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Facile fabrication of PEG-coated PLGA microspheres via SPG membrane emulsification for the treatment of scleroderma by ECM degrading enzymes

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ABSTRACT

We developed a facile fabrication method for preparing poly(ethylene glycol)(PEG)-coated poly (lactic-co-glycolic acid) (PLGA) microspheres with homogeneous size distribution via a combination of mPEG-*b*-PLGA and Shirasu Porous Glass membrane emulsification. Subsequently, extracellular matrix (ECM) degrading enzymes, collagenase (COLase) or hyaluronidase (HAse) were loaded into the microspheres. The obtained microspheres exhibited a sustained release of COLase or HAse over 10 days. The degradation of ECM polymers by the released COLase and HAse was confirmed *in vitro*. Reversal of established dermal fibrosis via degradation of over-deposited ECM is a promising treatment for scleroderma. The therapeutic effects of COLase- and HAse-loaded PLGA microspheres on scleroderma were evaluated *in vivo* following their intradermal administration to a bleomycin-induced mice model of scleroderma. COLase- and HAse-loaded PLGA microspheres decreased scleroderma dermal thickness without altering the mechanical properties of skin, whereas the administration of free COLase and HAse solution induced overdecomposition of skin ECM and α-SMA expression. The facile one-pot synthesis of PEG-coated PLGA microspheres with high colloidal stability and narrow size distribution could be employed as a drug carrier for various diseases in future.

1. Introduction

Poly (lactic-co-glycolic acid) (PLGA) is widely used as a drug carrier owing to its biocompatibility and controllable biodegradability [1]. PLGA-based microspheres have been extensively investigated for their application in localized and sustained drug release, such as in the lungs, dermis, and muscles [2–5]. In such applications, homogeneous size distribution of microspheres is a key factor in ensuring proper injectability, homogeneous drug release, and effective interaction with cells and tissues. Shirasu Porous Glass (SPG) membrane emulsification is a technique that facilitates the fabrication of micro-sized emulsions and particles with narrow size distribution via uniform-sized membrane pores [6–9]. The sizes of formed emulsions/particles can be precisely tuned by changing the size of membrane pores. Makino et al used SPG membrane emulsification and the solvent evaporation techniques to fabricate drug-loaded PLGA microspheres with homogeneous size distribution [10,11].

Surface coating with poly (ethylene glycol) (PEG) has been extensively used to provide high colloidal stability and biocompatibility to drug carriers. Surface modification via covalent bonds promotes stable PEGylation of particles [12,13] although this modification requires multi-step procedures. A more facile way is to use surface adsorption of PEG [10,14] but the stability of the coating is not as high as in covalent bonding. In addition to these post-synthesis processes, one-pot synthesis of PEG-coated PLGA nanoparticles has been reported by using amphiphilic block copolymers of PEG and PLGA (mPEG-*b*-PLGA) [15,16]. The combination of mPEG-*b*-PLGA and SPG membrane emulsification technique could facilitate facile one-pot production of PEG-immobilized PLGA microspheres with high colloidal stability and homogeneous size distribution. However, to the best of our knowledge, there is no report that explored the combined use of mPEG-*b*-PLGA and SPG membrane emulsification. In addition, if numerous kinds of unstable proteins such as collagenase (COLase) or hyaluronidase (HAse) can be encapsulated in these PEG-immobilized PLGA microspheres

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during fabrication, it could greatly benefit controlled release technologies.

Scleroderma is a multisystem connective tissue disease characterized by immune abnormalities, vasculopathy, and excessive fibrosis of skin lesions and internal organs due to fibroblast proliferation and excessive production of ECM [17]. Its symptoms include Raynaud's syndrome and skin ulceration. Previous reports have indicated a prevalence rate of 50–300 cases per 1 million persons [18,19]. However, currently, there is no effective treatment for scleroderma although several therapeutic methods have been attempted, including administration of immunomodulatory agents.

Since fibrosis is a major cause of morbidity and mortality in scleroderma, recent studies have suggested that therapeutics targeting fibrosis are one of the potential treatment options for scleroderma [20–25]. Use of antifibrotic agents [22–24], integrin modulation [20], and targeted apoptosis of myofibroblasts [21] have been explored as strategies for reversing established dermal fibrosis in scleroderma. A possible method to modulate the ECM environment in scleroderma is the use of enzymes that degrade ECM. ECM degrading enzymes, such as COLase and hyaluronidase HAse, are abundant in our body and are clinically used for ECM-related diseases [26–34]. For example, degradation of collagen via COLase produced by *Clostridium histolyticum*, Xiaflex®, has been used for the treatment of Dupuytren's contracture, in which fingers are permanently bent in a flexed position [26,27]. HAse has been used to enhance drug penetration through skin [28,30,34]. HAse is also used to promote intratumoral penetration of anti-cancer drugs, and clinical trials of PEGylated recombinant HAse for pancreatic cancer are underway [31–33].

In the present study, for the first time, we develop uniform-sized PLGA microspheres with high colloidal stability via a combination of mPEG-*b*-PLGA and SPG membrane emulsification. COLase or HAse was loaded into the microspheres for the treatment of scleroderma via ECM degradation. Release of COLase and HAse from PLGA microspheres and the resultant degradation of ECM were evaluated *in vitro*. Their therapeutic effects on scleroderma via the degradation of over-deposited ECM were evaluated by their intradermal administration to a bleomycin-induced mouse model of scleroderma (Fig. 1).

