ACS Macro Letters

Letter

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Injectable Degradation-Resistant Hyaluronic Acid Hydrogels Cross-Linked via the Oxidative Coupling of Green Tea Catechin

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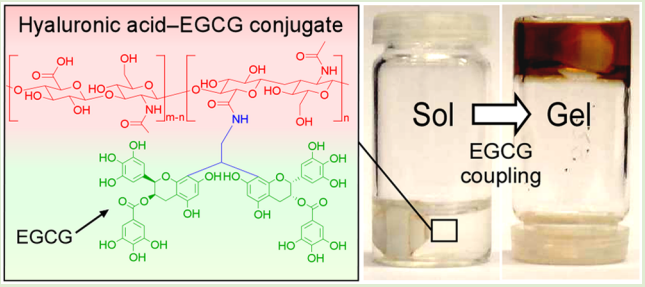
Supporting Information

ABSTRACT: The oxidative coupling of phenols by horseradish peroxidase (HRP) is widely utilized to cross-link polymer−phenol conjugates for hydrogel formation. Phenols containing one aromatic ring are most commonly used, and the addition of hydrogen peroxide (H_2O_2) is an indispensable step in catalyzing the enzymatic reaction. We describe here a hydrogel composed of polyphenol as the cross-linking moiety. (−)-Epigallocatechin-3-gallate (EGCG), a green tea catechin, was conjugated to hyaluronic acid (HA) to form HA-EGCG conjugates. Addition of HRP to a solution of HA-EGCG conjugates at pH 7.4 induced gelation in 7 min. Notably, the addition of exogenous H_2O_2 was not required, as H_2O_2 was generated via EGCG autoxidation. Moreover, cross-linking between HA-EGCG conjugates occurred in situ through EGCG quinone formation, even when no HRP was added. This approach of forming hydrogels circumvented the safety concern associated with HRP due to its plant origin. Furthermore, the EGCG moieties endowed the hydrogels with resistance toward hyaluronidase-mediated degradation in vivo.



Hydrogels are physically or chemically cross-linked polymeric networks swollen in water. The 3D porous structure and aqueous environment of hydrogels rendered them particularly suitable for the encapsulation of cells and biomolecules for tissue engineering and drug delivery applications.[1] Among the various cross-linking strategies, horseradish peroxidase (HRP)-mediated oxidative coupling of phenols has received much attention.[2] HRP catalyzes the oxidation of phenols, generating phenolic radicals which are coupled through either a C−C linkage between the ortho-carbons of the aromatic ring or a C−O linkage between the ortho-carbon and the phenolic oxygen.[3] Capitalizing on this efficient enzymatic reaction, various polymers have been modified with simple phenols containing one aromatic ring, such as tyramine,[4] 3,4-hydroxyphenylpropionic acid,[5] and catechol,[6] in order to form chemically cross-linked hydrogels. The addition of hydrogen peroxide (H_2O_2) is an indispensable step in the preparation of hydrogels, as it is the oxidant for the enzymatic reaction cycle. The advantages of HRP-mediated hydrogel formation include in situ gelation at physiological conditions, with the gelation time ranging from a few seconds to several minutes and tunable cross-linking density.[7] In addition to catalyzing the oxidative coupling of phenols, HRP reacts with thiols in the presence of H_2O_2 to form thiyl radicals, which dimerize to form disulfide bonds. It was reported that HRP induced the gelation of a solution of thiol-functionalized linear poly(glycidol) (SH-PG) within 4 h.[8] In contrast, no gel was formed in the absence of HRP. Importantly, the addition of H_2O_2 was not necessary in this system because it was produced during the autoxidation of thiols under aerobic condition. This simplifies the gel preparation process, as it only requires the mixing of two components, namely, the thiol-functionalized polymers and HRP. However, HRP-mediated gelation of thiol-functionalized polymers was much slower compared with the phenol-functionalized counterpart. This was attributed in part to the lower rate constant for the reaction between HRP and thiol than that between HRP and phenol.[9] In this study, we describe a hydrogel that is formed by HRP-mediated coupling of catechins. This hydrogel system exhibits fast gelation that is typical of hydrogels formed via HRP-mediated coupling of phenols while, at the same time, avoiding the need for exogenous H_2O_2. Furthermore, we demonstrated that hydrogels could be formed through EGCG quinone formation in the absence of HRP.

