Self-Healing Materials Hot Paper

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Enzymetically Regulating the Self-Healing of Protein Hydrogels with High Healing Efficiency**

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Abstract: *Enzyme-mediated self-healing of dynamic covalent bond-driven protein hydrogels was realized by the synergy of two enzymes, glucose oxidase (GOX) and catalase (CAT). The reversible covalent attachment of glutaraldehyde to lysine residues of GOX, CAT, and bovine serum albumin (BSA) led to the formation and functionalization of the self-healing protein hydrogel system. The enzyme-mediated protein hydrogels exhibit excellent self-healing properties with 100% recovery. The self-healing process was reversible and effective with an external glucose stimulus at room temperature.*

The ability to spontaneously heal injury and recover functionality are the key features that increase the survivability and the lifetime of the organism.[1] However, synthetic materials usually fail after being damaged or fractured. Inspired by nature, the demand for self-healing materials is rapidly developing to offer a new strategy toward safer, longer-lasting products and lower production costs.[2] Over the past few decades, three kinds of conceptual self-healing systems, namely the capsule system, vascular system, and intrinsic system, have been reported.[3] For the capsule system and vascular system, self-healing materials mainly relied on the encapsulated healing agents in the cavities. When the capsules or vessels were damaged, the healing agents were released to heal them through the formation of covalent bond.[4] For intrinsic self-healing materials, self-repair occurs by the inherent reversibility of chemical bonds or physical interactions between the damaged regions. The easy preparation and modification of the intrinsic self-healing materials as well as their stimuli-responsive self-healing properties led to them attracting much more attention. Up to now, various non-covalent interactions, such as hydrogen bonds, π–π stacking interactions, host–guest interactions, and ionic interactions have been used for the development of stimuli-responsive healable materials, such as self-healing films and rubbers.[5] However, how to enhance the structural stability and mechanical strength of the materials is still a big challenge for the self-healing systems.

To address this problem, self-healing materials based on dynamic covalent bonds which employ the reversible but relatively strong covalent bonds that control the structure of the materials by the equilibrium of bond breaking and re-forming[6] have been widely used. For most designed materials, external stimuli are required to achieve healing.[7] For example, thermally reversible covalent bonds have been introduced into polymers. Upon heating and cooling, they can reversibly rupture and re-form to achieve self-healing capacity. Recently, a transparent organic polymeric material was shown to thermally repeatedly mend or re-mend itself based on Diels–Alder reactions.[8] Furthermore, the redox stimuli are also used to develop healing materials. In the redox system, the disulfide bonds were cleaved and re-formed under the redox conditions.[9] Acidity reversible covalent bonds were also used to construct healing materials owing to controllable properties and easy adjustment. The acylhydrazone bonds and imine bonds were frequently used as reversible covalent bonds.[10] These covalent bonds show reversibility by breaking down the network and regenerating the starting reagents by acid catalysis. These are relatively simple stimulate processes; however, in living organisms, the healing process should be complicated and hierarchically controlled. Therefore, ways to simulate tissue repair and construct enzyme-mediated self-healing systems is a very new field that has strongly aroused our interest.

Herein, we report a new strategy for the construction of self-healing protein hydrogels based on imine bond that is regulated by two synergetic enzymes: glucose oxidase (GOX) and catalase (CAT). As shown in Scheme 1a, the reversible covalent attachment of glutaraldehyde to lysine residues of GOX, CAT, and bovine serum albumin (BSA) is suitable for the formation and functionalization of the self-healing protein hydrogel system. The BSA scaffold supports the hydrogel system and the GOX as a catalytic center plays a key role to adjust the pH of the system by the addition of extra traces of glucose. First, glucose is oxidized to gluconolactone by GOX catalysis. Then gluconolactone hydrolyzed to gluconic acid to decrease the pH value of the hydrogel system. The H_2O_2 generated from the catalytic process will be decomposed into H_2O and O_2 by the enzyme CAT to avoid the imine bonds being oxidized. The generated O_2 is re-utilized by GOX and can accelerate the whole catalytic reaction (Scheme 1b). With the change of pH, the imine bonds provide the opportunity to heal the protein hydrogel (Scheme 1c).

