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Enzymatic preparation of a redox-responsive hydrogel for encapsulating and releasing living cells

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Horseradish peroxidase-mediated oxidative cross-linking of a thiolated poly(ethylene glycol) is promoted in the absence of exogenous hydrogen peroxide, by adding a small amount of phenolic compound under physiological conditions. The prepared hydrogel can encapsulate and release living mammalian cells.

Hydrogels have received much attention in biopharmaceutical, biomedical, and bioengineering applications, because of their useful properties including high water content, high permeability, and biocompatibility.[1, 2] Various approaches have been used to prepare hydrogels. Enzyme-mediated hydrogelation has received recent attention,[3] because it can be conducted under mild conditions compatible with drugs, proteins, and living cells. Horseradish peroxidase (HRP)-catalyzed gelation systems have been proposed as an effective method for preparing hydrogels.[4-6] HRP is an enzyme that efficiently catalyzes the radical coupling of phenol and aniline derivatives, with the aid of exogenous hydrogen peroxide (H_2O_2).[7, 8] Aqueous H_2O_2 is usually directly supplied into the reaction system, to activate HRP for the enzymatic hydrogelation of phenol-containing polymeric substrates. Excess H_2O_2 can inactivate HRP, because of the formation of the inactive intermediate, compound III.[9] Directly supplying aqueous H_2O_2 may also negatively affect either the homogeneity of the resultant hydrogel network, or the activity of biological entities subsequently encapsulated in hydrogel.

An HRP-mediated cross-linking method requiring no added H_2O_2 has been proposed. Sakai and coworkers reported that the glucose oxidase (GOx) catalysis of glucose generated and supplied H_2O_2, for the HRP-mediated hydrogelation of an aqueous phenolated polymer.[10] GOx is an oxidoreductase that catalyzes the oxidation of glucose to H_2O_2 and glucono-δ-lactone. In the reported system, H_2O_2 was gradually generated by the GOx-catalyzed oxidation of glucose, and generated H_2O_2 was rapidly consumed by the HRP-catalyzed *in situ* hydrogelation. The inactivation of HRP was suppressed, and the obtained hydrogel had excellent mechanical properties because of its high cross-linking density.[11] Groll and coworkers reported the HRP-mediated preparation of a redox-sensitive disulfide-cross-linked hydrogel, without requiring exogenous H_2O_2.[12] H_2O_2 was formed early in the reaction, by the auto-oxidation of thiol substrates under aerobic conditions. HRP catalysis was subsequently initiated by the generated H_2O_2, producing thiyl radicals from the reaction of thiol and thiolate. Disulfides were formed after reaction with molecular oxygen. Additional H_2O_2 was not needed, because H_2O_2 was generated in the HRP cycle during disulfide bond formation. This gelation system is very simple, requiring only the mixing of aqueous HRP and thiolated polymer solutions to trigger hydrogel formation. However, it requires basic pH conditions (pH 8.5). The gelation time of the polymer solution is also slow (> 110 min), even with a high HRP concentration and a high concentration of polymeric substrate (30 wt.%). This was possibly because of an extremely low bimolecular rate constant for the reaction of activated HRP with thiol substrates to generate thiyl radicals, compared with that of phenolic substrates. For example, the second-order rate constants for cysteine with HRP reactive intermediates, compounds I and II, are 240 and <50 M^{-1} s^{-1}, respectively.[8]

Herein, in this study we report a dramatic enhancement of the HRP-catalyzed hydrogelation of polymeric thiol substrates. This yields a redox-responsive hydrogel, that can effectively encapsulate and release living cells.

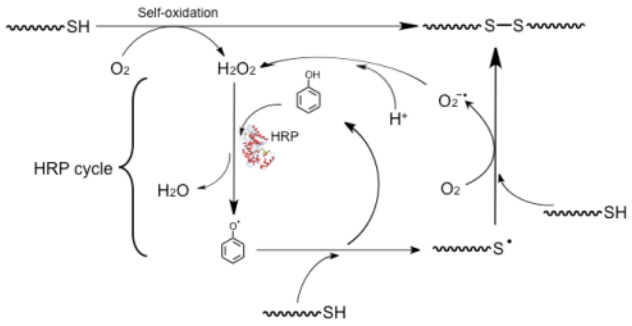

Figure 1. Proposed scheme of the HRP-mediated cross-linking of the thiolated polymer.