Catechins are a group of polyphenols found in green tea, which are known for their anticancer and anti-inflammatory properties.[10] Among the various green tea catechins, (−)-epigallocatechin-3-gallate (EGCG) is the most abundant constituent.[11] EGCG undergoes autoxidation at aerobic condition, producing H_2O_2 as a byproduct (Figure S1a, Supporting Information).[12] The generated H_2O_2 could serve as the oxidant in HRP-mediated reaction (Figure S1b, Supporting Information). While it has been demonstrated previously that EGCG coupling occurred upon the addition of HRP, exogenous H_2O_2 was added concurrently.[13] Herein, we proposed that the addition of HRP alone would be sufficient to catalyze the cross-linking of polymer-EGCG conjugates via the coupling of

Received: August 4, 2015
Accepted: August 18, 2015
Published: August 21, 2015

DOI: 10.1021/acsmacrolett.5b00544
ACS Macro Lett. 2015, 4, 957−960

EGCG moieties (Figure 1a). To this end, hyaluronic acid−EGCG conjugates (HA-EGCG, Figure 1b) were synthe-

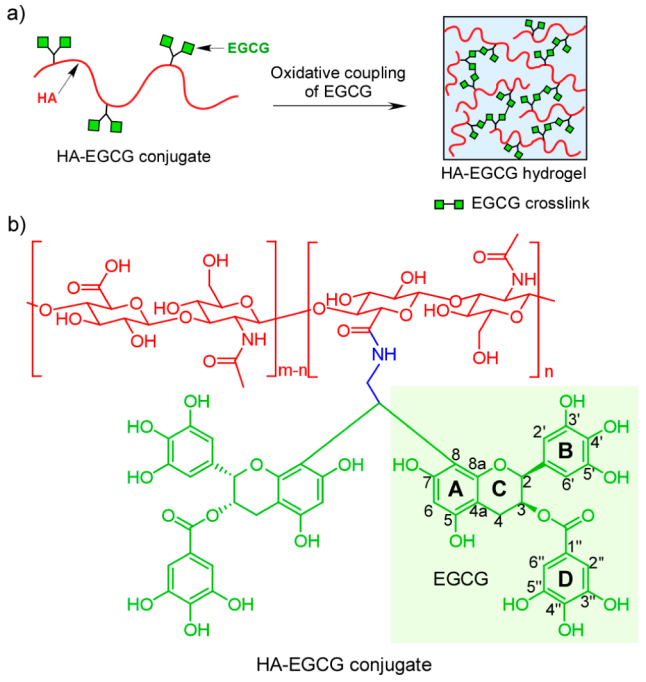


Figure 1. (a) Formation of hydrogels through the oxidative coupling of EGCG moieties. (b) Chemical structure of HA-EGCG conjugates.

sized for gel formation studies. HA is a nonsulfated glycosaminoglycan found in the extracellular matrix (ECM) and widely used to form hydrogels due to its low-immunogenicity, biodegradability, and versatility in chemical modification.[14] An aldehyde-mediated condensation reaction, which selectively reacts with the A ring of EGCG, was utilized to form ethylamine-bridged EGCG dimers.[15] For the conjugation to HA, EDC and NHS were utilized to facilitate amide bond formation between EGCG dimers and HA. For the isolation of the HA-EGCG conjugates, NaCl and ethanol were added to the reaction mixture to precipitate the conjugates. After centrifugation, the unconjugated EGCG dimers in the ethanol phase were decanted. The HA-EGCG conjugates were redissolved in H_2O and precipitated again for a total of three cycles. After which, the conjugates were dialyzed against H_2O and freeze-dried. The presence of aromatic protons in ^1H NMR spectroscopy of purified HA-EGCG conjugates confirmed the conjugation of ethylamine-bridged EGCG dimers to HA (Figure S2a). Conjugation was also confirmed by absorbance measurement (Figure S2b). A characteristic peak at 273 nm was observed, which indicated the presence of EGCG. The degree of substitution (DS, the number of EGCG dimers conjugated in every 100 disaccharide units) was determined based on the absorbance at 273 nm and was found to be 0.8.

First, we measured the amount of H_2O_2 generated by HA-EGCG conjugates. HA-EGCG conjugates were dissolved in deionized water before mixing with phosphate buffer to bring to a specific pH. Oxidation of EGCG by molecular oxygen occurs at the trihydroxyl B ring, which results in the formation of EGCG semiquinone radical and a superoxide radical ($^{\bullet}O_2^-$). The $^{\bullet}O_2^-$ can oxidize another EGCG molecule and generate H_2O_2 in the process (Figure S1a, Supporting Information).[12] Autoxidation of EGCG is known to depend on the pH of the medium; a decrease in pH prevents acid dissociation of the hydroxyl groups of B ring.[12b,c] Thus, the generation of H_2O_2 is inhibited at acidic condition. Indeed, H_2O_2 was produced by HA-EGCG conjugates at a much slower rate at pH 5 than pH 7.4 (Figure 2a). The concentrations of H_2O_2 generated by HA-

