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ORIGINAL CONTRIBUTION

Glucose-triggered release from liposomes incorporating poly(*N*-isopropylacrylamide-co-methacrylic acid-co-octadecylacrylate) and glucose oxidase

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Abstract In order to design liposomes which release their contents in a glucose-sensitive manner, the surfaces of egg phosphatidylcholine (egg PC) liposomes or dioleoylphosphatidylethanolamine (DOPE) liposomes were modified with the copolymer of *N*-isopropylacrylamide/methacrylic acid/octadecylacrylate and hydrophobically modified glucose oxidase (EC 1.1.3.4.). Whichever the liposomes were prepared with egg PC or DOPE, an extensive release of calcein was observed at acidic conditions. And DOPE liposomes were more pH sensitive than egg PC liposomes in terms of the release. In glucose-dependent calcein release experiment, there was no release for 180 min when the suspension of liposome was free of glucose. When the glucose concentration was 50 mg/dl, no appreciable amount of calcein was released for the first 50 min, but a significant release was observed for the last 130 min. At glucose concentration of 200 mg/dl, calcein release became more extensive and the releases for 180 min from egg PC and DOPE liposome were 84% and 98%, respectively.

Keywords Liposomes · Glucose oxidase · *N*-isopropylacrylamide · Methacrylic acid · Glucose-sensitive release

Introduction

Several kinds of glucose-sensitive carriers have been proposed for the treatment of type I diabetes. Glucose-sensitive membranes were developed by immobilizing glucose oxidase (GOD; EC 1.1.3.4.) in pH-sensitive polymers [1–6]. Glucose is converted to gluconic acid and hydrogen peroxide by the enzymatic reaction of GOD. The pH of release medium decreases with time due to the increased amount of gluconic acid. As a result, the release is triggered by the swelling and the deswelling of the pH-sensitive polymer membrane. Proposed were other glucose-sensitive systems which took advantage of the competitive binding of glycosylated insulin and free glucose to concanavalin A (Con A). Glycosylated insulin bound to Con A was immobilized either in polymer beads or in membranes [7–12]. At abnormally high concentrations of free glucose, glycosylated insulin tends to be detached from Con A and it releases from the beads or the membranes. Another example is based on the competitive binding of glucose attached to a polymer and free glucose to Con A [7–12]. The insulin release was controlled by a glucose concentration-dependent sol–gel transition of the polymer system. Until now, few studies have been done on glucose-sensitive liposomes. Liposomes are composed of phospholipids and they are known to be non-toxic in the human body. Liposomes can be functionalized by modifying the surface with stimuli-responsive molecules. The specific functions of liposomes are induced by direct interaction with target sites [13, 14], environmental pH [15, 16], and temperature changes [17–22]. In this study, a novel glucose-sensitive liposome was proposed by modifying the surface of egg phosphatidylcholine (egg PC) or dioleoylphosphatidylethanolamine (DOPE) liposome with hydrophobically modified glucose oxidase (HmGOD) and the copolymer of *N*-isopropylacrylamide/methacrylic acid/octadecylacrylate (P(NIPAM-co-MAA-co-ODA)). Our idea to achieve glucose-sensitive release is based on a mechanism proposed as follows. The pH of liposomal suspension

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decreases due to the increased amount of gluconic acid when glucose concentration increases. As a result, the copolymers would take a contracted form since the carboxylic groups are protonated and an intramolecular electrostatic repulsion force is weakened. The contraction of copolymers may give a mechanical stress to liposomal membranes, leading to an extensive release from the liposomes.

Experimental

Materials

DOPE was purchased from Fluka (Buchs, Switzerland). Egg PC, MAA, deoxycholate (DOC), GOD from *Aspergillus niger* Type X, palmitic acid *N*-hydroxysuccinimide ester, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), calcein, sodium citrate, citric acid, β-D-glucose, and Bradford reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Octadecylacrylate (ODA) was purchased from Aldrich Chemical. *N*-isopropylacrylamide (NIPAM) was purchased from TCI (Japan). All other reagents were in analytical grade.

