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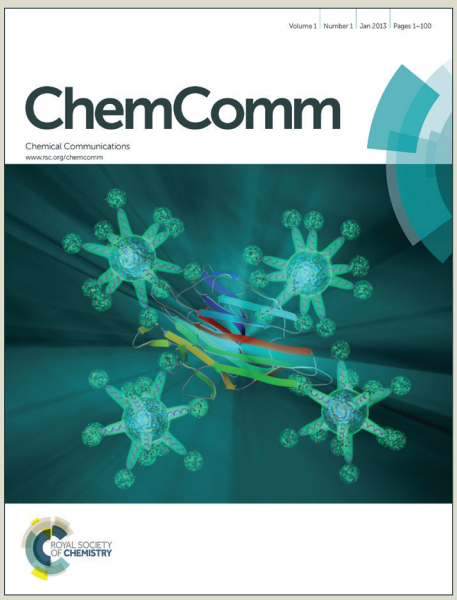

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In Situ Forming Enzyme-free Hydrogels via Ferromagnetic Microbeads-assisted Enzymatic Cross-linking

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In situ **formation of horseradish peroxidase (HRP)-free gelatin hydrogels was achieved via ferromagnetic microbeads-assisted enzymatic cross-linking. Gelation time and mechanical stiffness of the hydrogels could be tuned *in situ*, which make HRP-free gelatin hydrogels suitable for injectable cell delivery.**

In situ forming enzymatically cross-linked hydrogels (ECH) have recently received much attention as one of the most fascinating injectable platforms in biomedical fields.[1] Among several enzymatic cross-linking systems producing ECH, a horseradish peroxidase (HRP)-catalyzed cross-linking reaction has been extensively employed. The most attractive feature of the HRP-catalyzed cross-linking reaction is that various hydrogel properties such as gelation time, stiffness and degradation rate are highly tunable *in situ*,[2] which makes ECH highly beneficial for regulating cellular functions *in vitro* and *in vivo*. To date, significant progress has been made in developing HRP-based ECH for tissue engineering[3] and drug delivery applications.[4] Although many have shown great promise for the future, some key challenges for successful clinical translation of ECH remain.

The HRP cross-linking system allows rapid formation of ECH when HRP, hydrogen peroxide (H_2O_2), and phenol-rich polymers meet together in aqueous media. During the gelation, HRP and H_2O_2 are inevitably incorporated within hydrogels. Although most of H_2O_2 used in the reaction is converted to water, its excessive use may lead to a cytotoxic effect resulting in apoptosis of encapsulated cells.[5] This issue can be resolved using a minimum amount of H_2O_2 required for gelation.[2c] Rather, a major challenge involves using a plant-derived HRP that is a prerequisite for the formation of ECH but is not yet clinically available.[6] Moreover, a few reports have described adverse effects of HRP itself.[7] As the incorporated HRP that may potentially raise safety issues could be a major hurdle to the success of clinical translation of ECH, it is imperative to develop ECH not possessing HRP.

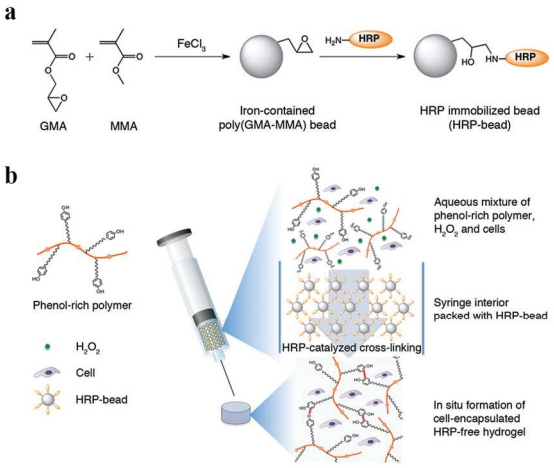

Fig. 1. (a) Surface immobilization of HRP onto ferromagnetic microbeads. (b) *In situ* formation of cell-encapsulated HRP-free hydrogels.

