initiating chain polymerization of water-soluble vinyl monomers. An eminent enzyme-mediated radical initiation system involving glucose oxidase and Fe^{2+} can generate a cytocompatible hydrogel within minutes.[9] Another example, HRP, can also initiate the polymerization of acrylamide via catalyzing the oxidation of acetylacetone (ACAC) by H_2O_2. However, this radical initiation system has not yet been applied for hydrogelation, possibly due to an irregular long inhibitory period before polymerization. This inhibitory period is considered unavoidable because one cannot ensure elimination of residual O_2, resulting from H_2O_2 dismutation, in the system, even by a degassing procedure.[10]

Here, we report on the preparation of tough multifunctional nanocomposite hydrogels triggered by a modified HRP-mediated radical initiation system within several minutes at room temperature (approx. 25°C). In our experiment, a typical hydrogel can be prepared from a homogeneous aqueous solution of silica nanoparticles (SNPs), acryloylated human serum albumin (HSA) (ESI†), and *N*, *N*-dimethylacrylamide (DMAA), with the pH value adjusted to approximately 6.0. In such a weakly acidic environment, HRP (isoelectric point 7.2) exhibits high catalytic activity and a positively charged surface that facilitates its adsorption on the negatively charged surfaces of the SNPs (isoelectric point approx. 2), as well as acryloylated HSA (isoelectric point approx. 4.7), through electrostatic interactions.[11] The successive introduction of ACAC, HRP and H_2O_2 ([ACAC]/[HRP]/[H_2O_2] = 42.0/0.056/15.7 mM) leads to the generation of a yellowish, transparent tough hydrogel after approximately 6 min (Fig. 1). Interestingly, the molar ratio of ACAC to H_2O_2 in the ternary initiation system is crucial because we chose a fixed [H_2O_2] / [HRP] ratio of 280 for all of the experiments according to the literature.[10] We observed no gelation at a [ACAC] / [H_2O_2] ratio below 2.6, regardless of the monomer concentrations.

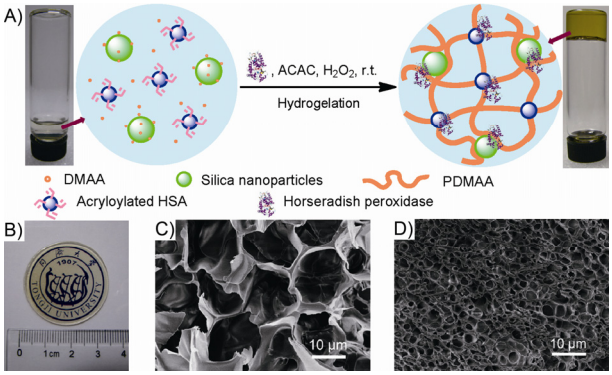

Fig. 1 A) Schematic illustration of nanocomposite hydrogel preparation via HRP-mediated polymerization to immobilize the enzyme *in situ*. B) Photograph of a free-standing gel film. C,D) SEM images of the cryo-dried hydrogels without C) and with D) SNPs.

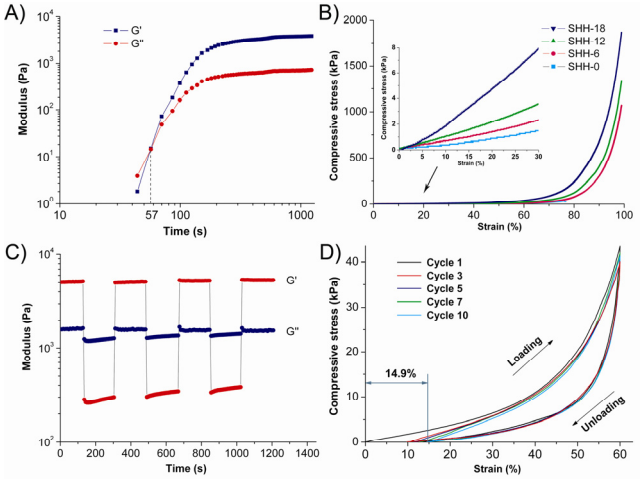

Fig. 2 Rheological and mechanical properties of hydrogels. A) Dynamic time sweep. B) Mechanical behaviour during compression measurements. C,D) Mechanical behaviour during continuous step strain sweep C) and cyclic compression D) measurements.

To understand the gelation kinetics of such a system, we conducted time sweep measurements using a rheometer, for monitoring the storage modulus (G') and loss modulus (G''), as a function of time (Fig. 2A). A crossover point between G' and G'' curves occurs at 57 s, indicating that the gelation process of this system is fast. These moduli nearly reach equilibrium after 360 s, and the value of G' is 5 times larger than that of G'', indicating the formation of an elastic hydrogel. The nuclear magnetic resonance (NMR) data of the deuterium oxide-substituted sample confirm an approximately 99% conversion of vinyl double bonds after 6 min reaction time (Fig. S1, ESI†), which is consistent with the gelation kinetics characterization.