Methods

Preparation and characterization of NIPAM copolymers

Copolymers of NIPAM/MAA/ODA (P(NIPAM-co-MAA-co-ODA)) were prepared by a free radical reaction [23]. The molar ratio of NIPAM/MAA/ODA monomer was 79/20/1. The ^{1}H NMR spectrum of the copolymer was taken on a Varian VXR-500S spectrometer using $CDCl_3$ as a solvent [24]. The content of MAA in the copolymer was determined by titrating MAA residue [24]. Gel permeation chromatography was performed in a high performance gel permeation chromatograph system (Waters 2690) equipped with columns of styragel HR 5E (M.W. 2,000–4,000,000), styragel HR 4 (M.W. 5,000–600,000), styragel HR 3 (M.W. 500–30,000), and styragel HR 2 (M.W. 500–20,000). The eluent was tetrahydrofuran and polymethylmethacrylate was used as a standard polymer. In order to investigate the pH sensitivity of P(NIPAM-co-MAA-co-ODA), the surface tensions of the copolymer solutions in distilled water were measured at various pH. Measurements were made using the ring method with a tension meter (SEO DST60A, Korea) [23].

Modification and characterization of GOD

GOD was hydrophobically modified with a palmitic acid ester, following a method described in a previous report [25]. In the reaction mixture, the molar ratio of GOD to the palmitic acid ester was adjusted to 1:40. The number of palmitic acid residue conjugated to GOD was determined by TNBS method [25, 26]. Enzymatic activity was determined on a UV spectrophotometer and the detailed procedure was described elsewhere [27].

Preparations of liposome incorporating HmGOD and NIPAM copolymers

A solution consisting of 1.4 ml DOPE (10 mg/ml) or 0.14 ml egg PC (100 mg/ml) in chloroform was put into a 25-ml round bottom flask. The solvent was evaporated in a rotary evaporator under reduced pressure to obtain a dry thin film of phospholipid. The dry film was dispersed into 2 ml of HEPES buffer (pH 8.0) containing calcein (50 mM), DOC (0.09%), HmGOD, and P(NIPAM-co-MAA-co-ODA). The weight ratio of copolymer to phospholipids was 0.2 and the molar ratio of HmGOD to phospholipids was 0.0001. The mixture was sonicated for 15 min with a bath-type sonicator (Ultrasonic processor, VC 505, Sonics & Materials, USA). The liposomal suspensions were allowed to stand at room temperature for 12 h. DOC, unentrapped calcein, and unbound enzyme and copolymer were removed by gel permeation chromatography using Sephadex G-100 column (1.6×40 cm). The final concentrations of phospholipid were adjusted to 2 mg/ml. The fluorescence quenching of calcein entrapped in liposomes was determined by the formula % quenching=$(1-F_i/F_f)\times 100$, where F_i is the initial fluorescence after removing free calcein and F_f is the total fluorescence after DOC is added to the suspension of calcein-containing liposomes so that final DOC concentration is 0.2%. The fluorescence intensities were measured at room temperature.

Determination of size of liposome

The size of liposomes was measured by a dynamic lighter scattering technique using a particle size analyzer (Plus 90, Brookhaven, USA). Moreover, 100 μl of a liposome suspension (2 mg/ml) was put into a cuvette and then filled up with HEPES buffer (10mM, pH 4.0, 5.0, 6.0, or 7.0) to 3.0 ml. The suspension was equilibrated for 5 min at 25°C. The measurement was done with three runs where each run consists of ten single gauging.

pH-triggered calcein release

A calcein-containing liposome suspension (0.15 ml, 2 mg lipid/ml in HEPES; pH 8.0) was injected into a fluorescence cell containing 2.0 ml of HEPES buffer or sodium citrate buffer, pre-adjusted to pH ranging from 4.0 to 7.5. The change in fluorescence was monitored with time at

