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Hyaluronidase-incorporated hyaluronic acid–tyramine hydrogels for the sustained release of trastuzumab

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ABSTRACT

We developed an injectable hydrogel system for the sustained release of protein drugs that incorporated both protein drugs and hyaluronidase. Trastuzumab and hyaluronidase were incorporated in hydrogels composed of hyaluronic acid–tyramine (HA–Tyr) conjugates through the enzymatic crosslinking utilizing hydrogen peroxide (H_2O_2) and horseradish peroxidase (HRP). Through electrostatic interactions with the HA, trastuzumab was retained in the hydrogel to minimize its burst release. Hyaluronidase was incorporated in the hydrogel to release trastuzumab from the hydrogels. The hydrogels were degraded and showed sustained release of trastuzumab in phosphate buffer over four weeks *in vitro*. Both the rates of drug release and gel degradation were controlled by the concentration of hyaluronidase. Trastuzumab released from the hydrogels inhibited the proliferation of BT-474 cells *in vitro*. In an animal model, the single subcutaneous injection of a mixture solution of HA–Tyr conjugates, H_2O_2, HRP, trastuzumab and hyaluronidase inhibited tumor growth significantly, whereas injection of trastuzumab alone at the same dose failed to do so. Compared to trastuzumab alone, the hyaluronidase-incorporated HA–Tyr hydrogels improved the pharmacokinetic profile of trastuzumab in the plasma of mice. Furthermore, they were fully degraded over two weeks, and the formation of fibrous capsules was not observed in mice.

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1. Introduction

Recently, injectable hydrogels have attracted much attention for the delivery of therapeutic proteins [1–3]. By preserving the native structures of proteins in a water-abundant matrix, hydrogels are ideal reservoirs for protein drugs and are expected to improve the drug release profiles. Injectable hydrogels are especially useful in clinical applications, as surgeries are not required and their administration is simple. The utilization of hydrogels with an optimized drug release profile would enhance the efficacy of the drug, reduce the frequency of drug administration, and improve the patient's compliance. Although significant progress has been made in the field [4–7], several challenges still exist that need to be fully addressed. Firstly, as the mesh size of most hydrogels is much larger than the hydrodynamic diameters of proteins [8], it is often difficult to retain drugs in the hydrogel matrix and minimize their burst release. Secondly, since proteins are fragile and prone to denaturation, crosslinking processes need to be optimized to ensure the intactness of the protein. Finally, fibrous capsules are often formed around the hydrogels *in vivo*, which could hinder the release of drug, lower the degradability of hydrogel, and potentially cause chronic inflammation [9].

The demand for a better drug delivery system has surged with the development of new drugs in the pharmaceutical industry. Monoclonal antibodies have been widely used in the treatment of cancer, yet problems such as their high costs and side effects hinder the advancement of these drugs in clinical practice. Trastuzumab, also known as Herceptin, represents one class of antibody drugs widely used for the treatment of breast cancers [10–12] which are human epidermal growth receptor 2 (HER2)-positive [13,14]. It is administered once every three weeks over a period of one year for patients diagnosed with early-stage breast cancer [15]. The usual route of administration is intravenous (IV) infusion, which requires trained personnel and a dedicated infusion facility. It usually takes 30 to 90 min for one infusion, and additional time is required for post-infusion observation. Furthermore, infusion-related reactions and complications can occur in patients. Recently, subcutaneous (SC) injection has been explored as an alternative method for the administration of trastuzumab. Ismael G. et al. have reported a Phase III clinical trial that showed SC administration of trastuzumab, with recombinant human hyaluronidase (rHuPH20) [16] as excipient, offered a pharmacokinetic profile, efficacy and safety that was not inferior to IV administration [17]. Compared to IV infusion, SC treatment is less technically demanding and takes less than 5 min. Furthermore, 88.9% of patients preferred SC treatment in a study that examined patients' preferences of SC administration versus conventional IV infusion, whereas only 9.6% preferred IV treatment [18]. To further enhance the efficacy of trastuzumab through the SC route, we utilized hydrogels as

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the delivery vehicles and exploited the enzyme-triggered trastuzumab release from the hydrogels.

Hyaluronic acid (HA) is a naturally-occurring, biodegradable polymer with well-known biocompatibility [19,20]. We have reported an injectable hydrogel system composed of HA–tyramine (HA–Tyr) conjugates for various biomedical applications [21,22]. The hydrogels were formed through the oxidative coupling of Tyr moieties, which were catalyzed by hydrogen peroxide (H_2O_2) and horseradish peroxidase (HRP). In this study, we designed an injectable HA–Tyr hydrogel system that incorporates both trastuzumab and hyaluronidase for the sustained release of trastuzumab for breast cancer treatment. Under physiological conditions, HA–Tyr is highly anionic, whereas trastuzumab with an isoelectric point of 8.45 is cationic [23]. Therefore, it is expected that the initial burst release of trastuzumab from HA–Tyr hydrogels would be minimized by immobilization of the protein in the hydrogel matrix through electrostatic interactions. Although hyaluronidase is present in the human bodies, its concentration in the plasma is only 60 ng/ml [24]. Thus we incorporated hyaluronidase into the hydrogel to control the degradation of hydrogel, which in turn would promote the release of trastuzumab. We expect that HA–Tyr hydrogels can release trastuzumab in a sustained manner through the enzyme-triggered degradation of hydrogels (Fig. 1), as the hyaluronidase catalyzes the hydrolysis of α-*N*-acetyl-D-glucosaminidic linkages in HA. The advantages of this injectable system are: (i) minimized burst release through electrostatic interaction between HA and trastuzumab; (ii) tunable rates of drug release and gel degradation by controlling the hyaluronidase concentration; and (iii) transient presence of the hydrogel which avoids long-term discomfort and possible side effects for patients.

