Injectable biodegradable hydrogels composed of hyaluronic acid–tyramine conjugates for drug delivery and tissue engineering

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Received (in Cambridge, UK) 19th May 2005, Accepted 29th June 2005
First published as an Advance Article on the web 28th July 2005
DOI: 10.1039/b506989k

The sequential injection of hyaluronic acid–tyramine conjugates and enzymes forms biodegradable hydrogels *in vivo* by enzyme-induced oxidative coupling, offering high potential as a promising biomaterial for drug delivery and tissue engineering.

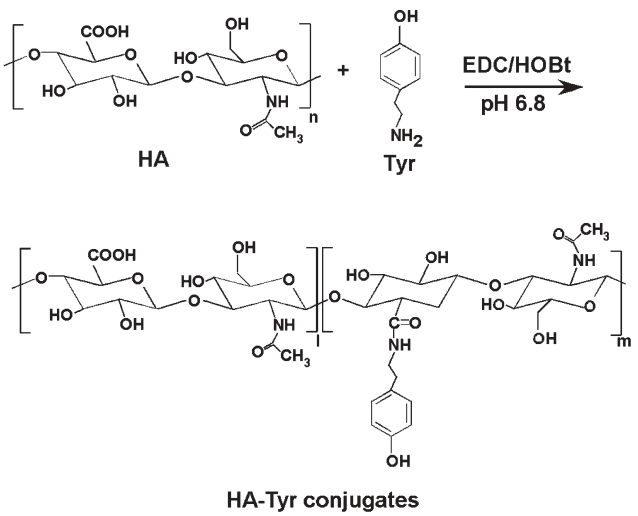
Hydrogels have been used extensively for the controlled release of bioactive molecules and the encapsulation of cells.[1] In particular, the use of hydrogels as scaffolds for tissue engineering is a promising method to achieve tissue repair or tissue regeneration in the body. However, most of the hydrogels to date require surgical implantation, which often results in tissue irritation and damage.[2] Therefore, the development of injectable *in situ* gel-forming polymeric hydrogel systems has received much attention.[3] Ideally, the injectable system should form a hydrogel within a narrow range of physiologically acceptable temperature and at a sufficiently rapid rate.[4] In addition, the reagents must be non-toxic, and the hydrogels formed should be degraded after the disease has been cured and/or tissue regeneration is complete. In order to avoid the use of toxic cross-linking agents, physically cross-linked hydrogels have been designed to utilize ionic interactions between polymer chains and the sol–gel transition of amphiphilic block coplymers.[2,5] Chemical cross-linking is a more versatile method to synthesize hydrogels in terms of mechanical stability and control of degradability. However, the method is inadequate to apply to injectable systems because toxic chemical agents are usually used in hydrogel synthesis and induce undesirable reactions with bioactive molecules, such as growth factors, and cells.

In this study, we propose the very simple and biocompatible *in situ* gel-forming system composed of hyaluronic acid–tyramine (HA-Tyr) conjugates (Scheme 1) using a peroxidase-catalysed oxidation reaction involved in some biosynthetic pathways such as melanin formation. Hydrogels were formed *in vivo* by injecting two solutions through syringes: (i) HA-Tyr solution containing H_2O_2 as an oxidant of horseradish peroxidase (HRP) and (ii) HRP as a model catalyst, which induces the oxidative coupling of the phenol moiety in the body (Scheme 2). HA, a major constituent of the extracellular matrix (ECM), is a glycosaminoglycan made up of repeating disaccharide units (β-1,4-D-glucuronic acid and β-1,3-*N*-acetyl-D-glucosamine).[6] We cast HA as the backbone polymer in the hydrogel because of its excellent biocompatibility and biodegradability.[7] Thus, this novel gel-forming system allows the formation of hydrogels without any inflammation and redundant reactions with bioactive agents loaded in the hydrogels. We believe that one promising way to achieve effective drug therapy and tissue regeneration is by enzyme-mediated HA gel formation *in vivo*.

