Polymer Journal (2019) 51:1223–1230
https://doi.org/10.1038/s41428-019-0232-1

ORIGINAL ARTICLE

Controlled release and targeting of polypeptide-deposited liposomes by enzymatic degradation

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Received: 6 April 2019 / Revised: 7 June 2019 / Accepted: 15 June 2019 / Published online: 4 July 2019

Abstract

We prepared biobased nanocapsules with enzymatic degradability, which were generated by the layer-by-layer deposition of enzymes and polypeptide over the liposomal surface. Here, we demonstrate two different systems based on the enzymatic degradation of polymer layers. First, the deposition of trypsin and polyarginine (P_{Arg}), which is cleavable by trypsin, was carried out over a negatively charged liposome. The enzymatic cleavage of P_{Arg} resulted in exposure of the lipid membrane, which facilitated release of the cargo. Next, we attempted to degrade the outer polymer layer of the multilayered capsule wall to display the inner polymer layer by enzymatic degradation. This approach enabled the accumulation and targeting of the nanocapsules through the affinity between the displayed polymer layer and the target hydroxyapatite (HAp). The polymer wall was constructed with an inner layer consisting of poly-L-glutamic acid (P_{Glu}) and an outer layer consisting of trypsin and P_{Arg} onto the liposome. The degradation of the outer P_{Arg} by trypsin allowed the surface to display the inner P_{Glu}, which has bone-targeting ability. In addition, the polymer wall was constructed from an inner layer of P_{Arg} and an outer layer of pepsin and P_{Glu}. The degradation of the outer P_{Glu} by pepsin led to inner P_{Arg} on the surface to achieve cell-penetrating activity.

Introduction

Bioderived and nanosized capsules (nanocapsules) hold great potential to serve as vehicles for applications in pharmaceutical, cosmetic and food fields due to their size and ability to encapsulate, deliver and release cargo [1, 2]. Many studies have focused on applying a liposome, which is composed of phospholipid bilayers, as a drug carrier due to its biocompatibility, low toxicity and biodegradability [3, 4]. Surface modifications of liposomes have been carried out to achieve mechanical robustness as well as functional properties, such as improved encapsulation, site-specific delivery and controlled release of substances [5–8]. For example, the coating of chitosan via electrostatic interactions [6–8] has been employed to render them mucoadhesive. In addition, liposome site specificity was achieved by grafting antibodies or ligand-immobilized hydrophilic polymers onto the liposome surface [9]. For programmed or controlled drug release, environmentally responsive phospholipids [10, 11] or polymers [12, 13] were chemically coupled onto the liposomal membranes. In recent years, a layer-by-layer (lbl) approach has been developed by Decher et al. to fabricate multilayer films through electrostatic interactions [14]. This method is attractive for creating polymeric shells over various colloidal templates [15–18]. Based on this coating technique, our research group has created nanocapsules by lbl deposition of polymers onto the liposomal surface [19–21]. In particular, biopolymers, such as polypeptides and polysaccharides, were employed to create a biosafe capsule, resulting in a robust capsule wall over the liposomal surface that suppressed leakage of the cargo. Additionally, it has been reported that the release of encapsulated cargo can be triggered by degradation of the capsule wall via enzymatic degradation and UV irradiation [22, 23]. Thus, we have focused on the enzymatic degradation of the capsule wall to

Supplementary information The online version of this article (https://doi.org/10.1038/s41428-019-0232-1) contains supplementary material, which is available to authorized users.

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create a liponanocapsule that exhibits self-degradability by the lbl deposition of an enzyme and its polymeric substrate. We selected trypsin and polyarginine (P_{Arg}), which is enzymatically cleavable by trypsin (Scheme 1a). The degradation of the capsule wall was evaluated by measuring the number of amine groups in the fragments of P_{Arg} that were cleaved by trypsin under different conditions (pH and temperature). In addition, we investigated the exposure of the lipid membrane and the release of the encapsulated cargo upon enzymatic cleavage of P_{Arg}.