2. Materials and methods

2.1. Materials

PLGA (PLA/PGA = 75/25, Mw = 75 kDa) was a kind gift from Mitsui Chemicals, Inc. (Tokyo, Japan). Hyaluronan (HA, MW = 2000 kDa) was kindly gifted from Denka Co. Ltd. (Tokyo, Japan). Porcine gelatin (G-2994 P, Mw = 160 kDa) was a kind gift from Nitta Gelatin Inc (Osaka, Japan). Dichloromethane (DCM), polyvinyl alcohol (PVA), and trehalose were purchased from Wako Pure Chemical Industries (Osaka, Japan). PEG, poly (ethylene glycol) methyl ether-block-poly(lactide-co-glycolide) (mPEG-b-PLGA, PEG: Mn = 5000, PLGA: Mn = 55,000), COLase from *Clostridium histolyticum*, HAse from bovine testes, monoclonal anti-actin, α-smooth muscle (α-SMA) antibody produced in mouse, and rhodamine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sunsoft was purchased from Taiyo Kagaku Co. (Tokyo, Japan). SPG membrane was purchased from SPG Technology (Miyazaki, Japan). Cellmatrix® Type I-A was purchased from Nitta Gelatin Inc (Osaka, Japan). Hematoxylin, eosin, and colloidal iron stain kit were purchased from Muto Chemicals Co. Ltd. (Tokyo, Japan). Alexa Fluor 488-conjugated goat anti-mouse IgG was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

2.2. Fabrication of COLase- and HAse-loaded PLGA microspheres

First, 1 ml of 2 wt% HAse aqueous solution containing 5 wt% trehalose was emulsified in 5 ml DCM containing 450 mg PLGA, 50 mg mPEG-b-PLGA, and 100 mg sunsoft by sonication to obtain W/O emulsions. Subsequently, the obtained w/o emulsions were further pressurized using nitrogen gas through SPG membrane to 2 wt% PVA aqueous solution containing 0.026 wt% PEG under stirring at 150 rpm, resulting in the formation of W/O/W emulsions. SPG membranes of 4, 5, and 10 μm pore sizes were used. Then, DCM was removed using an evaporator on ice to obtain COLase- and HAse-loaded PLGA

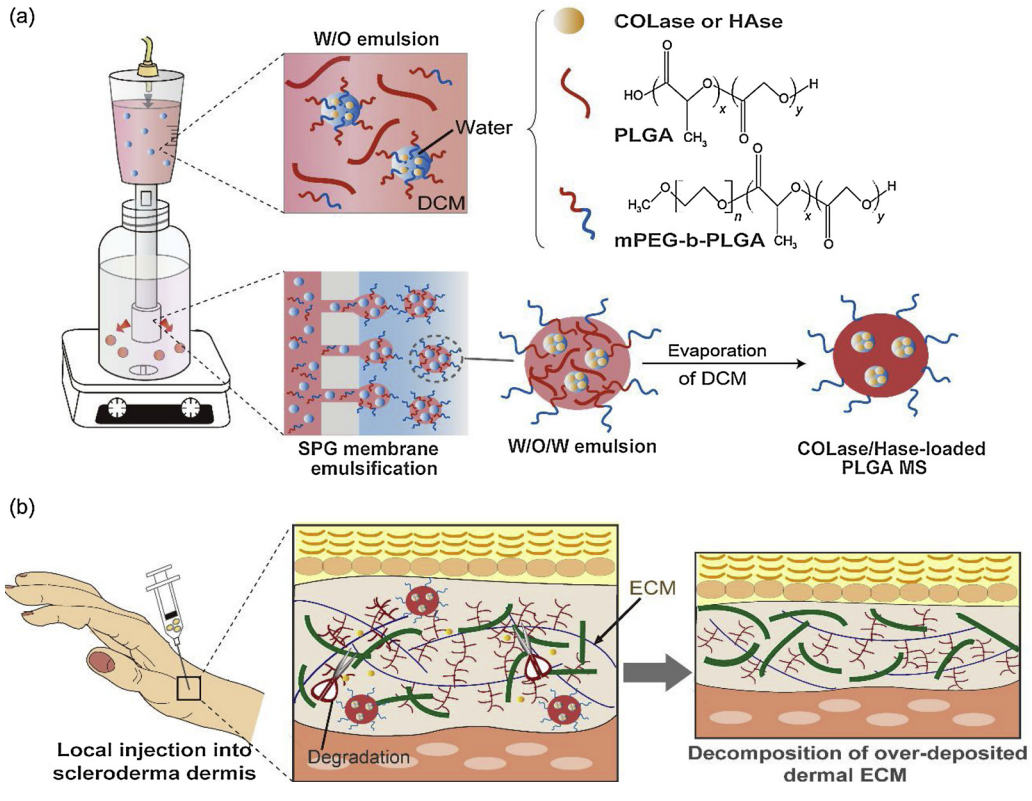


Fig. 1. Schematic illustration of (a) fabrication of COLase- and HAse-loaded PLGA microspheres using SPG membrane emulsification and (b) their use in the treatment of scleroderma via intradermal injection. Sustained release of COLase and HAse from intradermally localized PLGA microspheres degrades over-deposited ECM in scleroderma skin.

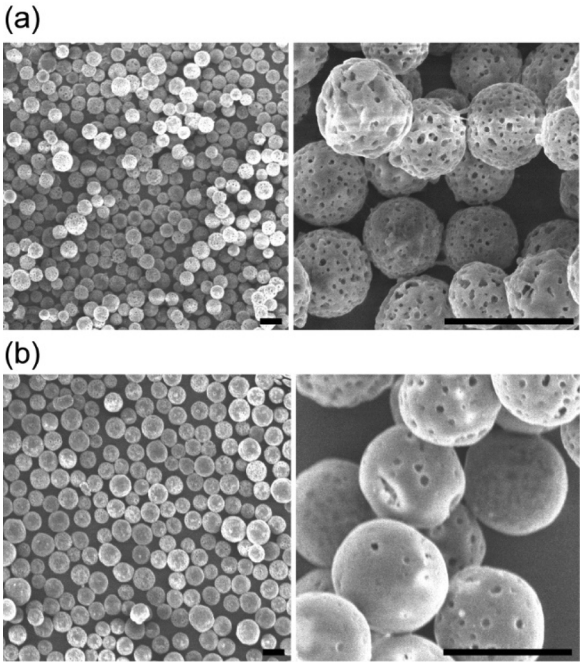


Fig. 2. SEM images of (a) COLase-loaded and (b) HAse-loaded PLGA microspheres. Scale bar is 10 μm.

microspheres. The microspheres were washed twice with pure water using centrifugation at 2500 rpm for 5 min, followed by preservation at −80 °C until use. In the case of microsphere fabrication without mPEG-*b*-PLGA, 500 mg PLGA was used instead of 450 mg PLGA. All other procedures were performed as described above. Scanning electron microscopy (SEM) (S-900; Hitachi, Japan) was used to observe the obtained microspheres. A laser diffraction particle size analyzer (LA-350; Horiba, Japan) was used to measure their size distribution.