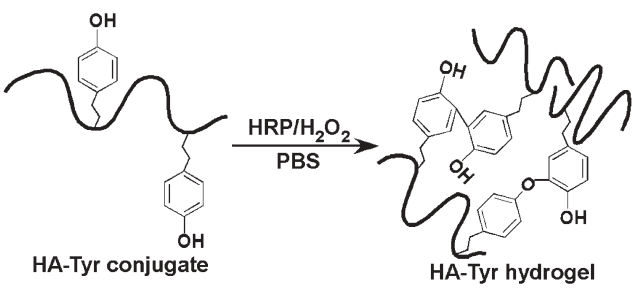
Here, we report the hydrogel formation of HA-Tyr by a catalytic enzyme, HRP *in vitro* and *in vivo*, and the biodegradability of the hydrogels with the objective of developing an ideal implant system for drug delivery and tissue engineering.

HA-Tyr conjugates were successfully synthesized by a general carbodiimide/active ester-mediated coupling reaction[8] in distilled water.† From ^{1}H NMR measurements, there were 9 tyramine molecules present per 100 repeat units of HA.

The HA-Tyr hydrogels were synthesized by the enzymatic oxidative reaction of tyramine moieties using H_2O_2 and HRP.‡



Scheme 1 Synthesis of HA-Tyr conjugates.



Scheme 2 *In situ* gel forming of a polymeric hydrogel composed of HA-Tyr conjugates by an enzyme-catalysed oxidation reaction.

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Peroxidase is frequently used as a catalyst for oxidative coupling of phenol derivatives under mild reaction conditions.[9] It is known that the oxidative coupling of phenol proceeds at the C–C and C–O positions between phenols, as shown in Scheme 2. Fig. 1 shows the dependence of the gelation time on the concentration of catalysts (H_2O_2 and HRP). The gelation time increased as the H_2O_2 concentration increased (Fig. 1a). In addition, the gelation time was almost constant in a range of H_2O_2 concentration between 2.4 and 20 mmol L^{-1}. Also, no gel formation was observed when the concentration of H_2O_2 was not only 0 mmol L^{-1} but also above 100 mmol L^{-1} due to excessive oxidation. In contrast, the gelation time decreased as the concentration of HRP increased, and was almost constant at HRP concentrations above 1.25 unit ml^{-1} (Fig. 1b). Furthermore, the hydrogel mechanical strength was related to the gelation time: the gels formed for shorter time showed higher mechanical strength (data not shown). Based on these results, the gelation time as well as gel strength can be controlled by varying the concentrations of H_2O_2 and HRP. The hydrogels were formed within 20 seconds at 1.25 unit ml^{-1} of HRP and 2.4 mmol L^{-1} of H_2O_2.

We assessed *in vitro* enzymatic degradation of hydrogels (20 × 20 × 1.2 mm in size) in terms of weight loss[10] (Fig. 2). The degradation of the hydrogels was studied at different concentrations of hyaluronidase (0, 10, 25, 50 and 100 unit ml^{-1}) as a model enzyme for hydrolyzing the hyaluronic acid chain in the body.§ All the hydrogels completely degraded in the presence of hyaluronidase, while no significant change in weight was observed in the negative control hydrogels which were incubated in PBS alone.

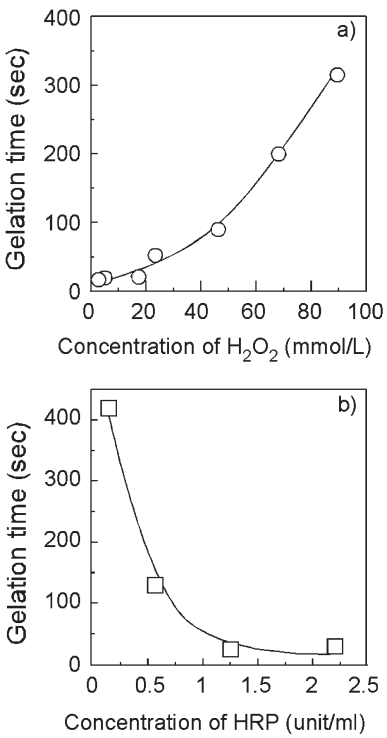

Fig. 1 Dependence of gelation time of HA-Tyr hydrogels on catalyst concentrations. (a) Effect of H_2O_2 with 1.25 unit ml^{-1} of HRP; (b) effect of HRP with 2.4 mmol L^{-1} of H_2O_2.