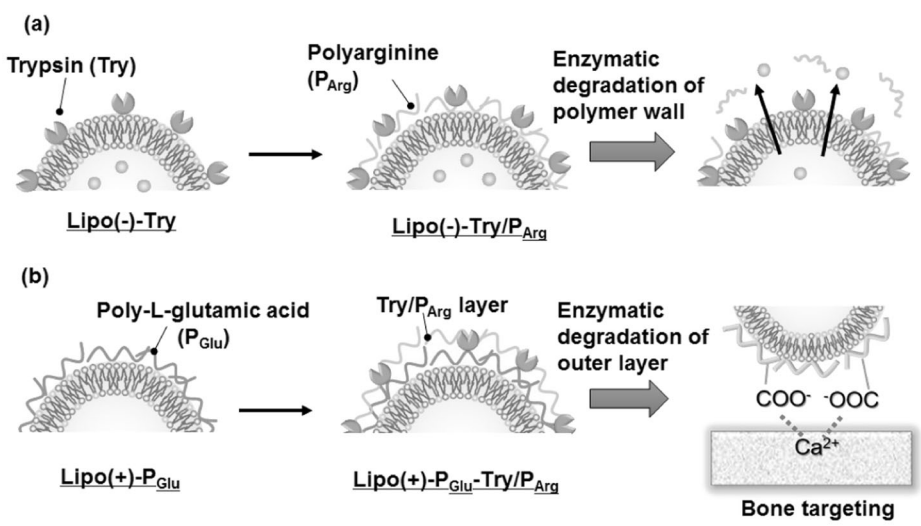
Next, we intended to display the inner polymer layer with new and useful functions on the capsule surface by enzymatic degradation of the outer polymer layer. Poly-L-glutamic acid (P_{Glu}) was deposited over a cationic liposome as the inner layer; then, the outer layer was fabricated by the deposition of trypsin and P_{Arg} (Scheme 1b). Taking advantage of enzyme specificity, we attempted to degrade the outer P_{Arg} layer of the polymer wall. Therefore, we investigated the surface exposure of the inner P_{Glu}, which is often used as a bone-targeting moiety [24, 25], and its accumulation on powdery hydroxyapatite (HAp), which is the main constituent of bone. Another degradable capsule was prepared by deposition of pepsin and its substrate, P_{Glu}, onto the P_{Arg}-deposited liponanocapsules. Using this type of nanocapsule, we intended to expose the inner P_{Arg}, which possesses cell penetration activity, by enzymatic degradation of the inner P_{Arg} layer.

Materials and methods/ experimental procedure Materials

Dilauroyl phosphatidic acid (DLPA) and 1,2-dimyristoyl-sn-glycero-3-ethyl phosphocholine (DMEPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). P_{Arg} (M.W. 15,000–70,000), P_{Glu} (M.W. 50,000-100,000), and 1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Trypsin from porcine pancreas, pepsin from porcine stomach mucosa, dimyristoyl phosphatidylcholine (DMPC), 2-morpholinoethanesulfonic acid monohydrate (MES), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) and 2,4,6-trinitrobenzensulfonic acid sodium salt dihydrate (TNBS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Pyrene was purchased from Tokyo Chemical Industries, Ltd. (Tokyo, Japan). The Micro BCA Protein Assay Kit was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). All chemicals were used as received. The water used in all experiments was prepared in a water purification system (WT-100, Yamato Scientific Inc., Tokyo, Japan) and had a resistivity higher than 18.2 MΩcm.

Preparation and characterization of Lipo(−)-Try/P_{Arg}

Trypsin (Try), which is a basic protease with an isoelectric point (pI) at pH 10.5, and polyarginine (P_{Arg}) (pKa = 12.5),



Scheme 1 Schematic illustrations of the preparation of the self-degradable capsule wall over the surface of liposomes by the layer-by-layer depositions of polypeptides and enzymes. **a** A protease (trypsin) and polyarginine (P_{Arg}), which is cleavable by trypsin, were deposited over an anionic liposome (Lipo(−)-Try/P_{Arg}). Enzymatic cleavage of P_{Arg} is expected to promote the release of cargo. **b** As the first layer, poly-L-glutamic acid (P_{Glu}) was deposited over a cationic liposome (Lipo(+)- P_{Glu}). Then, depositions of trypsin and P_{Arg} were carried out to construct the second layer (Lipo(+)-P_{Glu}-Try/P_{Arg}). Enzymatic degradation of P_{Arg} from the capsule wall is expected to allow for the surface display of P_{Glu}, which can target bone