Herein we report a new enzymatic cross-linking strategy based on HRP immobilized ferromagnetic beads (HRP-beads) to produce HRP-free hydrogels. We hypothesized that HRP-free hydrogels might be formed when a phenol-rich polymer solution containing H_2O_2 was injected through a syringe packed with HRP-beads (Fig. 1). To test this concept, we synthesized a few hundred micrometer-sized ferromagnetic beads as a solid support for HPR immobilization so that cells of 10−100 μm in diameter could pass through void spaces between the microbeads packed inside a syringe. Ferromagnetic microbeads of poly(glycidyl methacrylate-co-methyl methacrylate) (poly(GMA−MMA)) with 300-500 μm in diameter were prepared as previously reported,[8] followed by immobilizing

HRP onto the microbeads through epoxy-amine coupling to obtain HRP-beads (Fig. 1a). Consequently, a series of HRP-beads were prepared by adding different amounts of HRP and iron (III) chloride (See details in ESI). It was found that the immobilized amounts of HRP decreased slightly as the iron content increased (Table S1). We also observed that the amount of immobilized HRP could be increased with increasing the amount of added HRP (Table S2).

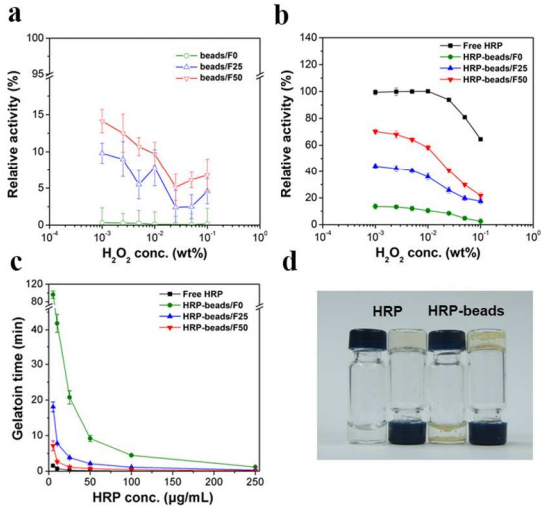

Fig. 2. H_2O_2-dependent catalytic activity of (a) ferromagnetic microbeads with varied iron content and (b) HRP-beads. The catalytic activity of HRP was set to 100% for comparison. (c) Gelation time of GPT with varying HRP concentrations. (d) GPT hydrogels formed after mixing with HRP or HRP-beads/F25.

Next, catalytic activity of HRP-beads toward H_2O_2 was measured using 2,2′-azino-bis(3-ethyl-benzothiasoline-6-sulfonate) (ABTS) as a chromogenic substrate. This assay monitors changes in the absorbance of the radical cation form of ABTS oxidized as a result of consumption of H_2O_2 by HRP,[9] thus identifying enzymatic capability of materials used for HRP-catalyzed crosslinking. Note that 0.001 wt% H_2O_2 is the minimum concentration where soluble HRP exerts favorable catalytic activity. As shown in Fig. 2a, it was found that ferromagnetic microbeads themselves exhibited intrinsic catalytic activity depending on the iron content. This might be probably due to peroxidase-like activity of iron oxide particles incorporated in the microbeads.[10] Fig. 2b shows H_2O_2-dependent activity of HRP-beads with varied iron contents but similar HRP amounts. The soluble HRP had the highest activity but increasing H_2O_2 concentrations caused a gradual decrease in activity, which is consistent with previous reports demonstrating that a high concentration of H_2O_2 impedes a HRP-catalyzed crosslinking reaction.[11] Interestingly, we found that surface immobilization of HRP onto ferromagnetic microbeads could lead to pronounced increases in the catalytic activity. Although the HRP-beads/F50 had the lowest amount of immobilized HRP (0.75 ± 0.09 mg of HRP/g of HRP-bead), the value at 0.001 wt% H_2O_2 was nearly 5-fold higher than the beads/F50. Other HRP-beads with lower iron contents also showed similar trends. This result indicates that there is a significant correlation between the iron content in microbeads and HRP activity for facilitating HRP-catalyzed cross-linking reactions.