The electron paramagnetic resonance (EPR) measurement detected the formation of a carbon-centered radical derived from the ACAC molecule[12] in the ACAC/HRP/H_2O_2 ternary system aqueous solution (Fig. S2, ESI†). The ACAC radicals are supposed to consume the residual O_2 and promote the propagation of the polymer chain. Furthermore, according to the catalytic mechanism of HRP/ACAC/H_2O_2 system, the insufficient ACAC concentration can also induce the deactivation of HRP by H_2O_2 in the initial stage of polymerization, which is detrimental to hydrogelation.[13] Therefore, our modified ACAC/HRP/H_2O_2 ternary initiation system, which employs an ACAC concentration high enough to counteract the inhibitory effect of residual O_2 and the deactivation of HRP by H_2O_2, induces hydrogelation rapidly without intentional degassing treatment, while the ternary system reported in the literature[10] causes no gelation.

The compression tests reveal that the incorporation of SNPs dramatically enhances the compressive properties of the hydrogel (Fig. 2B and Table S1 in ESI†). The hydrogel with silica content as low as 3.0% sustains a compressive strain (ε) up to 95%, whereas the control gel without silica (SHH-0) fractures at a strain of 76%. With a further increase of the silica content, the nanocomposite hydrogels can tolerate a compressive strain as high as 99%, and they almost recover their original volume within 10 min after the compression loading is released. The hydrogel prepared with 18.0% SNPs, 3.0% acryloylated HSA and 5.0% DMAA (SHH-18) shows an astonishing compressive toughness in comparison with the SHH-0 gel. The maximum compressive strength and modulus for SHH-18 achieve 1884.5 kPa and 21.6 kPa, which are 52.5 times and 5.2 times higher than those of SHH-0, respectively. As shown in Fig. 2C, this gel exhibits very rapid recovery of its mechanical strength after a large-amplitude oscillatory breakdown, and can realize rubber-like recovery when subjected to a fatigue cyclic compression test (at ε = 60%) with at least 10 successive loading/unloading cycles. The thickness reduction after the fatigue test, as indicated in Fig. 2D, is only approximately 14.9%. The excellent mechanical strength and fast-recovery properties of the nanocomposite hydrogels could be attributed to the SNPs, which facilitate the uniform cross-linking (Fig. S3, ESI†) of hydrogel by serving as additional physical cross-linking sites via non-covalent interactions with PDMAA chains as well as protein molecules (Table S2, ESI†) to produce a strong, self-recovering matrix in the hydrogel. We noticed that lowering the concentration of the ternary initiation system can also lead to the formation of tough nanocomposite hydrogels. However, a decrease in compressive strength and an increase in gelation time are observed (Fig. S4, ESI†). Thus, the mechanical properties, the gelation time, and the enzyme loading in the hydrogel could be tuned and optimized for specific applications.

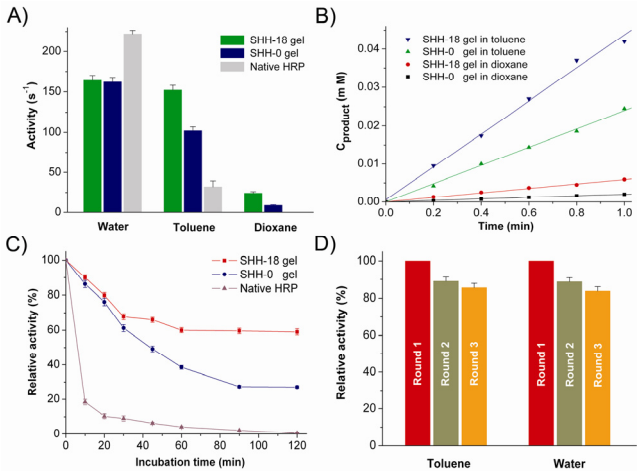

Fig. 3 A) Comparison of the activities of catalysts in different media. B) The initial reaction courses in the first minute of OPD (10 mM) and H_2O_2 (20 mM) catalyzed in organic media. C) The remaining activities of SHH-18, SHH-0 and native HRP in water after incubation at 70°C for different periods of time. D) The relative activities of SHH-18 over the course of three rounds of successive reactions.