which is a cationic polypeptide that is cleavable by trypsin, were deposited onto an anionic liposome (Lipo(−)). The preparation of liposomes and layer-by-layer deposition of polymers to produce liponanocapsules were carried out as described in our previous reports [19, 21, 26]. Briefly, DMPC and DLPA were dissolved in methanol at a lipid ratio of 0.5:0.5. The removal of methanol was performed by rotary evaporation to yield a thin lipid membrane. 1 mL of a 10 mM MES buffer solution (pH 5.0) or a 10 mM HPTS aqueous solution was added to the lipid membrane in the flask to afford a lipid suspension using a bath-type sonicator at 50 °C. After three freeze-thaw cycles, the liposome suspension was extruded 20 times through a membrane with 100 nm pores using a LipoFast Basic instrument (Avestin Inc., Ontario, Canada). Then, adsorption of trypsin was carried out by addition of 500 μL of a 20 or 50 ppm trypsin solution (pH 5.0) to the liposome suspension at a final lipid concentration of 0.38 mM followed by stirring at 700 r.p.m. for 30 min at 20 °C. To determine the surface coverage of trypsin, the supernatant solution was collected via centrifugal separation at 70000 r.p.m. for 45 min. Then, the residual amount of trypsin in the supernatant was measured by using a micro BCA protein assay, which is based on the reduction of Cu^{2+} to Cu^{+} by protein and the colorimetric detection of the cuprous cation (Cu^{+}) by bicinchoninic acid. The surface coverage was expressed in terms of the number of trypsin molecules adsorbed per 100 nm^2 of liposomal surface, assuming that trypsin has a spherical shape and a hydrodynamic diameter of 1.9 nm, as reported in the literature [27]. The number of trypsin molecules adsorbed per 100 nm^2 was determined to be 0.7 and 1.7 at feeding concentrations of 20 and 50 ppm, respectively. After the adsorption process, the excess trypsin was removed by repeated ultrafiltration (10 times) of the suspension using the VIVASPIN filtration membrane (M.W. cut-off 300 kD, Sartorius Stedim Biotech, Goettingen, Germany) for 2 min at 15000 r.p.m. Two sets of liponanocapsules were prepared and are referred to as Lipo(−)-$Try_{0.7}$ and Lipo(−)-$Try_{1.7}$ based on their surface coverage (0.7 and 1.7, respectively), as described above. Subsequent adsorption of P_{Arg} was performed by adding 500 μL of 1000 ppm P_{Arg} (pH 5.0) to the Lipo(−)-Try suspension. The control nanocapsule, which is referred to as Lipo(−)-P_{Arg}, was also prepared in the same manner as described above without the deposition of trypsin. The hydrodynamic diameter and zeta potential of the nanocapsules were evaluated by using a Malvern Zetasizer Nano-ZS (Malvern Instruments Ltd., Worcestershire, UK). The morphologies of the nanocapsules were observed with a transmission electron microscope (TEM, Tecnai Spirit TEM, FEI Co., Portland, OR, United States). The samples were prepared by depositing an aqueous suspension of nanocapsules on a carbon-coated copper grid, which was followed by air drying for 5 min.

Enzymatic degradation of the P_{Arg} layer by trypsin

Enzymatic degradation of the P_{Arg} layer and its desorption from the liposomal surface were evaluated as described below. Each suspension was poured into a dialysis unit (M.W. cut-off 35 kD, Slide-A-Lyzer MINI, Pierce Biotechnology, Inc., USA) and dialyzed against 3.0 mL of each 10 mM buffered solution at pH 2.0, 5.0, 7.4 and 10.0 for 7 days at 37 °C. At the predetermined time intervals, each release medium from the dialysis unit was collected to trace the proteolytic cleavage of amide bonds by measuring the number of amine groups in the fragments of degraded P_{Arg}. The number of amine groups was measured by using a TNBS assay, which can spectrophotometrically detect the primary amine groups. Furthermore, the change in the zeta potential of the nanocapsules after enzymatic degradation of the P_{Arg} layer was measured in the same manner as described above.

Release of cargo from Lipo(−)-Try/P_{Arg} triggered by enzymatic degradation of the P_{Arg} layer

The effect of enzymatic degradation of the capsule wall on the membrane fluidity of the liposome was evaluated in terms of the pyrene excimer/monomer intensity ratio. Pyrene was loaded onto the lipid bilayer by incubation at 50 °C for 30 min at a final probe to lipid ratio of 1:200. The temperature of the nanocapsule suspension was increased stepwise from 10 °C to 50 °C in a thermostat quartz cuvette, and the fluorescent intensities were measured every 10 °C. The samples were excited at 342 nm, and the fluorescent intensities at 386 nm (I_M) and 480 nm (I_E) were detected. These intensities correspond to the excitation of the monomer and excimer, respectively.