We first explored the impact of pH in the protein hydrogel systems. Mechanical properties of protein systems under

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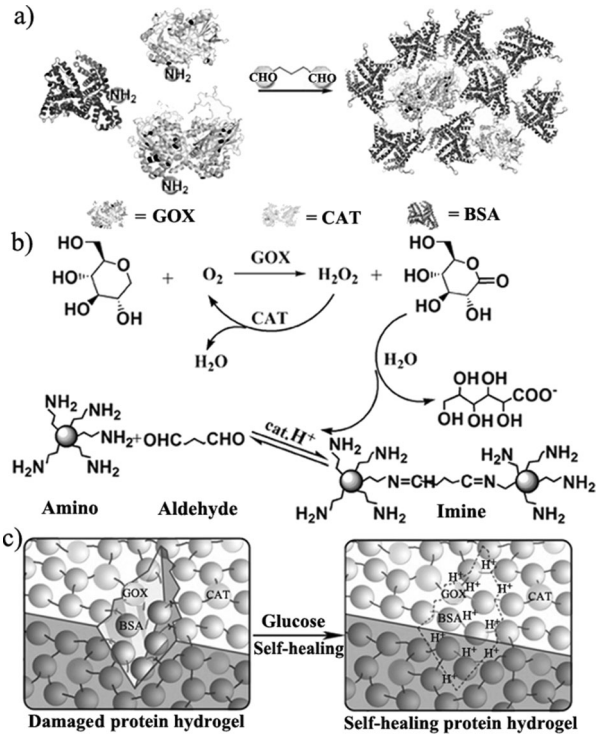

Scheme 1. The enzyme-mediated self-healing of the protein hydrogel system. a) Protein hydrogel system; b) the mechanism of enzyme-regulated catalytic reactions; c) the procedure of self-healing in the protein hydrogel system.

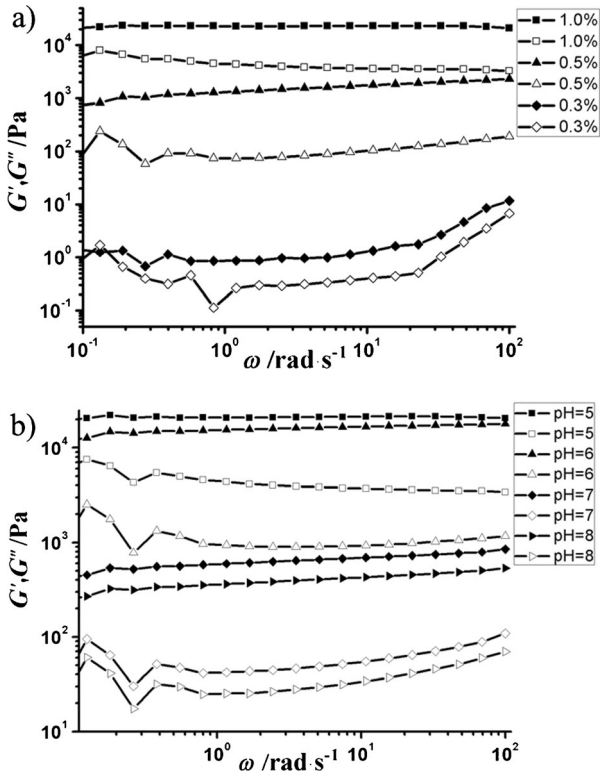

Figure 1. a) Storage modulus (G') and loss modulus (G'') versus angular frequency (ω) of the protein hydrogel (protein concentration 12 wt%) with different glutaraldehyde concentrations at 25 °C. b) Storage modulus (G') and loss modulus (G'') vs angular frequency (ω) of the protein hydrogel (protein concentration 12 wt%) with different pH values at 25 °C. Solid symbols represent G', and open symbols represent G''.