2.3. Amount and retained activity of loaded COLase and HAse

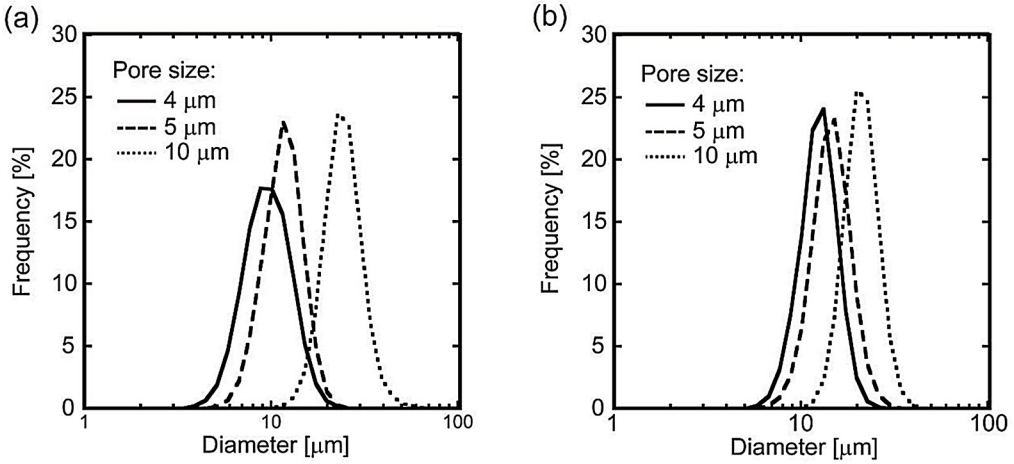
For PLGA degradation, 1.0 ml of 1 N sodium hydroxide was added to 40 mg of COLase- or HAse- loaded PLGA microspheres. After all microspheres had dissolved, the amount of loaded COLase and HAse was measured using the bicinchoninic acid (BCA) assay. Retained activity of loaded COLase was also measured using a commercial COLase activity assay kit (CLN100, Collagen Research Center, Tokyo, Japan), according to the manufacturer's instructions, and that of loaded HAse was turbidimetrically measured according to previously reported procedures [5].

2.4. Release behavior of COLase and HAse from PLGA microspheres

40 mg of COLase- and HAse-loaded PLGA microspheres were dispersed in 1 ml PBS, and the mixture was incubated at 37 °C. At various time points, microspheres were centrifuged at 15,000 rpm for 5 min, followed by collection of all supernatant and subsequent addition of fresh 1 ml PBS. Concentration of COLase or HAse in the collected media was measured using the BCA assay to evaluate the release behavior of COLase- and HAse-loaded PLGA microspheres. We conducted four independent experiments for each condition. Data are expressed as means ± standard deviation.

2.5. Degradation of gelatin and HA by released COLase and HAse

0.5 wt% COLase-loaded PLGA microspheres were incubated in 10 ml of 2 wt% gelatin dissolved in phosphate buffer (200 mM. pH 7.5) at 37 °C. Similarly, 0.6 wt% HAse-loaded PLGA microspheres were incubated in 5 ml of 1 wt% HA dissolved in phosphate buffer. At various time points, 100 μl of the incubation media was collected and preserved at - 80 °C until the measurements were performed. To evaluate the



(c)

Membrane Pore size [μm]	COLase-loaded PLGA MS		HAse-loaded PLGA MS	
	Mean diameter [μm]	CV [%]	Mean diameter [μm]	CV [%]
4	8.9	28.9	11.7	22.8
5	12.7	20.6	13.6	23.9
10	21.0	25.1	21.1	24.8

Fig. 3. Size distribution of (a) COLase-loaded and (b) HAse-loaded PLGA microspheres prepared using SPG membranes of different pore sizes, measured via laser diffraction. (c) Summary of the mean diameters and CV values of COLase- and HAse-loaded PLGA microspheres.

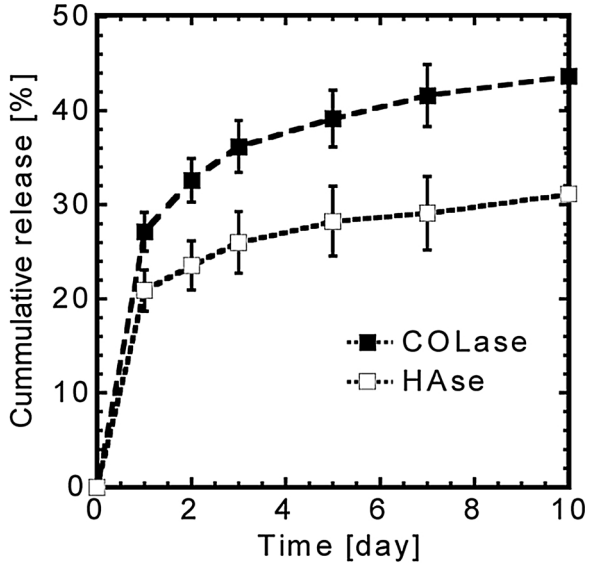


Fig. 4. Release of COLase and HAse from PLGA microspheres in PBS at 37 °C. Data were expressed as means ± standard deviation (n = 4).

degradation behavior of gelatin and HA by COLase- and HAse-loaded PLGA microspheres, changes in their molecular weight were measured using gel permeation chromatography (GPC) equipped with an LC-10ADVP pump (Shimadzu, Tokyo, Japan) and 830-RI differential refractive index detector (JASCO, Tokyo, Japan). Separation was performed at room temperature with a TSK-Gel GMPWXL column (TOSOH, Tokyo, Japan) at a flow rate of 0.2 mL/min. Phosphate buffer was used as the eluent. The molecular weights were determined relative to dextran standards (Extrasynthese, Genay, France).