A fluorescent substance, HPTS, was used as a model drug to trace the release profile from the nanocapsules. The preparation of liposomes was carried out in the presence of HPTS and was followed by lbl deposition of polymers to incorporate HPTS into Lipo(−)-$Try_{0.7}$/P_{Arg}, Lipo(−)-$Try_{1.7}$/P_{Arg} and Lipo(−)-P_{Arg}. Then, a 1% Triton X-100 aqueous solution (0.10 mL) was added to 1 mL of each capsule suspension. The fluorescent intensity (I_0) of each lysate was measured to estimate the incorporation of HPTS. Then, each of the suspensions was poured into a dialysis unit and dialyzed against 10 mL of each 10 mM buffered solution at pH 2.0, 5.0, 7.4 and 10.0 for 7 days at 37 °C. After a prescribed time interval, the fluorescence intensity (I) at 512 nm was recorded at an excitation wavelength of 413 nm using a spectrofluorometer (FP-6500, JASCO, Tokyo, Japan) to measure the HPTS released into the HEPES

buffer. HPTS release was calculated from I and I_0 using the following equation:

$$\text{HPTS release}\,(\%) = I/I_0 \times 100$$

Preparation and characterization of Lipo(+)-P_{Glu}-Try/P_{Arg}

Next, we wanted to endow the liponanocapsule with the ability to unveil the inner layer of the capsule wall via degradation of its outer layer. The preparation and characterization of liposomes and nanocapsules were conducted in the same manner as described above, except a cationic lipid, DMEPC, was used instead of DLPA to prepare cationic liposomes (Lipo(+)); DMPC and DMEPC were mixed at a lipid ratio of 0.5:0.5. Poly-L-glutamic acid (P_{Glu}) (pKa = 4.3), which is known as a ligand that strongly binds to hard tissue, such as bone, was deposited onto Lipo(+) to produce the inner layer, and the resulting nanocapsule is referred to as Lipo(+)-P_{Glu}. Then, trypsin and P_{Arg} were deposited onto Lipo(+)-P_{Glu} to construct the outer layer. Here, the surface coverage of trypsin was determined to be 2.3 per 100 nm^2, and the nanocapsule is referred to as Lipo(+)-P_{Glu}-$Try_{2.3}$/P_{Arg}. In addition, the control nanocapsule, denoted as Lipo (+)-P_{Glu}-P_{Arg}, was prepared without the deposition of trypsin.

Surface display of an inner P_{Glu} layer of Lipo (+)-P_{Glu}-Try/P_{Arg} in response to enzymatic degradation of the outer P_{Arg} layer

Enzymatic degradation of the outer P_{Arg} layer and its desorption from the surface of the nanocapsules along with exposure of the inner P_{Glu} layer were evaluated according to the above-mentioned procedure. As a proof of concept, binding of Lipo(+)-P_{Glu}-$Try_{2.3}$/P_{Arg} to powdery HAp after unveiling the inner P_{Glu} layer was evaluated. Prior to the addition of nanocapsules to HAp, the inner P_{Glu} layer was exposed (Lipo(+)-P_{Glu}-$Try_{2.3}$/P_{Arg} (d)) by incubating for 7 days at pH 10.0 and 37 °C to allow for enzymatic degradation of the outer P_{Arg} layer. Then, a series of nanocapsules, including Lipo(+)-P_{Glu}, Lipo(+)-P_{Glu}-P_{Arg} and Lipo(+)-P_{Glu}- $Try_{2.3}$/P_{Arg}(d), were suspended in 10 mM HEPES buffer; subsequently, the nanocapsules were added to 20 mg of powdery HAp and stirred for 24 h at pH 7.4. At predetermined time intervals, each suspension was centrifuged at 15000 r.p.m. for 2 min, and the supernatant solution was collected to measure the scattering intensity to estimate the amount of unbound nanocapsules (S_u). The amount of adsorption was estimated based on the following equation:

$$\text{Adsorption to HAp}\,(\%) = (S_0 - S_u)/S_0 \times 100$$

where S_0 is the initial scattering intensity of each suspension. After a 24 h incubation, the nanocapsules bound to HAp were stained with Nile red, which is commonly used for lipid staining, and observed with a fluorescence microscope (BX60, Olympus, Tokyo, Japan).

Preparation and characterization of Lipo(−)-P_{Arg}-Pep/P_{Glu}

As an alternative combination of enzyme and polypeptide, pepsin and P_{Glu} were selected and deposited onto Lipo(−)-P_{Arg} to construct the self-degradable outer layer (Lipo(−)-P_{Arg}-Pep/P_{Glu}). The nanocapsules were prepared in the same manner as described above, except 25 ppm of pepsin was used rather than trypsin. The surface coverage of pepsin was estimated as 1.05 pepsin/100 nm^2, assuming that pepsin is spherical with a hydrodynamic diameter of 6.08 nm [28].

Results and discussion

Preparation and characterization of Lipo(−)-Try/P_{Arg}

Trypsin is a protease that catalyzes the hydrolysis of arginine derivatives. Although no activity with P_{Glu} was observed irrespective of the pH value, the rate of enzymatic hydrolysis of P_{Arg} increased with the pH value and reached a maximum at pH 10.0 (Fig. S1). Adsorption of trypsin on the surface of negatively charged Lipo(−) (−28.7 ± 0.8 mV) was performed at pH 5.0, which is lower than its isoelectric point (pH 10.5) and is well below the optimum pH for its activity.