In this study, we first examined the drug release and degradation profiles of HA–Tyr hydrogels with varying concentrations of hyaluronidase. Next, we studied the anti-proliferation effects of trastuzumab released from these hydrogels using breast cancer cell line BT-474 *in vitro*. In a BT-474-xenografted nude mouse model, we investigated the inhibitory effect of HA–Tyr hydrogels incorporating hyaluronidase in comparison to free trastuzumab on tumor growth. Histological and immunohistochemical analyses were utilized to examine the proliferation and apoptosis of tumor cells in the tissue. Pharmacokinetic studies were performed to measure the plasma concentration of trastuzumab after the treatment with hyaluronidase-incorporated hydrogels. Finally, we assessed the tissue reaction to HA–Tyr hydrogels with or without hyaluronidase.

2. Materials and methods

2.1. Materials

Sodium hyaluronate (HA) (MW = 90 kDa, density = 1.05 g/cm^3) was kindly donated by JNC Corporation (Tokyo, Japan). Hydrogen peroxide (H_2O_2, 30 wt.%) and hyaluronidase (439 unit/mg) from bovine testes were purchased from Sigma-Aldrich (MO, USA). Horseradish peroxidase (HRP, 100 unit/mg) was obtained from Wako Pure Chemical Industries (Osaka, Japan). RPMI-1640 medium was purchased from Lonza (Basel, Switzerland). Fetal bovine serum (FBS) and the alamarBlue assay kit were obtained from Life Technologies (CA, USA). Trastuzumab was purchased from Roche (Basel, Switzerland). The human IgG ELISA kit was purchased from ICL lab (OR, USA). Phosphate buffered saline (PBS, 150 mM, pH 7.3) was supplied by media preparation facility in Biopolis, Singapore. Hyaluronic acid–tyramine (HA–Tyr) conjugates were synthesized and characterized by ^{1}H NMR as previously described [22].

2.2. Hydrogel preparation and rheology measurement

HA–Tyr conjugate was dissolved in PBS, and mixed with trastuzumab, HRP and H_2O_2 solutions to prepare the HA–Tyr hydrogels. The final concentrations of HA–Tyr, trastuzumab, HRP and H_2O_2 were 1.75 wt.%, 0.3 mg/ml, 0.124 unit/ml and 437 μM respectively. For hydrogels incorporating hyaluronidase, 2.5 or 5 μl of 1000 unit/ml hyaluronidase was added into 1 ml of mixture solutions to achieve a final concentration of 2.5 or 5 unit/ml respectively. Rheological measurements of the hydrogels were performed with a HAAKE Rheoscope 1 rheometer (Karlsruhe, Germany) as described previously [25].

2.3. Incorporation and release of trastuzumab from HA–Tyr hydrogels

The mixture solution containing 1.75 wt.% HA–Tyr, 0.3 mg/ml trastuzumab, hyaluronidase of varying concentrations (0, 2.5 and 5 unit/ml), 0.124 unit/ml HRP abd 437 μM H_2O_2 were gently mixed by pipetting and then injected between two parallel glass plates clamped 1.5 mm apart. Gelation was allowed to proceed at 37 °C for 2 h. Hydrogel disks with a diameter of 1.6 cm, were then cut from the hydrogel slab using a circular mold. To measure the percentage of trastuzumab that can be recovered from hydrogels, the disks were immersed in 3 ml PBS buffer containing 200 unit/ml hyaluronidase. Hydrogels were degraded overnight at 37 °C, and trastuzumab concentration was measured by human IgG enzyme-linked immunosorbent assay (ELISA). The percentage of trastuzumab retrieved from hydrogels was then calculated. For cumulative release studies, hydrogel disks (1.6 cm in diameter, 1.5 mm in thickness) were placed in a plastic net and immersed in 20 ml of PBS buffer containing 0.05% sodium azide and 0.5% bovine serum albumin (BSA). At selected time points, 200 μl of the solution was withdrawn and replaced with an equal volume of fresh buffer solution to maintain a constant total volume. The collected samples were stored in LoBind tubes (Eppendorf, Germany) at 4 °C until measurement with a Human IgG ELISA kit.

2.4. Degradation of hydrogel in vitro and in vivo

For the degradation of hydrogels *in vitro*, hydrogel disks were prepared according to the protocols described in Section 2.3. Each disk was placed in a plastic net and immersed in 20 ml PBS buffer containing 0.05% sodium azide and 0.5% BSA. At selected time points, the hydrogel disks were taken out of solution and excess water was soaked away with tissue paper. Then gel disks were weighed and returned to the buffer solution.

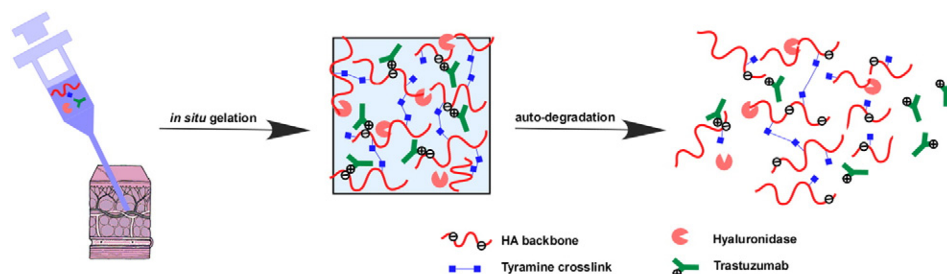

Fig. 1. The design of an auto-degradable HA–Tyr hydrogel that incorporates hyaluronidase for the sustained release of trastuzumab.

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