The ECM degrading property of COLase-loaded PLGA microspheres was examined further by applying them to collagen hydrogels, which mimics the ECM environment. 600 µl of collagen gel was fabricated in a plastic cuvette using Cellmatrix® Type I-A, according to the manufacturer's instructions. Subsequently, 200 µl of 6.4 wt% COLase-loaded PLGA microspheres were added on the formed collagen hydrogel and incubated at 37 °C. PLGA microspheres without COLase loading were also added as control (blank PLGA microspheres). In the present experiment, PLGA microspheres were fluorescently labeled by adding 5 mg rhodamine to DCM at the time of emulsification for visualization. Degradation of the collagen hydrogel was monitored in real time by measuring the height of the interface, from which the degraded volume of the collagen hydrogelgel was calculated.

2.6. Evaluation of therapeutic effects on a mouse scleroderma model

All animal experiments were performed in accordance with the Guidelines of Animal Experiments of the University of Tokyo, and the protocols were approved by the animal care committee of the University of Tokyo. Seven-week-old specific pathogen-free conditioned female C57BL/6 mice were maintained in a temperature-controlled room under a light-cycle. At eight weeks of age, 200 µl of 1.0 mg/ml bleomycin dissolved in PBS was intradermally injected into a single location on the backs of the mice daily for 3 weeks to construct a scleroderma model. Subsequently, COLase- or HAse-loaded PLGA microspheres dispersed in saline were intradermally administered with a bolus injection to the model mice at a dose of 10 U COLase/mouse and 20 U HAse/mouse, respectively. For comparison, 10 U COLase or 20 U HAse dissolved in saline were also intradermally administered with a bolus injection to the model mice. The above doses of COLase and HAse were similar with the clinical dose of COLase for Dupuytren's contracture (Xiaflex®: 10,000 U/person [35]) in terms of body weight-based extrapolation, although it was much higher than the clinical dose

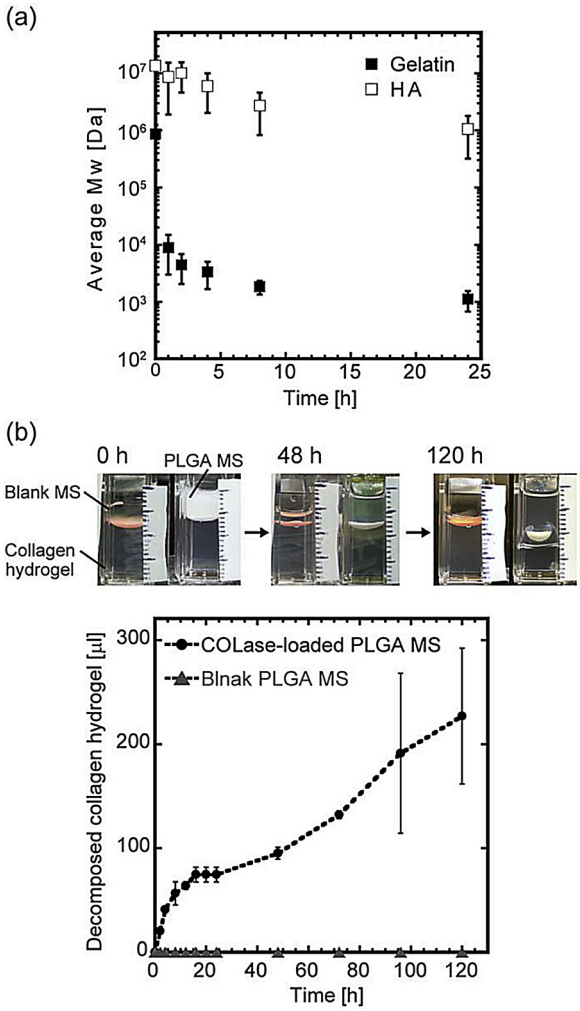


Fig. 5. (a) Time change in the average Mw of ECM polymers incubated with the ECM degrading enzyme-loaded PLGA microspheres. Gelatin and HA were incubated with COLase- and HAse-loaded PLGA microspheres, respectively. Raw data of the molecular weight distribution measured by GPC are also shown in Fig. S3. Data are expressed as means ± standard deviation (n = 5). (b). Degradation of the collagen hydrogel applied with COLase-loaded PLGA microspheres (MS). PLGA MS without COLase loading (blank MS) was also used for comparison. Data are expressed as means ± standard deviation (n = 3).

of HAse for enhanced drug permeation (Hylenex®: 50–300 U/person) [34]; assuming that the average body weight of human and mice is 60 kg and 25 g, the dose of Xiaflex® can be converted into 4.16 U/mouse. Since 2 out of 2 mice died by the intradermal administration of 20 U free COLase in our preliminary experiment, 10 U/mouse was chosen as the dose of COLase. On the other hand, because intradermal administration of 20 U free HAse did not affect the mice mortality, 20 U/mouse was chosen as the dose of HAse. After 1 week, the mice were euthanized using carbon dioxide, followed by the collection of skin sections. The sections were stained with hematoxylin and eosin (H&E), sirius red, and colloidal iron. Immunohistochemistry was performed for α-smooth muscle actin (α-SMA) using a mouse anti α-SMA monoclonal antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG [21]. Dermal thickness was examined as described previously [36,37]. Tensile test equipment (CR-3000EX-S, Sun Scientific Co., Tokyo, Japan) was used to evaluate the mechanical strength of skin. The collected skin was cut into dumbbell shape with a 16 mm gage length and 4 mm width using a punch. After clamping each end of skin samples, they were stretched until brake with a crosshead speed of 2.0 mm/min. Young's modulus, tensile strength, and strain at break were calculated from the