Fig. S2 shows the adsorption isotherm of trypsin, where the amount of adsorbed trypsin increased with its concentration and leveled off when the equilibrium concentration was approximately 80 ppm. To prepare two sets of nanocapsules with a low trypsin surface coverage, adsorption was carried out with an amount of trypsin less than that needed for saturated adsorption. The number of adsorbed trypsin was calculated to be 0.7 molecule/100 nm^2 and 1.7 molecule/100 nm^2 for 26 ng/cm^2 and 65 ng/cm^2, respectively. The zeta potentials of Lipo(−)-$Try_{0.7}$ and Lipo(−)-$Try_{1.7}$ retained their negative charges of −31.3 ± 1.5 mV and −26.6 ± 2.7 mV, respectively. Therefore, the adsorption of P_{Arg} should proceed on these two nanocapsules through electrostatic interactions. As a result, the adsorption of P_{Arg} onto Lipo(−) increased with its concentration (Fig. S3). After the saturated adsorption of

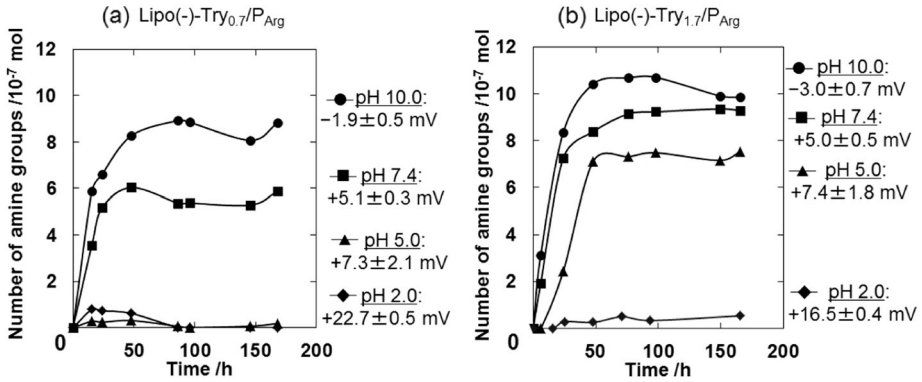


Fig. 1 Changes in cleavage of peptide bonds and zeta potential of Lipo(−)-Try$_{0.7}$/P$_{Arg}$ (left) and Lipo(−)-Try$_{1.7}$/P$_{Arg}$ (right) upon proteolytic cleavage of the capsule wall at 37 °C under different pH conditions

cationic P$_{Arg}$, the surface charges became positive, and the surface potentials were determined to be +26.2 ± 3.0 mV and +26.9 ± 0.8 mV for Lipo(−)-Try$_{0.7}$/P$_{Arg}$ and Lipo(−)-Try$_{1.7}$/P$_{Arg}$, respectively. The obtained nanocapsules remained spherical in shape (Fig. S4a and b) and monodisperse throughout the sequential adsorption (Table S1 and Fig. S5).

Enzymatic degradation of the P$_{Arg}$ layer by trypsin

The Lipo(−)-Try/P$_{Arg}$ suspensions were dialyzed at various pH values to estimate the proteolytic cleavage of peptide bonds by measuring the number of amine groups in the fragments of degraded P$_{Arg}$. Figure 1a shows the time courses of the number of amine groups detected from Lipo(−)-Try$_{0.7}$/P$_{Arg}$ during incubation at different pH values. The number of amine groups was barely detectable at pH 2.0 and 5.0, but this value increased substantially at pH 7.4 and reached the highest level at pH 10.0, which is the optimum pH for the enzymatic activity of trypsin. This result indicates that the P$_{Arg}$ layer was degraded by trypsin. Furthermore, the surface charge decreased from + 22.7 ± 0.5 mV to −1.9 ± 0.5 mV after incubation at pH 10.0 for 168 h. The degradation of Lipo(−)-Try$_{1.7}$/P$_{Arg}$ was faster than that of Lipo(−)-Try$_{0.7}$/P$_{Arg}$, and its surface charge became more negative (−3.0 ± 0.7 mV) after incubation at pH 10.0 for 168 h. This result is most likely due to the former containing more trypsin than the latter. Furthermore, the P$_{Arg}$ layer was degraded even at pH 5.0. These results suggest that enzymatic degradation of the P$_{Arg}$ layer can be modulated by the surface coverage of trypsin. The TEM images indicated that the spherical shape and hollow structure were retained despite degradation of the capsule wall for at least 7 days (Fig. S4c).