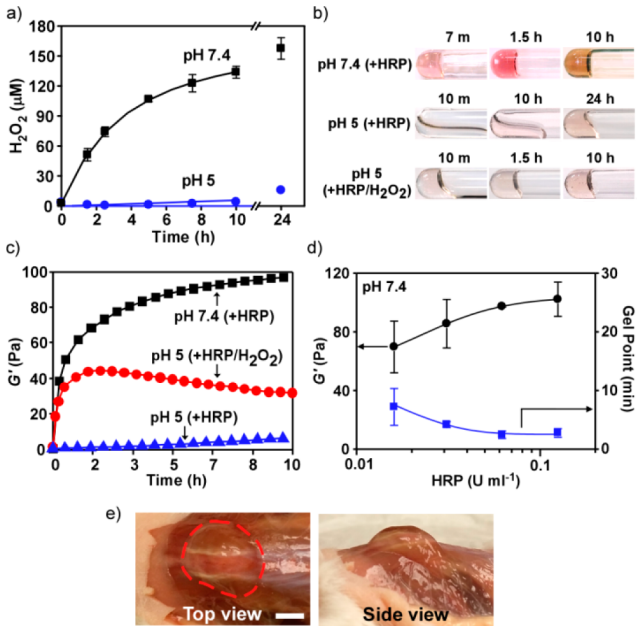


Figure 2. (a) Generation of H_2O_2 by HA-EGCG conjugates (1 mg mL^{-1}). (b) Photographs of HA-EGCG hydrogels (10 mg mL^{-1}) formed by adding HRP at pH 7.4 (first row), pH 5 (second row), or by adding both HRP and H_2O_2 at pH 5 (third row). (c) Evolution of G' of the corresponding hydrogels. (d) The effects of HRP concentration on the G' and gel point of hydrogels formed at pH 7.4 (n = 3, mean ± s.d.). (e) In vivo formation of HA-EGCG hydrogel with HRP at pH 7.4 (scale bar = 0.5 cm).

EGCG conjugates (1 mg mL^{-1}) after 24 h incubation were 15 and 150 μM at pH 5 and 7.4, respectively. Having confirmed that H_2O_2 is being produced by HA-EGCG conjugates at physiological pH, we proceeded to form hydrogels by adding HRP. When HRP was added to a solution of HA-EGCG conjugates (10 mg mL^{-1}) at pH 7.4, a hydrogel was formed within 7 min at 37 °C (Figure 2b, first row). Gelation time was determined by tilting the test tube occasionally until the solution stopped flowing. In contrast, when HRP was added at pH 5, a hydrogel was not formed until 24 h later (Figure 2b, second row). On the other hand, when exogenous H_2O_2 was added together with HRP at pH 5, a hydrogel was formed within 10 min (Figure 2b, third row). The results clearly demonstrated that the slow rate of H_2O_2 generation at pH 5 limited the rate of HRP-mediated cross-linking reaction. The color of the hydrogels darkened progressively. At 10 h, the hydrogel formed at pH 7.4 was reddish brown, which resembled the color of black (fermented) tea, indicating the presence of oxidized catechins.

Next, the gelation process was examined by oscillatory rheometry. The storage modulus (G') was lower than the loss modulus (G'') at the beginning of the measurement, except for the hydrogel formed at pH 7.4, in which crossover between G' and G'' occurred prior to the start of measurement (Figure S3, Supporting Information). At pH 7.4, G' increased to 80 Pa in 3 h, which was followed by a slower phase of increment, reaching

DOI: 10.1021/acsmacrolett.5b00544
ACS Macro Lett. 2015, 4, 957−960

100 Pa at 10 h (Figure 2c). At pH 5, G' increased more rapidly when both HRP and H_2O_2 were added than when only HRP was added. It was reported that the gelation rate of polymer−phenol conjugates increases with increasing HRP concentration.[5,7] Indeed, as the concentration of HRP increased, the gel point, defined as the crossover between G' and G'', of HA-EGCG conjugates at pH 7.4 decreased, while the G' at 10 h increased (Figure 2d). In order to demonstrate that the HRP-mediated gelation could likewise occur in vivo, a solution of HA-EGCG conjugates (10 mg mL^{-1}) at pH 7.4 was mixed with HRP (0.062 U mL^{-1}) and immediately injected into the subcutaneous tissue of mice. A hydrogel was successfully formed (Figure 2e).