Fig. 2c shows the dependence of gelation time on the HRP concentration. Previously we have shown that aqueous gelatin-poly(ethylene glycol)-tyramine (GPT) solutions can be cross-linked and formed a hydrogel upon mixing with HRP and H_2O_2.[12] It is also reported that higher HRP concentration leads to faster gelation, whereas higher H_2O_2 concentration makes gelation slower. In the gelation test, different amounts of HRP-beads with varied iron contents were mixed with aqueous solutions of 5 wt% GPT containing 0.01 wt% H_2O_2, as shown in Fig. 2d. Although the gelation rate of GPT triggered by HRP-beads was initially much slower compared with soluble HRP, we were able to accelerate it by increasing the amount of HRP-beads. In addition, GPT hydrogels were formed most rapidly by mixing with the HRP-beads/F50. These results suggest that the amount of HRP-beads as well as their iron content would play a significant role in the HRP-catalyzed gelation process. The following experiments, therefore, were carried out using the HRP-beads/F25 with approximately 40% activity (1.03 ± 0.18 mg of HRP/g of HRP-bead) and 0.01 wt% H_2O_2, allowing easy operability of enzymatic gelation using a syringe as we hypothesized earlier. Further characteristics of HRP-beads/F25 were described in ESI (Fig. S1 and S2).

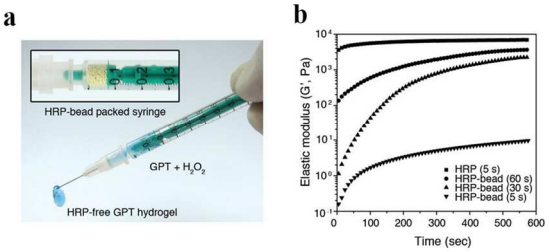

Fig. 3. (a) HRP-free GPT hydrogel prepared by using a HRP-beads/F25 (20 mg) packed syringe. Toluidine blue was mixed with GPT solutions for easy visualization of the transparent hydrogel. (b) Time-course elastic modulus of GPT hydrogels formed using a HRP-beads/F25 packed syringe. An equivalent amount of HRP (20 μg) was used as a control.

An aqueous mixture of 5 wt% GPT and 0.01 wt% H_2O_2 was loaded into a syringe packed with 20 mg of HRP-beads/F25 (Fig. 3a). Prior to the solution loading, HRP-beads/F25 was first hydrated with PBS to increase mobility of immobilized HRP in aqueous environment. The time-course elastic modulus of GPT solutions injected through a syringe packed with HRP-beads/F25 was recorded (Fig. 3b). In the case of soluble HRP, the immediate gelation was observed upon injection, reaching a plateau of 6900 Pa. In contrast, GPT hydrogels cross-linked by HRP-beads/F25 showed relatively slow increases in the elastic modulus, and were dependent on the injection time intervals. The elastic modulus of the GPT solution injected after 5 s was increased steadily and reached 10 Pa after 10 min, indicating that immediate injection was not sufficiently long enough to trigger gelation. When the GPT solution was injected after 30 s, the elastic modulus considerably increased and reached 2200 Pa. The hydrogel with a much higher modulus (3700 Pa) was obtained by injecting after 60 s. We also confirmed that increasing elastic modulus leads to more compact structures of hydrogels (Fig. S3). In general, a conventional enzymatic cross-linking system is based on a homogeneous catalytic reaction where three components (HRP, H_2O_2, and phenol-rich polymer) interact, generate radicals, and induce cross-linking. In our system, HRP-beads would probably lead to an interfacial cross-linking reaction. Thus, the HRP-catalyzed cross-linking reaction can be initiated only when a polymer solution containing H_2O_2 makes contact with HRP-beads. The number of radicals generated by HRP-beads is most likely to affect the contact time-dependent elastic modulus. Our result clearly demonstrated that the contact time between HRP-beads/F25 and GPT plus H_2O_2 plays a crucial role for enzymatic cross-linking.