In the current work, the HRP enzyme plays an important role in the generation of radicals in the initiation stage and, in effect, by immobilizing itself into the hydrogel matrix *in situ*. By using the oxidation of *o*-phenylenediamine (OPD) as a model reaction, we examined the catalytic activity of HRP in different forms. All tests concerning catalysis were performed after the concentrations of HRP (whether immobilized or not) in all of the systems were adjusted to be identical (i.e., 0.2 mg L^{-1}). As shown in Fig. 3A, the HRP immobilized in SHH-18 gel has a catalytic activity similar to that immobilized in SHH-0 gel in aqueous buffer (0.05 M, pH 7.0, phosphate buffer solution). However, the SHH-18 gel exhibits notably higher activity than the SHH-0 gel and the native HRP in both nonpolar (*e.g.*, toluene, log P=2.5) and polar (*e.g.*, dioxane, log P=−1.1) organic solvents. According to Fig. 3B, the initial reaction rate of SHH-18 gel at 10 mM OPD concentration

is much larger than that of SHH-0 gel in the same solvent. From the Lineweaver-Burk plots constructed from the initial rates of reactions, we obtained the kinetic parameters of the reactions (Fig. S5 and Table S3, ESI†). In a hydrophobic toluene medium, the SHH-18 gel has the highest turnover number (K_{cat}=152 s^{-1}), which is approximately 4.8 times and 1.5 times higher than that of the native HRP and SHH-0 gel, respectively. In a hydrophilic dioxane medium, the native HRP hardly shows any catalytic activity ($K_{cat} < 0.02$ s^{-1}), due to the stripping of the essential enzyme-bound water by dioxane molecules. However, the SHH-18 gel exhibits a dramatic activity enhancement of approximately 1200 times and 2.7 times, relative to the native HRP and the SHH-0 gel, respectively. We suggest that the high activity of the SHH-18 hydrogel in organic solvents could be mainly ascribed to the following four factors: i) the hydrophilic gel matrix facilitates the substrate (i.e., OPD) to enter the gel and enrich in the aqueous microenvironment of HRP, ii) the micro-sized pores (Fig. 1D) in the gel network ease mass transport,[14] iii) the hydrogel network composed of cross-linked HSA with PDMAA can prevent the denaturing of HRP catalytic sites, and iv) the SNPs, forming electrostatic interactions with the HRP molecules in the hydrogel, can render protection from distortion for the essential water layer on the enzyme surface, especially in a polar solvent. This assumption could be supported by the approximative Michaelis-Menten constant (K_m) values for the SHH-18 hydrogel in different organic solvents (Table S3, ESI†). The similar K_m values indicate that the immobilized HRP in the SHH-18 gel should have a similar microenvironment in different organic solvents,[15] which confirms that attack from organic solvents could be relieved in the presence of SNPs.

The nanocomposite-hydrogel-immobilized HRP also exhibits high thermal stability and reusability. As shown in Fig. 3C, the SHH-18 gel maintains 90% of its initial activity after incubation in water at 70 °C for 10 min and 59% even after incubation for 120 min, while the free HRP loses approximately 82% of its initial activity after only 10 min incubation. The SHH-0 gel renders certain protection to the self-immobilized enzyme in the early stage. However, it only retains 27% of its initial activity after the extended 120 min incubation. To test the gel reusability, we compared the fresh and recovered SHH-18 gel during the 15 min oxidation of OPD in toluene and in water, respectively. Fig. 3D shows that the amount of product in the third run reaches 85.5% of the first run in toluene and 83.7% in water. Moreover, the reaction product can be collected and purified easily by decantation to remove the hydrogel. In contrast, the SHH-0 gel is not free-standing in solvents and is difficult to remove after reaction. Therefore, the mechanically strong nanocomposite hydrogel exhibits good reusability.

In summary, we have demonstrated that HRP-mediated radical polymerization offers a convenient and environmentally friendly strategy to prepare mechanically strong nanocomposite hydrogels that exhibit substantially enhanced enzymatic activity in organic media as well as high thermal stability and reusability. Our nanocomposite hydrogel provides an effective platform for immobilizing enzymes to attain high catalytic performance in organic media, and shows promise in achieving widespread application in industrial biotransformation, biosynthesis, biosensing and tissue engineering.

This work was supported by National Natural Science Foundation of China (21274111), the Program for New Century Excellent Talents in University of Ministry of Education of China (NECT-11-0386), the Fundamental Research Funds for Central Universities, the Pujiang talents program (11PJ1409500), and the Recruitment Program of Global Experts.

Notes and references

Department of Chemistry, and Advanced Research Institute, Tongji University, Shanghai 200092, PR China
E-mail: wangqg66@tongji.edu.cn

† Electronic Supplementary Information (ESI) available: Experimental details, supplementary figures and tables. See DOI: 10.1039/b000000x/

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Table of Content

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