We previously reported that the polymer coating over the liposome decreased the membrane fluidity of the liposome and suppressed cargo leakage [19, 21]. Here, the membrane fluidity of Lipo(−)-Try$_{1.7}$/P$_{Arg}$ was estimated by measuring the excimer-to-monomer fluorescent intensity ratio of pyrene in the lipid bilayer [29], as shown in Fig. S6. In principle, an increase in the membrane fluidity facilitates the lateral diffusion of lipids, promoting excimer formation of pyrene. As shown in Fig. 2, the membrane fluidity of Lipo(−)-Try$_{1.7}$/P$_{Arg}$ increased with enzymatic degradation over the entire temperature range. It is important to note that the I_E/I_M values were still lower than those of bare liposomes (Fig. S7), indicating partial degradation of the P$_{Arg}$ layer even after incubation at pH 10.0 for 170 h.

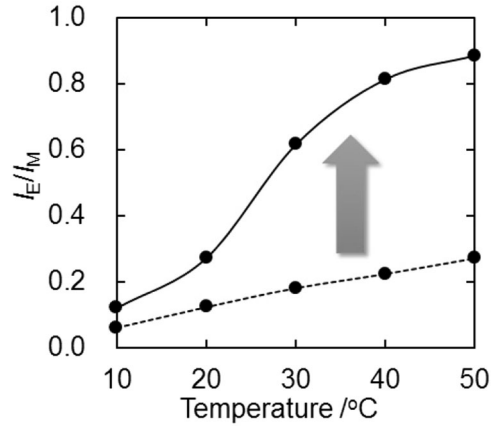


Fig. 2 Temperature-dependent membrane fluidity of Lipo(−)-Try$_{1.7}$/P$_{Arg}$ before (dotted line) and after (solid line) enzymatic degradation by incubation at pH 10.0 for 170 h

Release of cargo from Lipo(−)-Try/P$_{Arg}$ triggered by enzymatic degradation of the P$_{Arg}$ layer

Here, HPTS was selected as a model drug and incorporated into Lipo(−) and various liponanocapsules to evaluate its release behavior from their inner cavities. A bare liposome and Lipo(−)-Try$_{1.7}$ showed HPTS release due to its low trypsin surface coverage as shown in Fig. S8a and Fig. S8b, respectively. Previously, we reported that the polymer coating suppressed cargo leakage from the liposome [19, 21]. As expected, the release of HPTS from Lipo(−)-P$_{Arg}$ with a high P$_{Arg}$ surface coverage was suppressed over a wide range of pH values, as shown in Fig. 3a. The membrane fluidity of the lipid bilayer is a

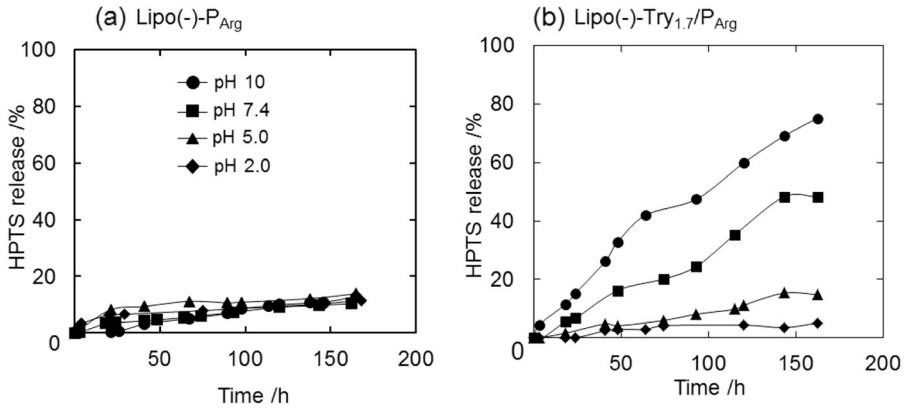


Fig. 3 Release profiles of HPTS from Lipo(−)-P_{Arg} (**a**) and Lipo(−)-$Try_{1.7}/P_{Arg}$ (**b**) at 37 °C under different pH conditions