different conditions were studied by dynamic rheological measurements. The storage moduli (G') and the loss moduli (G'') were presented at different concentration of glutaraldehyde and different pH values (Figure 1). In Figure 1 a, three pairs of G' and G'' curves were included with different glutaraldehyde concentrations with a constant protein concentration (12 wt%), and 20 min was required to form the hydrogels. With the increase of glutaraldehyde content, G' and G'' were significantly raised. When the glutaraldehyde concentration reached to 0.5 wt%, the G' value (average 1.5×10^3 Pa) is much larger than G'' (average 100 Pa), indicating the formation of strong chemical gel with covalent cross-linked network. In Figure 1 b, with a constant protein concentration (12 wt%) and glutaraldehyde concentration (0.5 wt%), after 40 min to form the hydrogels, four pairs of G' and G'' curves were included with different pH values (pH 5, 6, 7, and 8). These data showed that G' and G'' were significantly decreased with the increase of pH, suggesting the slow linkage reaction of amine with aldehyde, the extent of cross-linking decreasing, and also the reducing mechanical strength of the hydrogel.

The formation process of protein hydrogels with different pH values (the numbers 1 to 6 on the little vials corresponding to pH 8, 7, 6, 5, 4, and 3.5, respectively) was also investigated. Hydrogels were formed in 20 min at pH 5 (Supporting Information, Figure S1 b) and 40 min at pH 4 (Supporting Information, Figure S1 c) after allowing the solution to stand. The formation time of protein hydrogel was increased with the increase of pH (Supporting Information, Figure S1 d,e). However, at low pH of 3.5, no hydrogelation was observed even after 24 h of standing still (Supporting Information, Figure S1 f). These results suggest that the pH of the solution plays an important effect during imine formation. On one hand, the free H^+ facilitates this reaction. Therefore, from pH 8 to 5 the reaction rate was promoted with the increase of H^+ concentration. On the other hand, when the H^+ concentration is too high, the amino group would be protonated, and the nucleophilic attack occurs too slowly to inhibit the formation of the imine bond, leading to the reaction inefficiency. This is consistent with that observation where no hydrogelation took place at pH 3.5.

Next, the activity of the GOX in the hydrogel system was tested. The mechanism of the enzymatic catalysis is shown in the Supporting Information, Figure S2. To test the catalytic activity, hydrogel systems with different concentration of glutaraldehyde were employed in the catalytic system (Supporting Information, Figure S3). It can be seen from the experimental results that the activity of the GOX was gradually reduced with the increase of the glutaraldehyde content, but it still maintains a certain degree of activity under the hydrogel state. The gluconic acid can still be generated by the glucose/glucose oxidase system to regulate the pH of the system. At the same time, we also tested the relative GOX activity versus different pH values at 25 °C (Supporting

Information, Figure S4). The results show that the GOX remains in relatively stable catalytic activity with the change of pH from 3.5 to 7.5. The pH values versus time with different glutaraldehyde concentrations in the protein hydrogel system was also explored (Supporting Information, Figure S5).

Hydrogel systems with or without GOX were prepared to investigate whether the addition of GOX would promote gelation. While the protein hydrogels were formed only in the presence of 0.01 wt% GOX (Supporting Information, Figure S6a), we found that the pH value decreased from 6.5 to 5.5 and the solution color gradually turned deep yellow-brown during the formation process of protein hydrogel. In this case, the formed hydrogel was breakable with low elasticity. This is probably due to the oxidation of a part of imine bond caused by H_2O_2, resulting in the damage of the net structure of protein hydrogel. To demonstrate this conjecture, 0.05 wt% CAT was added to the above system to catalyze the decomposition of H_2O_2. As expected, the hydrogel color turned light (Supporting Information, Figure S6b) after the addition of CAT to clear out H_2O_2 and thus to prevent the oxidation of the imine bond. And we also test the activity of CAT in the hydrogel system with different concentrations of glutaraldehyde and different pH (Supporting Information, Figures S7 and S8, respectively). The result indicated that CAT could keep high activity in the protein hydrogel system.