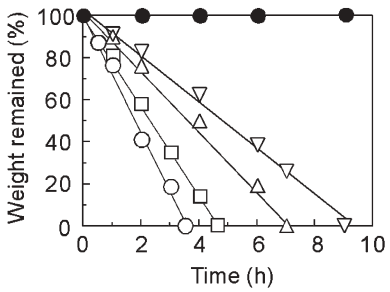

Fig. 2 *In vitro* enzymatic degradation of HA-Tyr hydrogels in PBS at 37 °C. Hyaluronidase concentration: ○, 100 unit ml^{-1}; □, 50 unit ml^{-1}; △, 25 unit ml^{-1}; ▽, 10 unit ml^{-1}; ●, 0 unit ml^{-1}.

The degradation rate (B) of the hydrogels can be calculated from the following equation:

$$B = h/2t_\infty \quad (1)$$

where h is the initial thickness of the slab and t_∞ is the time taken to complete degradation. This equation is based on the assumption that degradation takes place from both sides of the slab and not from the edges. The degradation rates increased with increasing hyaluronidase concentrations. Hyaluronidase concentrations of 0, 2.5, 10, 25 and 50 unit ml^{-1} caused degradation rates of 0, 1.8, 2.3, 4.1, and 5.5 × 10^{-6}cm s^{-1}, respectively. Moreover, the weight of these hydrogels decreased linearly with time, indicating that degradation proceeded *via* surface erosion only.[10] It is expected that this surface-controlled degradation of hydrogels can achieve the sustained release of bioactive molecules such as proteins which could be released only upon degradation of the surface.

To investigate the *in vivo* formation and degradation of the hydrogels, HA-Tyr was injected subcutaneously with and without H_2O_2/HRP into mice (Table 1).¶ Four methods of injection were used: (i) syringe A (HA-Tyr +H_2O_2) + syringe B (HRP), (ii) syringe A (HA-Tyr), (iii) syringe A (HA-Tyr) + syringe B (HRP), and (iv) syringe A (HA-Tyr + H_2O_2). Fig. 3a shows the hydrogels which formed *in vivo* and were collected from the mice 1 day after injection. The hydrogel formed by injection system (i): syringe A (HA-Tyr + H_2O_2) and syringe B (HRP), yielded the highest weight and mechanical strength; (i) > (iii) ≃ (iv) > (ii). This result demonstrated the synergistic effect of H_2O_2 and HRP, co-existing in high enough concentrations to induce the effective cross-link of the HA-Tyr hydrogels. Interestingly, hydrogels also formed upon injecting only HA-Tyr solution, suggesting that the oxidative coupling reaction was still able to proceed in the presence of the little amount of H_2O_2 and peroxidase available under physiological conditions even without injection of both H_2O_2 and HRP. There was not any visible inflammatory or irritative observation around

Table 1 Components of injection solutions[a]

Injection system	Syringe A	Syringe B
i	HA-Tyr + H_2O_2	HRP
ii	HA-Tyr	—
iii	HA-Tyr	HRP
iv	HA-Tyr + H_2O_2	—

[a] Hyaluronic acid–tyramine conjugates and enzyme were injected sequentially using two different syringes.

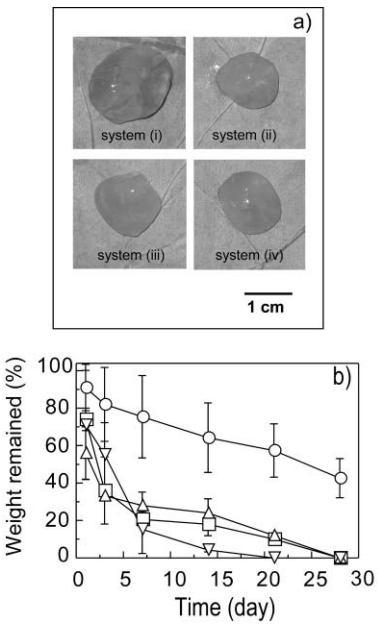

Fig. 3 Formation and degradation of hydrogels *in vivo*. (a) HA-Tyr hydrogels formed *in vivo*. (b) *In vivo* degradation of HA-Tyr hydrogels formed by different components of the injection solutions (n = 3): ○, syringe A (HA-Tyr + H_2O_2) and syringe B (HRP); □, syringe A (HA-Tyr); △, syringe A (HA-Tyr) + syringe B (HRP); ▽, syringe A (HA-Tyr + H_2O_2).

the tissue where all of these *in situ* gel-forming systems were administered by the four injection methods.