crucial factor that governs the permeability and is dependent on the temperature, lipid composition and surface coverage of the polymer. In addition, the presence of the polymer layer should provide a physical barrier for the diffusion of substances, which depends on the density and charge of the polymer layer. As shown in Fig. 3b, the HPTS release from Lipo(−)-$Try_{1.7}/P_{Arg}$ gradually increased at pH 7.4 and 10.0 as the pH approached the optimum pH (8.0–9.0) of trypsin. This is because the enzymatic degradation caused desorption of the cleaved P_{Arg} fragments and the increase in the membrane fluidity (Fig. 2), thus facilitating the release of the cargo. Although enzymatic degradation proceeded at pH 5.0 (Fig. 1b), the HPTS release was substantially suppressed (Fig. 3b), which is most likely due to enzymatic degradation causing a small amount of desorption and limiting the increase in the membrane fluidity. Because the physical barrier (the P_{Arg} layer) remained intact, this degradation was insufficient to trigger release of the cargo. Therefore, we successfully produced a degradable liponanocapsule from trypsin and its substrate P_{Arg}, and this nanocapsule is capable of releasing cargo upon proteolytic cleavage of the capsule wall in a pH-controlled manner. However, the effects of enzymatic degradation on the membrane fluidity and the physical barrier of the polymer layer must be investigated. More detailed studies on the permeability and releasability of the nanocapsule are required, for instance, by comparing the time course of I_E / I_M with the number of amine groups.

Unveiling the polymer layer of Lipo(+)-P_{Glu}-Try/P_{Arg} in response to enzymatic degradation

Taking advantage of site-specific degradation by enzymes, we intended to expose the properties and functionalities hidden in the inner layer of the liponanocapsules. Here, we focused on the ability of P_{Glu} to bind hydroxyapatite (HAp), which is the main constituent of bone. Adsorption of P_{Glu} onto Lipo(+) increased as its concentration increased (Fig. S9). To prepare the P_{Glu} layer on positively charged

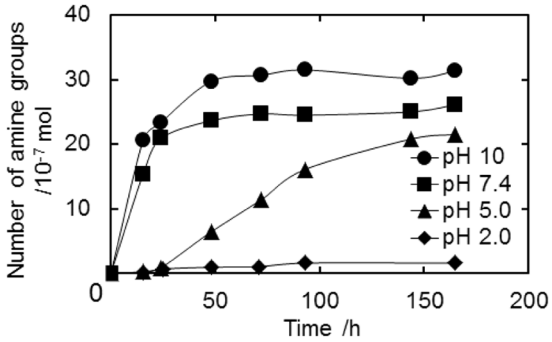


Fig. 4 Changes in cleavage of the peptide bonds of Lipo(+)-P_{Glu}-$Try_{1.7}/P_{Arg}$ upon proteolytic cleavage of the capsule wall at 37 °C under different pH conditions

Lipo(+) at + 29.9 ± 3.5 mV, deposition of P_{Glu} (pKa 4.3) was carried out at pH 5.0, and its concentration was set to 1000 ppm to achieve a high surface coverage. The resulting Lipo(+)-P_{Glu} became negatively charged (−18.0 ± 1.0 mV). Then, the outer layer was constructed by deposition of trypsin and P_{Arg} onto anionic Lipo(+)-P_{Glu}. As a result, a positively charged Lipo(+)-P_{Glu}-$Try_{2.3}/P_{Arg}$ (+21.7 ± 2.1 mV) was obtained. In addition, the shapes and sizes of the nanocapsules were maintained throughout the polymer depositions (Table S2, Figs. S10a-c and Fig. S11).

Degradation of the P_{Arg} layer by trypsin was confirmed by an increase in the number of amine groups that were generated by proteolytic cleavage of the amide bonds. As the pH increased, the release of amine groups increased, indicating that enzymatic degradation progressed smoothly (Fig. 4). Moreover, its surface charge changed from positive (+21.7 ± 2.1 mV) to negative (−8.8 ± 0.1 mV) after incubation at pH 10.0 for 168 h. In the absence of trypsin, the surface charge remained positive even after incubation at pH 10.0.

Lipo(+)-P_{Glu}-$Try_{2.3}/P_{Arg}$(d) was incubated with powdery HAp at pH 7.4 to elucidate its bone targeting ability. As shown in Fig. 5, nearly all Lipo(+)-P_{Glu}-$Try_{2.3}/P_{Arg}$(d) was adsorbed 5 h after incubation. However, only 20% of Lipo(+)-P_{Glu}-P_{Arg} was adsorbed after 24 h of incubation. In

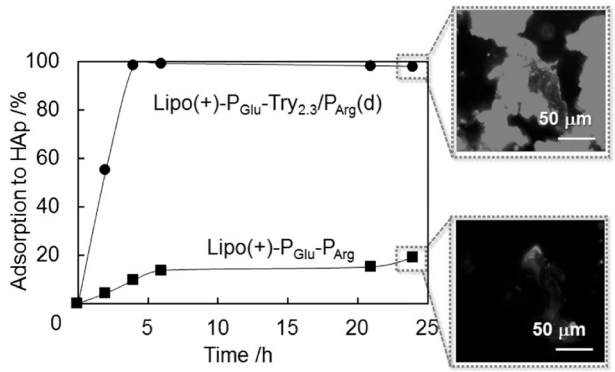


Fig. 5 Adsorption of Lipo(+)-P_{Glu}-P_{Arg} and Lipo(+)-P_{Glu}-$Try_{2.3}$/P_{Arg} (d) to powdery HAp during incubation at pH 7.4. Insets show fluorescence microscopy images of powdery HAp after incubation with each nanocapsule for 24 h. Nile red (red color) was used to stain phospholipids of each nanocapsule