One concern associated with the HRP-mediated injectable hydrogel system is the retention of the enzyme in the gel matrix, which can potentially induce immune reaction due to its plant origin. To overcome this issue, hematin-based HRP biomimetic has been developed as an alternative catalyst for the oxidative reaction.[16] In addition, enzyme-free hydrogels have been prepared by passing a mixture of polymer−phenol conjugates and H_2O_2 through a syringe packed of HRP-conjugated ferromagnetic microbeads.[17] It was reported that autoxidation of EGCG under aerobic condition results in EGCG semiquinone radical formation. When EGCG semiquinone radical is further oxidized by oxygen, EGCG quinone is formed, which leads to EGCG coupling via a linkage at the B rings (Figure S1c, Supporting Information).[12a] This suggests that cross-linking between HA-EGCG conjugates could potentially take place without HRP. To test this hypothesis, a solution of HA-EGCG conjugates at pH 7.4, containing no HRP, was added to a test tube and incubated at 37 °C. Gelation time was determined by tilting the tube periodically until a nonflowing gel was observed. The gelation time was 1.5 h (Figure 3a). The sol−gel transition was accompanied by a gradual change in color, from clear to pink to reddish brown, similar to hydrogels formed in the presence of HRP. However, rheological measurement showed that the G' increased at a slower rate compared with hydrogels that were formed in the presence of HRP at pH 7.4, reaching 50 Pa at 10 h (Figure 3b). Despite the slower rate of gel formation in the absence of HRP, when a solution of HA-EGCG conjugates (10 mg mL^{-1}) at pH 7.4 was injected subcutaneously, a hydrogel was successfully formed (Figure 3c). In contrast, a solution of HA-EGCG conjugates at pH 5 did not form a gel until 48 h (Figure S4, Supporting Information).

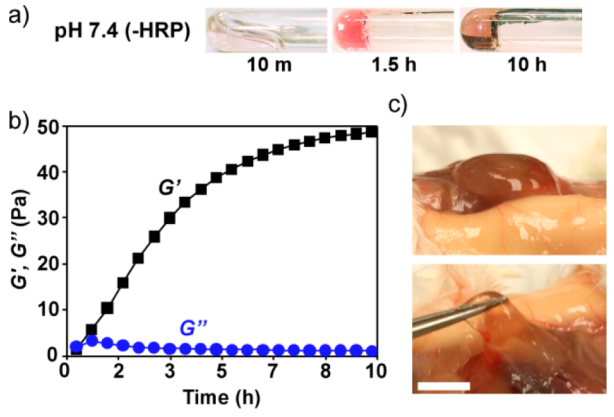


Figure 3. (a) Photograph of HA-EGCG hydrogel (10 mg mL^{-1}, pH 7.4) formed through quinone formation. (b) Rheological measurement of the hydrogel. (c) In vivo formation of HA-EGCG hydrogel at the subcutaneous environment (scale bar = 0.5 cm).

Green tea catechins are known to bind to proteins through hydrophobic interaction and hydrogen bonding.[18] These interactions have been associated with the anticancer and enzyme-inhibiting properties of tea polyphenols.[19] We have previously shown that the EGCG moieties endowed HA-EGCG conjugates with resistance toward enzymatic degradation by hyaluronidase.[15] In this study, the hydrogel degradation profiles in the presence of hyaluronidase were characterized. HA-EGCG and HA-tyramine hydrogels (both at 10 mg mL^{-1}) were prepared at pH 7.4; the latter were cross-linked by HRP/H_2O_2 and served as comparisons. The degree of substitution (DS) of HA-tyramine conjugates was 3, which was higher than that of HA-EGCG hydrogels (DS = 0.8). HA-tyramine hydrogels with higher G' values and lower swelling ratios than HA-EGCG hydrogels were prepared (Table 1). In 2.5 U mL^{-1} of hyaluronidase solution, HA-tyramine hydrogels were degraded at a faster rate than HA-EGCG hydrogels, as determined by weight measurement (Figure 4). The time needed to completely degrade HA-tyramine and HA-EGCG hydrogels were 8 and 33 h, respectively. When stiffer HA-

Table 1. Storage Modulus (G') and Swelling Ratio (Q_M) of Hydrogels Used in the Degradation Study

sample[a]	DS	G' (Pa)[b]	Q_M[c]
HA-EGCG gel (−HRP)	0.8	55 ± 19	234 ± 4
HA-EGCG gel (+HRP)	0.8	97 ± 1	212 ± 6
HA-tyramine gel[d] (+HRP/H_2O_2)	3.0	160 ± 15[e]	180 ± 6[e]

[a]All the samples were prepared with 10 mg mL^{-1} of conjugates. [b]G', storage modulus (n = 3, mean ± s.d.). [c]Q_M is the mass swelling ratio, which equals to the swollen weight of hydrogel divided by the dry weight (n = 2, mean ± s.d.). [d]The concentration of exogenous H_2O_2 added was 104 μM. [e]Values are significantly different from those of HA-EGCG gels formed with or without HRP (P < 0.05).