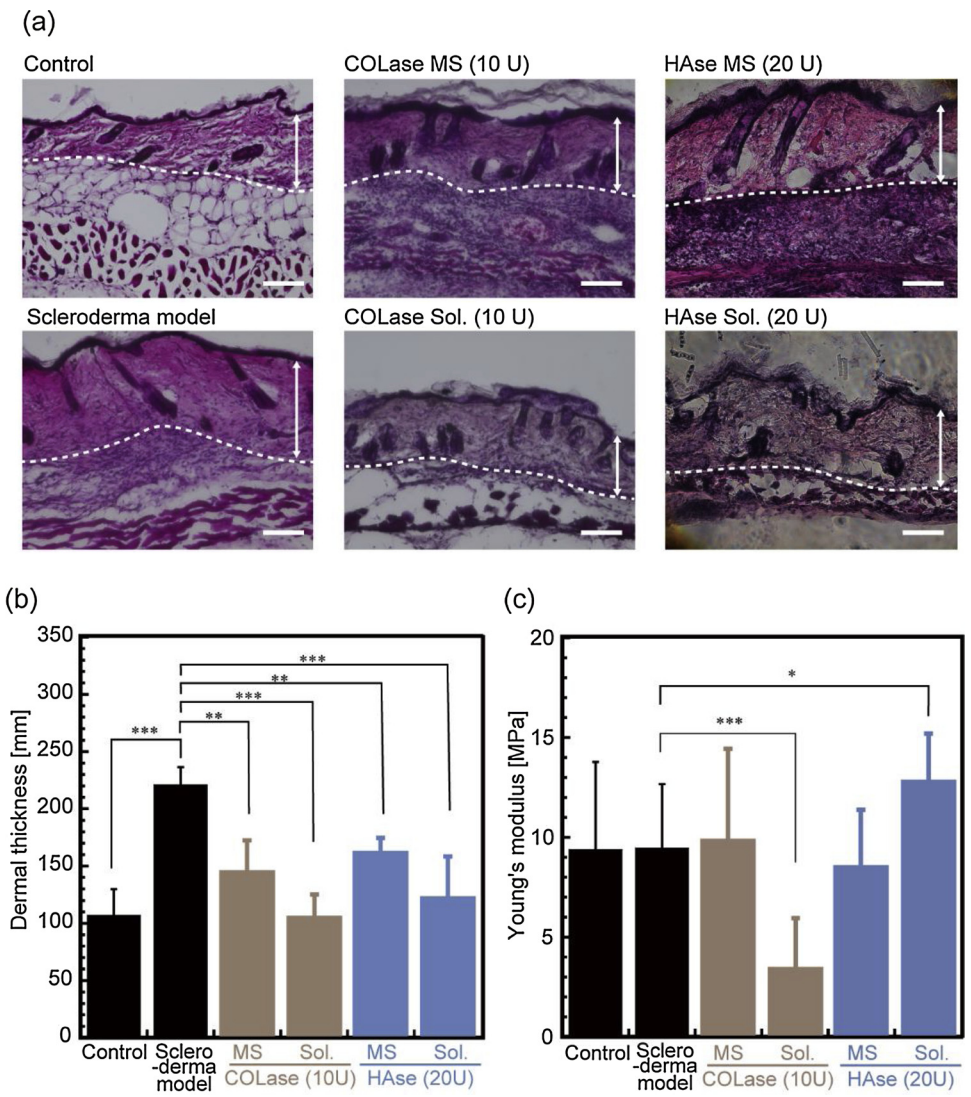


Fig. 6. (a) H&E staining of the skin sections of scleroderma model mice treated with COLase- and HAse-loaded PLGA microspheres. Results of control healthy mice and scleroderma model mice without material application are also shown for comparison. Dotted line shows the boundary between dermis and adipose tissue. Dermal thickness is indicated by arrow. Scale bar: 200 μm. (b) Dermal thickness of the mice analyzed from H&E staining images. Data are expressed as means ± standard deviation (n = 6). ***p < 0.001, **p < 0.01, *p < 0.05. (c) Young's modulus of the skin of scleroderma model mice treated with COLase- and HAse-loaded PLGA microspheres. Data are expressed as means ± standard deviation (n = 6). ***p < 0.001, **p < 0.01, *p < 0.05.

obtained stress-strain data. We conducted six independent experiments for each condition. Data were expressed as means ± standard deviation.

3. Results

COLase or HAse aqueous solution was first emulsified in DCM containing PLGA and mPEG-*b*-PLGA. Its further emulsification in 2 wt% PVA aqueous solution through SPG membrane generated COLase or HAse-encapsulated W/O/W emulsions (Fig. S1), and the subsequent evaporation of DCM resulted in the formation of COLase or HAse-loaded PLGA microspheres. Fig. 2 displays the SEM images of the obtained COLase and HAse-loaded PLGA microspheres. They appeared sphere in shape with uniform sizes. In addition, dimples were observed on the particle surfaces. Notably, even without using mPEG-*b*-PLGA, we could fabricate COLase and HAse-loaded PLGA microspheres. However, the formed microspheres caused aggregation in aqueous suspension (Fig. S2), whereas monodisperse microspheres were observed when fabricated with mPEG-*b*-PLGA (Fig. S2). The results suggested higher colloidal stabilities of COLase and HAse-loaded PLGA microspheres fabricated with mPEG-*b*-PLGA.