The self-healing property of the cross-linked protein hydrogel was investigated under a closed environment to minimize water evaporation at room temperature. At first, the self-healing property was demonstrated by connecting two pieces of protein hydrogel into one piece (Figure 2). The

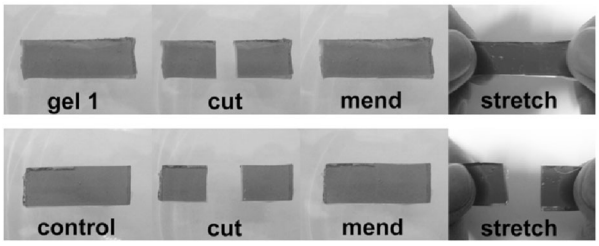

Figure 2. Photographs of the self-healing behavior of the protein hydrogel at room temperature: original, cut, mend, and stretched states, respectively.

gel was prepared in pure water with the protein concentration (12 wt%, GOX, CAT, and BSA for 0.1%, 0.5%, 11.4%, respectively), and the glutaraldehyde concentration was 0.5 wt%, and then the pH of the solution was adjusted to 7.0. The control gel was prepared under the same conditions only without GOX nor CAT. To allow the reaction reach the equilibrium, the mixture reacted for 20 h. As depicted in Figure 2, the gel samples with good visibility were prepared and cut with a blade into two pieces which were then brought together with a little pressure to ensure the surfaces were fully in contact. The contact samples with addition of glucose (0.2 mg) were kept at room temperature. After 5 h, self-healing of the contacted sample could be observed and the scars had almost disappeared. Even when manually stretching the sample, no rupture occurred. A similar self-healing test was performed on the sample without GOX or CAT; the control gel could not be coalesced and can be easily separated into two pieces by manually stretching the sample. When the coalesced gel was cut into separated pieces along the scar again and then put them together closely as before, the self-healing process occurred once again, proving that this process could be repeated.

Then the self-healing property of the gel in scars recovery was tested (Figure 3). It can be seen that when the gels were broken, after self-healing for 5 h, a very significant difference between the samples was observed in the present of GOX and CAT and without GOX nor CAT. After 5 h, the samples with GOX and CAT were almost completely repaired, but the samples without GOX nor CAT were repaired at a very low level.

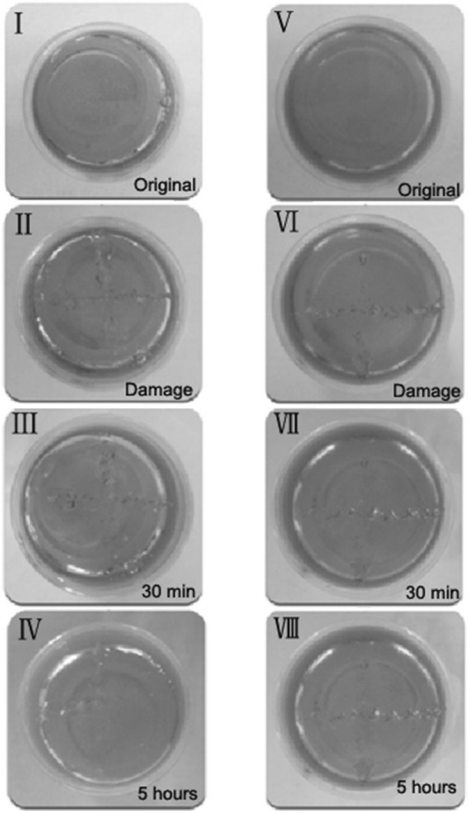

Figure 3. Photographs of the self-healing behavior of the protein hydrogel at room temperature; I–IV are the photographs of protein hydrogel with dual enzyme, I) original, II) after damage, III) after standing for 30 min, IV) after standing for 5 h, V–VIII are photographs of protein hydrogel without dual enzyme, V) original, VI) after damage, VII) after standing for 30 min, and VIII) after standing for 5 h.