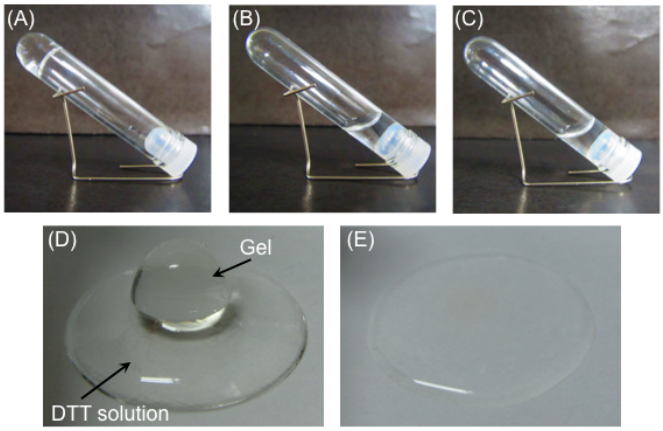

Figure 2. Photographs of (A–C) 4-arm PEG-SH solutions (pH 7.4) (A) in the presence of HRP and tyramine, and in the absence of (B) HRP or (C) tyramine, (D, E) 4-arm PEG-SH hydrogel (D) just after and (E) after 15 min soaking in DTT solution.

The concept is based on phenolic compounds promoting the HRP-catalyzed thiol oxidation.[8] During the HRP-catalytic cycle in the presence of thiol and phenol, the single electron oxidation of thiol occurs readily with enzymatically generated phenol radicals. Thiol radicals are then transformed into disulfides, after the reaction with molecular oxygen. The rate constants of the reaction between HRP compound II and the phenolic compounds are typically 10^3–10^7 M^{-1} s^{-1}.[13] The rate constant for the reaction between phenol radicals and thiols at pH 7.15 is reportedly 2×10^6 M^{-1} s^{-1}, when using tyrosine and glutathione as model substrates.[14] These rate constants are much higher than that of the reaction between HRP compound II and thiol. This means that phenol compounds should accelerate the HRP-mediated fabrication of disulfide-cross-linked hydrogels, without exogenous H_2O_2 (Fig. 1). A 4-arm poly(ethylene glycol) derivative possessing thiol moieties (4-arm PEG-SH, M_w: 20,000) was selected as the polymer backbone. Poly(ethylene glycol)-based hydrogels has been considered as potential materials for tissue engineering, regenerative medicine and drug delivery system due to their excellent biocompatible properties.[15] PEG-based hydrogels can be chemically functionalized by modifying the polymer networks, and thiol chemistry has proven to be effective for introducing biologically active entities such as synthetic peptides showing specific interactions with cells.[16, 17]

First, we selected tyramine as a phenolic promoter, and validated its potential in the hydrogelation of 4-arm-PEG-SH. An aqueous solution of 5 wt.% 4-arm PEG-SH was mixed with tyramine (5 mM) and HRP (5 U/mL) in phosphate-buffered saline (PBS, pH 7.4). The final mixture yielded a clear hydrogel (Fig. 2A). Nonetheless, polymer solutions did not render hydrogels within 24 h, in the absence of HRP or tyramine (Fig. 2B and 2C, respectively). The gelation of the polymer solution was probably prevented by the addition of catalase to the reaction system (Fig. S1†). This suggested that HRP was activated by H_2O_2 generated *in situ*, by the self-oxidation of thiol moieties. The obtained hydrogel was completely degraded by soaking in aqueous dithiothreitol (DTT) (Fig. 2D and 2E). We have also checked for the presence of free thiol groups using Ellman's method.[18] 5,5'-dithio-bis(2-nitrobenzoic acid) shows an absorbance at 412 nm after its reaction with –SH groups. The obtained hydrogel exhibited a very low absorbance at 412 nm, whereas the polymer solution before hydrogelation exhibited intense absorbance at this wavelength. The absorbance at 412 nm was completely recovered after the reductive degradation of the hydrogel using DTT (Fig. S2†). These results indicated that disulfide-bond formation was promoted by the HRP-catalyzed oxidation of tyramine. Other phenolic promoters including phenol, Gly-Tyr, resorcinol, and serotonin also resulted in gel formation (Fig. S3 and S4†).