Size measurement via laser diffraction revealed narrow size distribution of COLase and HAse-loaded PLGA microspheres (Fig. 3). Coefficient of variation (CV) values typically ranged from 20% to 30%. In addition, the average size of COLase- and HAse-loaded PLGA microspheres could be controlled by the pore size of SPG membrane, regardless of the type of encapsulated enzymes. Further, 4, 5, and 10 μm membrane pore sizes resulted in COLase-loaded microspheres of 8.9, 12.7, and 21.0 μm diameters, respectively, and HAse-loaded microspheres of 11.7, 13.6, and 21.1 μm diameters, respectively. Microspheres larger than 10 μm would be preferable to avoid the uptake from macrophages [38,39], while larger particles are more likely to cause clogging during the injection. Therefore, COLase-loaded microspheres with a diameter of 12.7 μm and HAse-loaded microspheres with a diameter of 13.6 μm were used for the subsequent experiments.

Amounts of COLase and HAse loaded into PLGA microspheres were 2.4 wt% and 3.3 wt%, respectively. In addition, measurement of enzyme activity revealed that 12% of loaded COLase and 10% of loaded HAse retained their activities after encapsulation, which were comparable with the results previously reported for enzyme-loaded carriers [40]. Fig. 4 presents the release curves of loaded COLase and HAse. As seen, they were gradually released from PLGA microspheres, reaching up to 30% of loaded COLase and 44% of loaded HAse in 10 days.

To examine the ECM-degrading activity of the released COLase and HAse *in vitro*, gelatin and HA solution were incubated with COLase- and HAse-loaded PLGA microspheres, respectively, during which the time change in their molecular weight was measured by GPC (Fig. 5a). Molecular weight of gelatin and HA decreased with time and gradually reached plateau values in 24 h, suggesting the degradation of gelatin and HA by COLase- and HAse-loaded microspheres, respectively. To

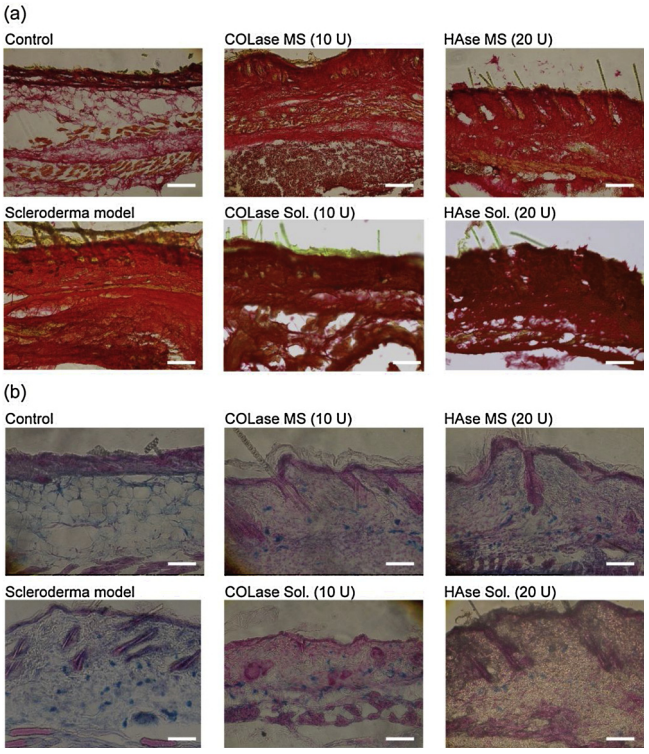


Fig. 7. (a) Sirius-red and (b) colloidal iron staining of the skin sections of the scleroderma model mice treated with COLase- and HAse-loaded PLGA microspheres. Results of the healthy control and scleroderma model mice without material application are also shown for comparison. Scale bar: 200 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

further examine the ECM degrading activity of COLase-loaded PLGA microspheres, suspensions of COLase-loaded PLGA microspheres were applied onto a collagen hydrogel fabricated in a cuvette, which mimics collagen matrix in ECM (Fig. 5b and c). Degradation of collagen hydrogel was evaluated by measuring its height in the cuvette. When blank PLGA microspheres without loaded COLase were applied as a control, degradation of the collagen hydrogel did not occur and its height remained unchanged. Conversely, when COLase-loaded PLGA microspheres were applied, rapid degradation of collagen hydrogel occurred within 24 h, followed by constant degradation over 120 h. This degradation behavior would be correlated with the release kinetics of COLase from PLGA microspheres, which showed similar initial burst and subsequent gradual release.

Therapeutic effects of COLase and HAse-loaded PLGA microspheres on scleroderma were examined by intradermal administration to the bleomycin-induced mouse model of scleroderma. Solutions of COLase and HAse were also used for comparison. Healthy control and scleroderma model mice without any treatment were also examined. Fig. 6a shows the H&E-staining pictures of mice 1 week after the administration of the material. The dermis of the scleroderma model mice was found to be much thicker than that of the healthy control mice. Conversely, the increase in dermal thickness was mitigated by the administration of COLase- and HAse-loaded PLGA microspheres. Similar results were obtained by the administration of free COLase and HAse solutions.

Change in dermal thickness following the application of COLase- and HAse-loaded PLGA microspheres was further evaluated quantitatively via image analysis of H&E staining images (Fig. 6b). The scleroderma model mice had more than 2 times higher dermal thickness than the healthy control mice, consistent with the findings of previous reports [37,41]. The increase in dermal thickness significantly decreased to a level similar to that in the healthy control mice following application of COLase- and HAse-loaded PLGA microspheres. Compared with the application of free COLase and HAse solution, microsphere application resulted in slightly higher dermal thickness in both types of enzymes.