To verify whether HRP was incorporated inside the formed GPT hydrogels, FITC-HRP was covalently bound to the beads by using the same immobilization protocol, and subjected to *in situ* gelation by using a FITC-HRP-beads/F25 packed syringe. Additionally, rhodamine phalloidin (Rho) was simply mixed with an aqueous mixture of GPT and H_2O_2 to visualize gel matrices under a fluorescence microscope. FITC-HRP-beads had a strong green

fluorescence (Fig. 4a, right inset image), indicating that FITC-HRP was successfully bound to bead surfaces. We prepared two kinds of GPT hydrogels using FITC-HRP and FITC-HRP-beads/F25, respectively. They both showed bright red fluorescence of Rho in gel matrices. The hydrogel prepared by soluble FITC-HRP had green fluorescence all over the matrix, demonstrating the presence of HRP in the hydrogel. However, no green fluorescence was observed in the hydrogel prepared by HRP-beads/F25. We found that the threshold concentration of HRP required for gelation was more than 100 ng/mL (Fig. S4). However, the rate of gelation was very slow. The elastic modulus of the hydrogel formed at this concentration was about 13 Pa, which was much lower than that of the hydrogel obtained by injecting after 30 s. These findings strongly support that immobilized HRP could catalyze a cross-linking reaction of GPT only during injection to form a HRP-free hydrogel. hDFBs encapsulated in two differently prepared GPT hydrogels were cultured in vitro for 7 days (Fig. 4b). We previously demonstrated that three different cells (MC3T3−E1, hMSCs and hDFBs) were viable for 24 h when cultured on the HRP-containing GPT hydrogels.[12] Moreover, it appeared that the encapsulated hDFBs were highly proliferative in the GPT hydrogel with a lower stiffness. Although the results showed that the incorporated HRP seems irrelevant to retaining cell viability, it may be problematic *in vivo* as we addressed earlier. Therefore, it is important to confirm that HRP-free hydrogels also can be used as an injectable cell delivery carrier. The experiment was carried out using 3 wt% GPT to make hydrogels less stiff, and two GPT hydrogels of similar stiffness (2000 Pa) were formed by either soluble HRP and HRP-beads/F25. Fig. 4c shows that cells were highly proliferative in both hydrogels, having no statistical differences. In addition, confocal images of hDFBs taken after 7 days of culture exhibited spindle-shaped cells in both hydrogels (Fig. 4c).

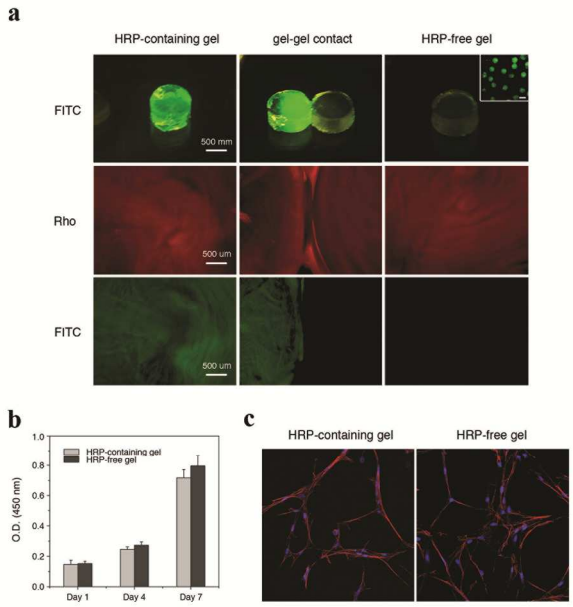

Fig. 4. (a) Fluorescent detection of HRP in GPT hydrogels prepared by using soluble FITC-HRP and FITC-HRP-bead (right inset image, scale bar = 500 μm). (b) Viability of hDFBs cultured in HRP-containing or HRP-free gel. (c) Representative confocal images of hDFBs in HRP-containing or HRP-free gels after 7 days of culture.

Conclusions

In summary, we have developed a new enzymatic cross-linking method based on HRP-beads to prepare HRP-free hydrogels. The obtained results support our hypothesis that HRP-beads can initiate an enzymatic cross-linking reaction upon contact with GPT solutions in the presence of H_2O_2. In addition, we showed that the iron content in microbeads contributes greatly to the HRP-catalyzed gelation process. The gelation time and mechanical stiffness could be controlled by the amount of HRP-beads as well as their contact time. Most importantly, HRP was not detected in the GPT hydrogels formed *in situ* by HRP-beads. *In vitro* cell studies revealed that the HRP-free GPT hydrogel could serve as a bioactive injectable matrix for cell delivery. We thus expect that the interfacial *in situ* enzymatic cross-linking system using HRP-beads can be used as a certain alternative to the conventional method that has been widely used to prepare ECH.

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Notes and references

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