Introduction

We have developed an injectable, biodegradable hydrogel system composed of hyaluronic acid–tyramine conjugates using a peroxide-catalyzed oxidation reaction for drug delivery and tissue engineering. Previous efforts in this area have been unsuccessful at addressing a number of problems, including the conventional use of toxic catalysts and chemicals, which affect the bioactivity of the drugs and cells, causing tissue damage. Also, most of the hydrogels to date require surgical implantation, which often results in tissue irritation and damage. Ideally, the reagents must be nontoxic, 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 copolymers. However, the physically cross-linked hydrogels formed by physical interactions such as hydrogen bonding and hydrophobic interaction might not be stable because biological compounds such as protein and sugars destabilize the gel formation in the body, even though such systems do not utilize toxic chemical cross-linkers. Therefore, we were motivated to form the chemically cross-linked hydrogel without the use of toxic chemical cross-linkers.
Our injectable system is a simple and biocompatible in situ gel-forming system composed of hyaluronic acid–tyramine (HA-Tyr) conjugates using a peroxidase-catalyzed oxidation reaction (Fig. 1). Hydrogels are formed in vivo by injecting two solutions through syringes (Fig. 2): a solution of horseradish peroxidase (HRP) as a model catalyst, which induces the oxidative coupling of the phenol moiety; and HA-Tyr solution containing H$_2$O$_2$ as an oxidant of HRP. The hydrogel that is formed does not need to be surgically removed after treatment, as it can be safely degraded in the body.

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). We cast HA as the backbone polymer in the hydrogel because of its excellent biocompatibility and biodegradability. Thus, this novel gel-forming system allows the formation of hydrogels without any inflammation and redundant reactions, with bioactive agents loaded in the hydrogels. Drugs are released from the hydrogel because of hydrogel degradation. Prolonged and sustained drug release can be achieved by controlling the hydrogel degradation. This is programmed through the design of the cross-link density.

**Formation of HA-Tyr Hydrogel**

The HA-Tyr hydrogels were synthesized by the enzymatic oxidative reaction of tyramine moieties using H$_2$O$_2$ and HRP. Peroxidase is frequently used as a catalyst for the oxidative coupling of phenol derivatives under mild reaction conditions. It is known that the oxidative coupling of phenol proceeds at the C-C and C-O positions between phenols, as shown in Fig. 1(b). The gelation time of the hydrogels was dependent on the concentration of the catalysts H$_2$O$_2$ and HRP. We could control the gelation time, which ranges from a few seconds to a few minutes, by varying the amount of H$_2$O$_2$ and HRP. The gelation time increased as the H$_2$O$_2$ concentration increased; in contrast, the gelation time decreased as the concentration of HRP increased. Furthermore, the hydrogel mechanical strength was related to the gelation time: the gels formed in a shorter time showed higher mechanical strength. Based on these results, the gelation time as well as gel strength can be controlled by varying the concentrations of H$_2$O$_2$ and HRP.

**Enzymatic Degradation of HA-Tyr Hydrogel in vitro**

We assessed the in vitro enzymatic degradation of hydrogels (20 x 20 x 1.2 mm$^3$ in size) in terms of weight loss (Fig. 3). The degradation of the hydrogels was studied at different concentrations of hyaluronidase (0, 10, 25, 50, and 100 unit/mL), which is used 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 that were incubated in phosphate-buffered saline (PBS) alone. Moreover, the weight of these hydrogels decreased linearly with time, indicating that degradation proceeds via surface erosion only. It is
expected that this surface-controlled degradation of hydrogels can achieve the sustained release of bioactive molecules such as proteins, which can be released only upon degradation of the surface.

**Formation and Degradation of Hydrogel in vivo**

To investigate the in vivo formation and degradation of the hydrogels, HA-Tyr was injected subcutaneously with and without \(\text{H}_2\text{O}_2/\text{HRP}\) into mice. Four methods of injection were used: (1) syringe A (HA-Tyr + \(\text{H}_2\text{O}_2\)) + syringe B (HRP); (2) syringe A (HA-Tyr); (3) syringe A (HA-Tyr) + syringe B (HRP); and (4) syringe A (HA-Tyr + \(\text{H}_2\text{O}_2\)). The hydrogel formed by injection system (1), syringe A (HA-Tyr + \(\text{H}_2\text{O}_2\)) and syringe B (HRP), yielded the highest weight and mechanical strength; (1) > (3) ß- (4) > (2). This result demonstrates the synergistic effect of the coexistence of \(\text{H}_2\text{O}_2\) and HRP, which, in appropriate concentrations, induced the effective cross-linking of HA-Tyr hydrogels. Interestingly, hydrogels also formed upon the injection of only HA-Tyr solution, suggesting that the oxidative coupling reaction is still able to proceed with a little amount of \(\text{H}_2\text{O}_2\) and peroxidase under physiological conditions, even without the injection of both \(\text{H}_2\text{O}_2\) and HRP.

There was no visible inflammatory and irritative reaction observed around 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. 4). The weight of the hydrogels formed using both HRP and \(\text{H}_2\text{O}_2\) 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 and valuable information to achieve optimal drug therapy and tissue regeneration, where controlling the degradability of hydrogels is critical.

**Protein Release from Hydrogel**

We also examined the protein release from HA-Tyr hydrogels. Only a small amount of the positively charged protein was released by diffusion, due to the ionic interaction between the carboxyl groups in HA and the positively charged amino acids in the protein. The negatively charged protein, on the other hand, was released simply by diffusion. However, when the protein release was performed in the presence of hyaluronidase, all of the protein was released from the HA-Tyr hydrogels by the degradation of the hydrogels. These results indicate that sustained protein release without burst release can be achieved by the degradation of the hydrogel, when we immobilize positively charged proteins in the HA-Tyr hydrogel.

Recently, we also developed an improved hydrogel system composed of HA–epigallocatechin gallate (EGCG) conjugates, which showed slower release of negatively charged protein compared to that of HA-Tyr hydrogel. As EGCG is a major component of green tea and has numerous biological activities such as antioxidant and antitumor properties, this HA-EGCG hydrogel is expected to act with multi therapeutic effects.
Perspectives

A simple and nontoxic injectable in situ hydrogel system was achieved using an enzymatic oxidative coupling reaction, a biosynthetic pathway. The high convenience and biocompatibility of this injectable in situ hydrogel system will offer great advantages in controlled drug delivery and tissue regeneration through the controlled release of bioactive molecules and/or cells. One of the most significant advantages of our system is its ability to incorporate therapeutic proteins, growth factors, and cells in the hydrogels without the risk of damage to these biological compounds.

Our hydrogel can be used as a safe and convenient protein/peptide delivery system for diseases such as prostate cancer, which require frequent administration of therapeutics, e.g., via injections that may lead to severe side-effects due to the high drug concentration that is injected. We are also studying the use of our injectable hydrogel as a scaffold for tissue/bone regeneration in vivo. This hydrogel can be embedded with biological components in a fluid state and can be shaped at any desired site by injection. It also offers a benign environment that is similar to the body. We expect such a system to lead to the development of potential scaffolds that can manipulate biological functions and interactions in three dimensions.

Acknowledgments

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References