In addition to dermal thickness, mechanical strength is a critical factor influencing skin condition. Young's modulus of skin 1 week after material administration was measured using a tensile test (Fig. 6c). Following the application of free COLase solution, Young's modulus significantly decreased to 3.3 MPa, which was ca. 30% of that of the healthy control mice (9.4 MPa). Conversely, no significant change was observed in terms of Young's modulus following the application of COLase-loaded microspheres. However, in the case of free HAse solution, Young modulus significantly increased to 12.7 MPa, which was 1.4 times higher than that in healthy control mice. The application of HAse-loaded PLGA microspheres did not induce significant changes in Young's modulus. Similar trends were observed in tensile strength of skins (Fig. S4), while no significant difference was observed in maximum strain at break (Fig. S5).

Fig. 7a shows the results of sirius red-staining. The dermis of the scleroderma model mice exhibited a stronger red color over a wider area compared with that of the control mice, suggesting the formation of dense and thick collagen networks. Further, collagen deposition decreased following the application of COLase-loaded PLGA microspheres and free COLase solution. Results of colloidal iron staining also revealed overdeposition of HA in scleroderma model mice, which was decreased by the application of HAse-loaded PLGA microspheres as well as HAse solution (Fig. 7b).

Fig. 8 shows immunohistochemical staining of the skin sections for α-SMA, a marker of mature myofibroblasts playing a key role in collagen synthesis during the development of scleroderma [42]. Application of free COLase and HAse solutions increased the expression of α-SMA in the dermis of the scleroderma model mice. However, no obvious increase in α-SMA expression was observed following the application of COLase- and HAse-loaded PLGA microspheres.

4. Discussions

In the present study, COLase- and HAse-loaded PLGA microspheres of uniform sizes were fabricated using a combination of SPG membrane emulsification and amphiphilic block copolymer mPEG-*b*-PLGA. The sizes of the obtained microspheres were tunable by membrane pore size and had CV values ranging from 20% to 30%, which are lower than the values reported for PLGA microspheres fabricated by mechanical agitation. For example, the CV value of HAse-loaded PLGA microspheres for the treatment of pulmonary fibrosis was ca. 50% in a previous report [5]. This narrow size distribution could facilitate homogeneous release of loaded enzymes and desired localization at the injected site.

In addition, combination with mPEG-*b*-PLGA facilitated facile and stable PEG coating during microsphere fabrication, resulting in suitable colloidal stability. PLGA block of mPEG-*b*-PLGA was considered to have been inserted into the oil phase during SPG membrane emulsification, followed by the precipitation of PLGA in the emulsions leading to the stable immobilization of PEG blocks on the microsphere surface through the solidified PLGA blocks. Another potential benefit of the PEG coating could be mitigation of immune responses induced by the PLGA microspheres. It has been reported that subcutaneously implanted PLGA microspheres induce foreign body reactions and are eventually encapsulated in fibrous capsules [43]. Since PEG coating mitigates the foreign body reaction associated with implant materials [44], the stable PEG immobilization presented here could also facilitate the inhibition of immune response and prolong the durability of COLase- and HAse-loaded PLGA microspheres.

It was also found that small dimples were formed on the surface of COLase- and HAse-loaded PLGA microspheres. The dimples had possibly formed through the coalescence of internal water droplets during

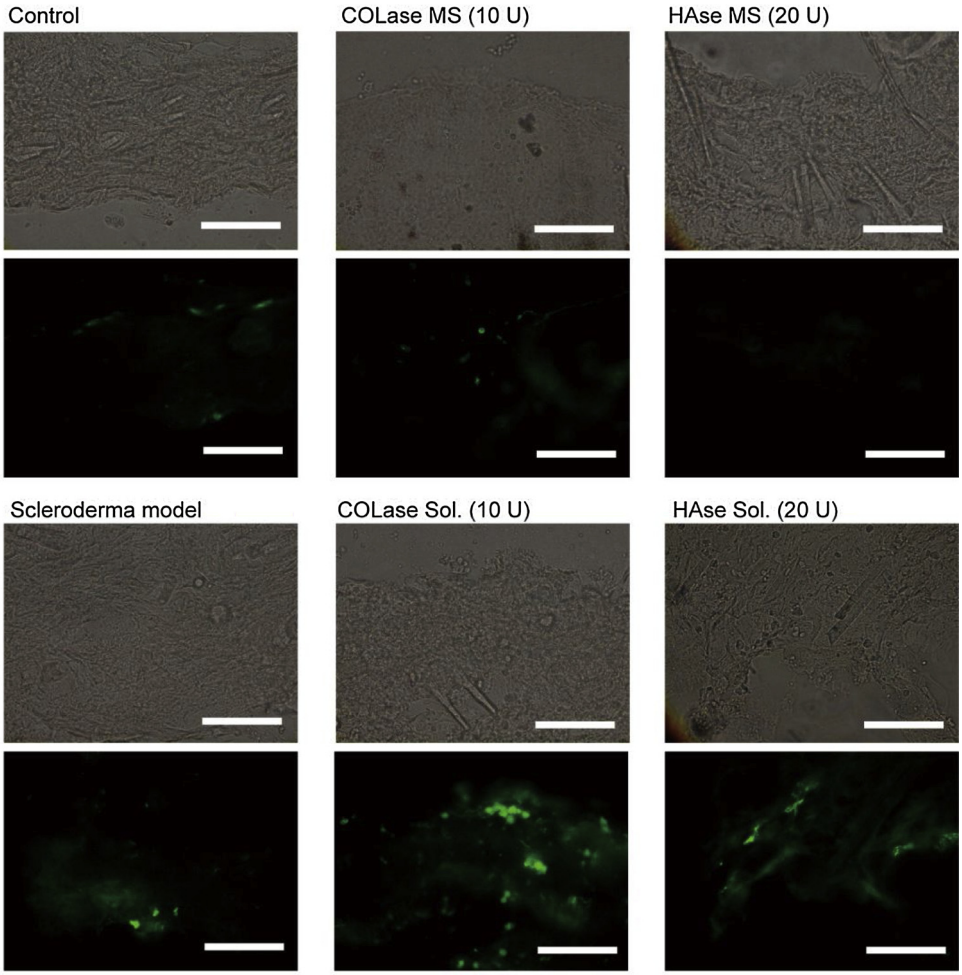


Fig. 8. Immunohistochemical staining of the skin sections of the scleroderma model mice treated with COLase- and HAse-loaded PLGA microspheres for α-SMA. Results of the healthy control and scleroderma model mice without material application are also shown for comparison. Scale bar: 50 μm.