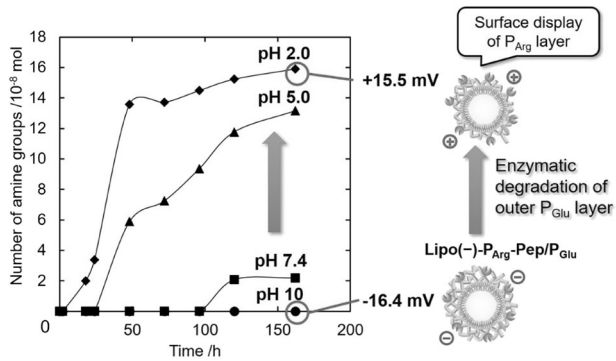


Fig. 6 Changes in cleavage of peptide bonds and zeta potential of Lipo(−)-P_{Arg}-Pep/P_{Glu} upon proteolytic cleavage of the capsule wall at 37 °C under different pH conditions

addition, fluorescent observation confirmed the selective accumulation of Lipo(+)-P_{Glu}-$Try_{2.3}$/P_{Arg}(d) on HAp (Inset photos in Fig. 5). These results suggest that trypsin can cleave the outer P_{Arg} layer to expose the inner P_{Glu} layer, which has a high affinity for HAp.

To demonstrate another application of the layer-specific degradation by an enzyme, pepsin (Pep) (pI = 3.0) was employed as a protease. After the deposition of cationic P_{Arg} onto anionic Lipo(−) as the inner layer to form Lipo(−)-P_{Arg}, Pep and its substrate, P_{Glu}, were further deposited as an outer layer to form Lipo(−)-P_{Arg}-Pep/P_{Glu} (Fig. S12). As shown in Fig. 6, enzymatic degradation of the outer P_{Glu} layer could allow for the unveiling of the polymer wall, thus exposing the inner P_{Arg} layer. The highest level of degradation was achieved under the optimum conditions for Pep (pH 2.0, 37 °C). Arginine oligopeptides are well known to exhibit cell-penetrating activity, which is expected to play a role in delivering various substances [30–32]. We previously demonstrated that surface deposition of P_{Arg} over a liposomal surface improved cellular internalization [26]. To elucidate this point, additional investigations of the activities of the exposed polymer wall, especially regarding the cell-penetration activity of the exposed P_{Arg}, must be performed in the future.

Conclusions

We developed biobased nanocapsules based on the lbl deposition of an enzyme and its substrate. Trypsin and its substrate, P_{Arg}, were deposited over a negatively charged liposome (Lipo(−)-Try/P_{Arg}). Enzymatic degradation of the P_{Arg} layer by trypsin was confirmed by an increase in the number of amine groups that were generated by the proteolytic cleavage of amide bonds. The rate of degradation reached the highest level under the optimum conditions for trypsin. Enzymatic cleavage of P_{Arg} resulted in exposure of the lipid membrane, which facilitated cargo release. Next, we attempted to unveil the polymer wall to expose a functional surface by enzymatic degradation. Here, the polymer wall was constructed from an inner layer consisting of poly-L-glutamic acid (P_{Glu}) and an outer layer consisting of trypsin and P_{Arg} on a positively charged liposome (Lipo(+)-P_{Glu}-Try/P_{Arg}). The degradation of the outer P_{Arg} by trypsin allowed for the surface display of the inner P_{Glu}, which has bone-targeting ability. To demonstrate another application of the layer-specific degradation by enzymes, pepsin was employed as a protease rather than trypsin. Here, the polymer wall was constructed with an inner layer consisting of P_{Arg} and an outer layer consisting of pepsin and P_{Glu} (Lipo(−)-P_{Arg}-Pep/P_{Glu}). The degradation of the outer P_{Glu} by pepsin led to the surface display of inner P_{Arg}, which has cell-penetrating ability.

Our developed nanocapsules exhibit layer-specific degradation by enzymes, which enables the release of cargo through degradation of the capsule wall as well as accumulation and targeting of nanocapsules through the emergence of special surface properties and functionalities mediated by unveiling an inner polymer layer.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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