We then investigated the gelation time of the polymer solution using different phenolic compounds (5 mM) at room temperature (*ca.* 22 °C). The gelation time varied according to which phenolic compound was added to the reaction (Table S1†). The shortest gelation time of ~30 min was observed using tyramine. Gelation using serotonin required >8 h. The reason for varying gelation times with phenolic compounds remains unclear. It may be attributed to the substrate specificity of HRP, and/or the stability of phenolic radicals generated by each phenolic compound. Figure 3 shows the effect of HRP and tyramine concentration on gelation. Gelation time increased with increasing HRP concentration. We considered that the phenol radical dimerization could be one of the reasons of increasing the gelation time. In fact, we observed an increase in the fluorescent intensity derived from the formation of di-tyramine using a small molecular model substrate (glutathione, GSH) at a higher HRP concentration (Fig. S5†). The gelation time decreased as the tyramine concentration increased to up to 50 mM. This suggests that tyramine radicals effectively promoted the gelation. The shortest gelation time was 26.4 ± 1.6 min at 5 U/mL HRP and 50 mM tyramine. Further increasing the tyramine concentration to 100 and 200 mM has slightly increased the gelation time to 27.1 ± 1.2 and 34.8 ± 1.1 min, respectively. Although we haven't had clear answer for this phenomenon yet, it was reported that the phenol radical coupling efficiency strongly depends on the relative concentrations of HRP, thiol and phenol in a reaction system.[8] Thus, controlling the phenol coupling reaction was important for efficient gelation. In terms of polymer concentration, gelation time was approximately the same (~ 30 min) in the range of 5 – 15 wt% 4-arm PEG-SH (Table S2†). This result hints that the coupling reaction between thiyl radicals would not be a rate-limiting step in gelation. The physical properties of the resultant hydrogel varied according to the polymer concentration. The storage modulus (G') of the hydrogel increased with increasing polymer concentration (Fig. S6†) and the equilibrium swelling ratio (Q_M) decreased with increasing polymer concentration (Table S3†). These results can be attributed to the increase of the cross-linking density as increasing the 4-arm PEG-SH concentration. In addition, the gel content of cross-linked hydrogels was >80% for all hydrogels prepared (Table S3†).

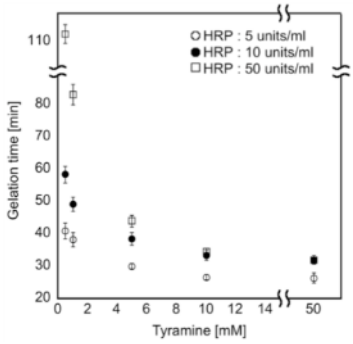

Figure 3. Effect of HRP and tyramine concentration on gelation time of the polymer solution at pH 7.4. Bars indicate standard deviations of n=3.

The present system provides gelation conditions suitable for encapsulating biological entities. Under physiological conditions (pH 7.4), simply mixing solutions of thiolated polymers, HRP, and phenolic compounds yields transparent hydrogels. The efficacy of thiol auto-oxidation

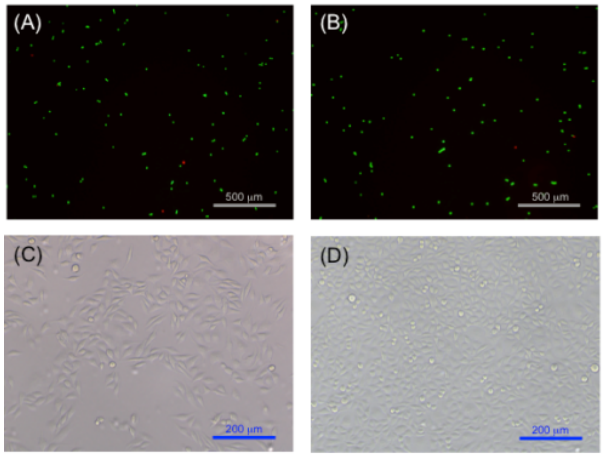

Figure 4. Photographs of L929 cells (A, B) encapsulated in 4-arm PEG-SH hydrogel after (A) 3 and (B) 24 h of cultivation, and (C, D) at (C) 4 and (D) 48 h after re-plating to the cell culture dish, after recovering from the 4-arm PEG-SH hydrogel by Cys reduction. Live and dead cells in (A, B) exhibit green and red fluorescence, respectively.