Finally, the tensile tests were performed to quantitatively evaluate self-healing properties of the original hydrogels and repaired samples with GOX/CAT and without GOX nor CAT (Figure 4). The healing process was performed as described before when connecting the two pieces of protein hydrogel into one piece. Figure 4a shows typical stress-strain curves for

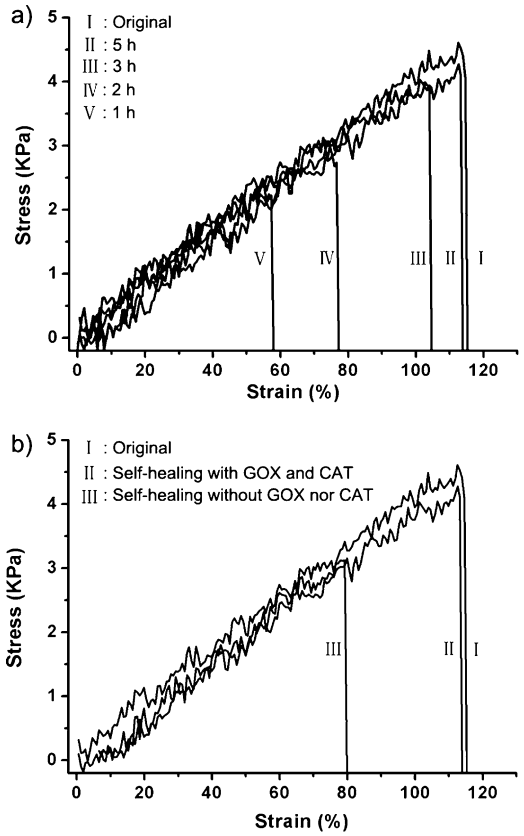

Figure 4. Typical stress–strain (stress–stretch ratio) curves of protein hydrogels. a) Self-healing with dual enzymes. I): Original; II)–V): the protein hydrogels were healed for different times. b) Self-healing with dual enzymes and without enzymes. The samples were cut into completely separate pieces using a razor blade and the cut faces were gently brought together with glucose at room temperature for different times.

the original self-healed samples with GOX/CAT and without GOX nor CAT after healing for different time. An important result is that the healed sample with GOX and CAT did not break at the contacted surface under the tensile testing. This observation indicated that the break stress and strain of the sample with added glucose can reach 100% of the original strength and elongation abilities, but the sample without GOX nor CAT only achieves 50% of the healing efficiency (Figure 4b). The mechanism of this self-healing property is related with the equilibrium of the imine bond formation, the reaction can be promoted forward by dual enzymes synergetic catalysis. The stretch experiments demonstrated that the GOX protein hydrogel without CAT exhibited a slightly higher tensile strength than that in the presence of CAT, but its self-healing ability is lower, only 75% recovery (Supporting Information, Figure S9). This is mainly due to its relatively lower reversibility resulted from the oxidation of a part of the imine bond without CAT.

In conclusion, we have prepared the healable dual-enzyme-mediated protein hydrogels and demonstrated that the self-healing nature of dynamic imine bond cross-linked chemical gels. The protein hydrogels exhibit excellent self-healing properties, which not only can be visually seen, but can also be studied by mechanical test, showing 100% recovery. The self-healing process was reversible and effective with an external glucose stimulus at room temperature. Moreover, the pH of this system was moderately adjusted by two enzymes: glucose oxidase and catalase. The study of the self-healing protein hydrogels by enzymatic manipulation of dynamic covalent bonds will bring light to the properties of dynamic and smart materials and will stimulate the development of the biocompatible materials that would have potential applications in biological repair smart materials.

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