The degradability of the hydrogels *in vivo* was dependent on the components of the injection solutions (Fig. 3b). The weight of the hydrogels formed using both HRP and H_2O_2 gradually decreased over one month. In comparison, other hydrogels were rapidly degraded. It is considered that the difference in degradability was affected by the degree of cross-linking of the hydrogels. These results will provide practical, valuable information in attempts to achieve optimal drug therapy and tissue regeneration, where controlling the degradability of hydrogels is critical.

In conclusion, a simple and non-toxic injectable *in situ* hydrogel system was achieved using an enzymatic oxidative coupling reaction: one of a biosynthetic pathway. The high convenience and biocompatibility of this injectable *in situ* hydrogel system will give great advantages to controlled drug delivery and tissue regeneration by the controlled release of bioactive molecules and/or cells.

This research was funded by the Agency for Science, Technology and Research, Singapore. We gratefully acknowledge Chisso Corporation, Japan for the gift of hyaluronic acid and Cindy Ren for her valuable comments.

Notes and references

† HA-Tyr was synthesized by following a general protocol. HA (0.5 g, 1.25 mmol; 800 kDa) was dissolved in 100 ml of distilled water. To this solution tyramine hydrochloride (4.68 g, 27.0 mmol) was added. The pH of the reaction mixture was adjusted to 6.8 by the addition of 0.1 M NaOH. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) (0.995 g, 5 mmol) and 1-hydroxybenzotriazole (HOBT) (0.675 g, 5 mmol) were dissolved in DMSO/distilled water (1 : 1, 10 ml). After mixing, the pH of the reaction was maintained at 6.8. The solution was kept at room temperature for 48 h under gentle stirring. The mixture was subjected to purification by dialysis (molecular weight cut off = 3500). The remaining solution was lyophilized to give HA-Tyr (yield 95%).^{1}H NMR (D_2O): δ 1.9 (s, C(=O)CH_3), 2.6–2.8 (br, ArCH_2CH_2), 6.7–7.2 (br, Ar).

‡ The gelation time of hydrogels was determined using a test tube inverting method reported by Jeong *et al.*[11] HA-Tyr (50 mg) was dissolved in 2.95 ml of PBS (phosphate buffer saline, pH 7.4) in a vial. Freshly prepared HRP and H_2O_2 solutions with different concentrations were added into the vial and gently vortexed. When no fluidity was visually observed upon inverting the vial, the gel state was determined. For *in vitro* degradation experiments, the slab-shaped hydrogels were prepared by injecting the HA-Tyr solution containing HRP and H_2O_2 into a spacer covered on both sides with glass plates. After the reaction was complete, the resulting hydrogels were placed in an excess amount of distilled water to remove the unreacted substances and was finally swollen in distilled water.

§ HA hydrogels (20 × 20 × 1.2 mm in size) were immersed in 100 ml of PBS to which hyaluronidase was added, and the mixture was stirred at 37 °C. The degree of degradation of the hydrogels was estimated by measuring the residual weight.

¶ Animal experiments were performed according to the international guiding principles for animal research. BALB/c mice that were supplied by the Laboratory Animal Center and bred in the Animal Holding Unit of The National University of Singapore were used at 6–8 weeks of age. HA-Tyr (10 mg ml^{-1}) was dissolved in PBS containing 70 mmol L^{-1} of H_2O_2, and 0.5 ml of the solution was then injected subcutaneously through a 22-gauge needle into the mice, followed by an injection of 50 μl of HRP (0.25 mg ml^{-1}) with a 27-gauge needle.

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