precipitation of PLGA. Similar surface dimples have been reported previously in PLGA microspheres fabricated via the W/O/W double emulsion–solvent evaporation technique [45–47]. Since the sizes and numbers of dimples could affect the release kinetics of encapsulated enzymes [48], their precise control would need to be investigated in future studies. The amounts of mPEG-b-PLGA, as well as the relative volumes of internal water phases, are suggested to be key factors. We note that the amount of mPEG-*b*-PLGA used in the present study relative to PLGA (1:9) was a value optimized during the development. Compared with the case of nano-sized particles [16], the above-mentioned amount of mPEG-*b*-PLGA was relatively low, because of lower surface to volume ratio. A higher percentage of mPEG-*b*-PLGA induced instability in emulsions, which resulted in the wide size distribution in the obtained microspheres (Fig. S6).

COLase and HAse released from PLGA microspheres degraded gelatin and HA in solutions *in vitro*. Degradation of gelatin hydrogels by COLase-loaded PLGA microspheres was also observed. Degradation of the gelatin hydrogels lasted over 5 days, suggesting the high stability of encapsulated COLase. One notable observation was the volumes of collagen hydrogels decreased almost linearly with time after the initial rapid degradation. This could be explained by slow diffusion of COLase, which was assumed to be the rate limiting process. A similar phenomenon might be observed in fibrosis tissues, such as in dermis of the scleroderma patients, in which tight and dense ECM would restrict the diffusion of released enzymes.

A concern in the use of ECM degrading enzymes for the treatment of scleroderma is their side effects, which are caused by the degradation of ECM in healthy tissues. Their controlled release by drug carriers can be a solution for the potential adverse effects. Our *in vivo* experiment showed that intradermally injected COLase- and HAse-loaded PLGA microspheres degraded dermal ECM in the scleroderma model mice. Consequently, the dermal thickness decreased to a level similar to that in healthy mice. The results suggest the effectiveness of COLase- and HAse-loaded PLGA microspheres in the treatment of scleroderma. While an increased expression of α-SMA was observed following the administration of free COLase and HAse solutions, comparison of free and encapsulated enzymes revealed that an increased expression of α-SMA was not observed in the case of COLase- and HAse-loaded PLGA microspheres. Furthermore, while the administration of COLase- and HAse-loaded PLGA microspheres did not induce significant differences in terms of dermal Young's modulus, it significantly decreased by the administration of free COLase solution and increased by the administration of HAse solution. The differences could be attributed to the overdecomposition of dermal ECM by free COLase and HAse solutions. Excess degradation of collagen would disrupt the ECM network structure resulting in a decrease in Young's modulus, while excessive degradation of HA would induce loss of water retention properties in ECM, which would lead to an increase in Young's modulus [49,50]. Since such changes in the mechanical microenvironment of ECM, in addition to the activation of matrix metalloproteinase, reportedly induced myofibroblastic differentiation [51–53], an increase in α-SMA expression would be observed following the application of free COLase and HAse solutions. Such an increase in the generation α-SMA positive myofibroblasts could result in the future recurrence of dermal fibrosis.

On the other hand, the loading of COLase and HAse into PLGA microspheres enabled their gradual release, which would result in milder degradation of ECM and consequent mitigation of the above adverse effects. In addition, sustained release of loaded COLase and HAse is expected to reduce the number of repeated administrations into tight skin for treatment, which would enhance the quality of life (QOL) of patients. These mitigations of potential adverse effects and improved patient QOL can be the advantages of COLase- and HAse-loaded PLGA microspheres over free enzyme administration, although the therapeutic efficacy was similar.

PLGA microspheres are safe, reliable, and effective for sustained drug deliveries in clinical settings. Although COLase and HAse are relatively easily deactivated, we successfully encapsulated them in the mentioned PEG-coated PLGA microspheres and demonstrated their therapeutic efficacy *in vivo*. Therefore, other proteins or low-molecular weight compounds could be similarly encapsulated. The present facile one-pot production of PEG-coated PLGA microspheres with high colloidal stability and narrow size distribution could be applied as potential drug carriers for various diseases in future.

5. Conclusions

A one-pot fabrication method was developed for the preparation of uniform-sized PEG-coated PLGA microspheres with high colloidal stability using a combination of mPEG-*b*-PLGA and SPG membrane emulsification. COLase or HAse was loaded into these microspheres for the treatment of scleroderma. Sustained release of COLase and HAse from the obtained microspheres was observed over 10 days. The released COLase and HAse degraded gelatin and HA dissolved in PBS *in vitro*. Degradation of collagen hydrogel that mimicked the ECM environment by COLase-loaded PLGA microspheres was also confirmed. Intradermal administration of COLase- and HAse-loaded PLGA microspheres decreased the dermal thickness of bleomycin-induced scleroderma model mice, while minimizing changes in skin mechanical strength and expression of α-SMA, which were observed following the application of free COLase and HAse solutions. Since the proposed method could facilitate the encapsulation of various drugs in a similar manner, it could be employed as a versatile tool for controlled drug release in a wide range of diseases.

Declarations of interest

None.

Contributions

Study concept and design were performed by HO, YA, SS, and TI; Acquisition of data was done by SO, MM, YK, XC, and MT; Analysis and interpretation of data were done by all authors; Writing and final approval of manuscript were done by all authors.

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Appendix A. Supplementary data

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