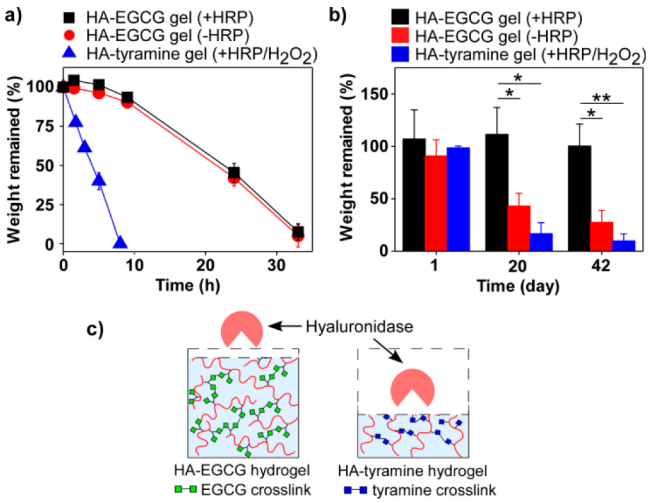


Figure 4. (a) In vitro degradation of HA-EGCG and HA-tyramine hydrogels in 2.5 U mL^{-1} of hyaluronidase solution (n = 2, mean ± s.d.). (b) In vivo degradation of hydrogels at the subcutaneous tissue of mice (n = 6, mean ± s.e.m.). (c) Schematic illustrating the slower rate of degradation of HA-EGCG hydrogels. *P < 0.05 and **P < 0.005.

DOI: 10.1021/acsmacrolett.5b00544
ACS Macro Lett. 2015, 4, 957−960

tyramine hydrogels (G' = 601 Pa) were tested, the degradation time was extended to 24 h, which was still shorter than the amount of time required to degrade HA-EGCG hydrogels (Figure S5a). A similar trend was observed when hyaluronidase concentration was increased to 10 U mL^{-1}; the degradation time of HA-EGCG hydrogels was longer than HA-tyramine hydrogels (Figure S5b, Supporting Information). The longer time required for HA-EGCG hydrogels to be fully degraded suggests the inhibition of hyaluronidase activity. The degradation of HA-EGCG hydrogels was further evaluated in vivo. A total of 42 days after injection into the subcutaneous tissue, the weight of HA-EGCG hydrogel formed with HRP remained close to 100%, while the weight of HA-EGCG hydrogel formed without HRP decreased to 28% (Figure 4b). In contrast, only 16% of the initial weight remained for HA-tyramine hydrogels with comparable stiffness. Taken together, the in vitro and in vivo results demonstrated that the EGCG moieties endowed the hydrogels with resistance toward degradation by hyaluronidase (Figure 4c).

In summary, we showed that the rate of H_2O_2 generation via EGCG autoxidation at pH 7.4 was sufficiently fast that the addition of HRP resulted in gelation within a few minutes. Coupling between EGCG moieties could also take place through EGCG quinone formation in the absence of HRP, albeit the gelation time was 1.5 h. Currently, most of the hydrogels formed by the oxidative coupling of phenols require the addition of both HRP and H_2O_2. The present study demonstrated the possibility of forming hydrogels via the oxidative coupling of EGCG without the need of adding H_2O_2 or both H_2O_2 and HRP. HA is widely used as dermal fillers for cosmetic application or as viscosupplements for osteoarthritis treatment.[20] However, in its free form, HA is rapidly degraded or drained from the injection site. The HA-EGCG hydrogel system, with in situ gelation and resistance toward hyaluronidase-mediated degradation, offers the advantages of injectability and prolonged residence time in the body.

■ ASSOCIATED CONTENT

Supporting Information

Materials, experimental procedures, and supplementary figures. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmacrolett.5b00544.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Institute of Bioengineering and Nanotechnology (Biomedical Research Council, Agency for Science, Technology and Research, Singapore (A*STAR)) and Science and Engineering Council (SERC) Personal Care Programme Grant, A*STAR. The authors thank JNC Corporation (Japan) for the gift of hyaluronic acid.

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■ NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on August 20, 2015 with incorrect labels in Figure 2 caption. The corrected version was reposted on August 21, 2015.

DOI: 10.1021/acsmacrolett.5b00544
ACS Macro Lett. 2015, 4, 957−960