to produce H_2O_2 is reportedly as very low at neutral pH.[19] This prevents the hydrogelation of thiolated polymers under physiological pH. In our system, the catalytic HRP cycle triggered by a small amount of H_2O_2 was significantly amplified in the presence of a phenolic compound. As a result, the phenolic compound promoted the gelation of 4-arm PEG-SH, and the required polymer concentrations could be significantly lowered to 5 wt.%.

Disulfide-cross-linked hydrogels are easily decomposed by cleaving the disulfide bond (–S–S–) to thiol groups (–SH HS–) with reductants. The degradability of the 4-arm PEG-SH hydrogel was characterized by measuring the change in hydrogel weight after soaking in aqueous L-cysteine (Cys) at 37 °C. Cys was used as the reductant, because it promotes the degradation of disulfide-cross-linked hydrogels under mild conditions.[20] All hydrogels completely degraded in the presence of Cys. No significant change in weight was observed when incubated in PBS alone (Fig. S7†). The time required for the complete degradation of hydrogels decreased with increasing Cys concentration. Cys concentrations of > 5 mM were sufficient to completely degrade 4-arm PEG-SH hydrogels within 30 min. The controllable gelation and degradation properties of the hydrogel are likely to be useful for drug delivery carriers and three-dimensional cell-culture scaffolds.

We investigated the cytocompatibility of the obtained redox-responsive hydrogel. L929 fibroblasts cells were mixed with 4-arm PEG-SH, HRP, and tyramine in PBS. After incubation at 37 °C for 1 h, the cells were encapsulated in the disulfide-cross-linked hydrogel. Figure 4A and 4B show fluorescence images of encapsulated L929 cells, after 3 and 24 h of cell culture, respectively. Cells were stained with fluorescent dyes within the hydrogel. The viabilities of encapsulated cells, calculated from the number of living (green fluorescence) and dead (red fluorescence) cells were 98.2 ± 0.5 and 98.9 ± 0.1% (mean ± standard deviation, n = 3) after 3 and 24 h of cell culture, respectively. A reported two-step approach for cell encapsulation involved cells sandwiched between two layers of hydrogel.[12] The present system encapsulated living cells within the hydrogel via a one-pot preparation. Hydrogelation in the previous report required high HRP concentrations (*ca.* 1400 U/mL, as calculated based on the HRP activity of 320 U/mg and enzyme concentration of 4.4 mg/mL[12]). The HRP concentration required in the present hydrogel was significantly lower (5 U/mL). These results are attributed to the HRP catalytic cycle being effectively amplified by the addition of phenolic compounds. Ultimately, we have explored the recovery of encapsulated cells from the 4-arm PEG-SH hydrogel. Cys solution (5 mM) was poured on the hydrogel containing the cells, and the viability and morphology of the released cells were evaluated after 30 min of incubation. The viability of the recovered cells as determined by trypan blue exclusion was 98.7 ± 0.5% (n = 3). After 4 h of seeding onto a cell culture dish, almost all of the released cells had adhered to the dish. The morphology of the spreading cells was comparable to that of untreated cells (Fig. 4C). Further cultivation of the adhered cells indicated the continued growth after 48 h (Fig. 4D). Thus, the gelation and degradation processes were sufficiently mild for compatibility with mammalian cells.

In conclusion, the role of phenolic compounds in the HRP catalytic cycle for preparing redox-sensitive disulfide-cross-linked hydrogels was demonstrated. The gelation and degradation processes can be kinetically controlled by the appropriate experimental conditions. The obtained hydrogel was validated as a potential matrix, for encapsulating and releasing mammalian cells. These results suggest that this gelation system has potential in biotechnological applications.

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Notes and references

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† Electronic Supplementary Information (ESI) available.

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