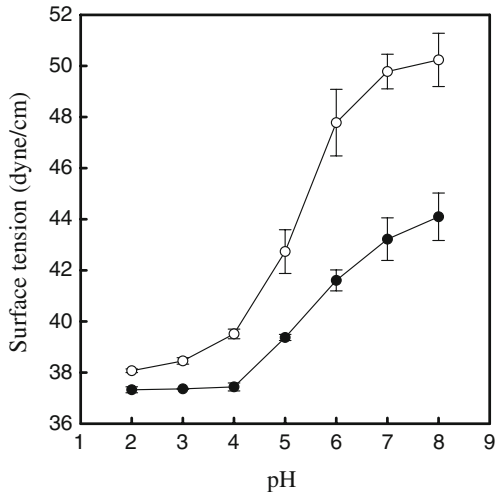

Fig. 1 Surface tensions of P(NIPAM-co-MAA-co-ODA) solutions at various pHs. The concentrations were 0.001% (○) and 0.004% (●)

524 nm with excitation at 494 nm. The percent release of calcein was determined as described elsewhere [28].

Glucose-triggered calcein release

A calcein-containing liposome suspension (0.5 ml, 2 mg lipid/ml in HEPES; pH 8.0) was put into 20 ml of glucose solution in isotonic saline (adjusted to pH 7.5 with 0.001 M NaOH). The final concentrations of glucose were 0, 50, and 200 mg/dl. The percent release of calcein was determined at predetermined time intervals. In parallel, the change in pH of the liposomal suspensions was measured during the release experiment.

Results and discussion

Characterization of NIPAM copolymers

In the spectrum of P(NIPAM-co-MAA-co-ODA), the peaks are assigned as follows. Peak around 1.1 ppm: methyl proton of isopropyl groups; 3.67 ppm: methylene proton of vinyl groups; 4.0 ppm: C-2 proton of isopropyl group; 0.85 ppm: terminal methyl proton of ODA; 1.25 ppm: methylene proton of ODA; 11.65 ppm: carboxylic proton of MAA. According to the result of titration of MAA residue, the content of MAA in P(NIPAM-co-MAA-co-ODA) was 18.5%. The number average molecular weight (Mn), the weight average molecular weight (Mw), and polydispersity index (Mw/Mn) were 6,258, 18,102, and 2.89, respectively. Figure 1 shows the surface tension variations of P(NIPAM-co-MAA-co-ODA) solutions with pH. The surface tensions increased in a sigmoidal manner with increasing pH. It means that the surface activities of the copolymers decrease with increasing pH. At acidic pH such as pH 2.0, 3.0, and 4.0, carboxyl groups of the copolymer tend to be unionized. Thus, the copolymers will be strongly surface active at acidic pHs since the hydrophobicity of ODA dominates the hydrophilicity of the unionized carboxylic group. The surface tensions at pH 2.0 were about 38.0 and 37.4 dyn/cm when the concentrations were 0.001% and 0.004%, respectively (Fig. 1). On the contrary, the carboxyl groups of the copolymer are likely to be ionized at higher pHs such as pH 6.0, 7.0, and 8.0. In this circumstance, the hydrophilicity of ionized carboxylic group dominates the hydrophobicity of ODA. As a result, the copolymers would become more soluble and they lose their surface activity at higher pH. The surface tensions at pH 8.0 were about 50.0 and 44.0 dyn/cm when the concentrations were 0.001% and 0.004%, respectively (Fig. 1). Obviously, the surface tensions at pH 8.0 were much higher than those at pH 2.0, indicating that the surface activities of the copolymers decrease with increasing pH. Following the results of pH-dependent surface tensions described above, it could be concluded that the surface activity of P(NIPAM-co-MAA-co-ODA) is sensitive to pH change.

Fluorescence quenching

The percent quenching of calcein encapsulated in liposome is a measure of the formation of liposomes. The values were 69% for egg PC liposomes and 67% for DOPE liposomes. This high percent quenching means that closed phospholipid bilayer vesicles, liposomes, have been successfully formed.

pH-triggered calcein release

Figure 2 shows the degrees of calcein release from liposomes incorporating HmGOD and P(NIPAM-co-MAA-co-ODA). Egg PC liposomes exhibited no significant release in the pH

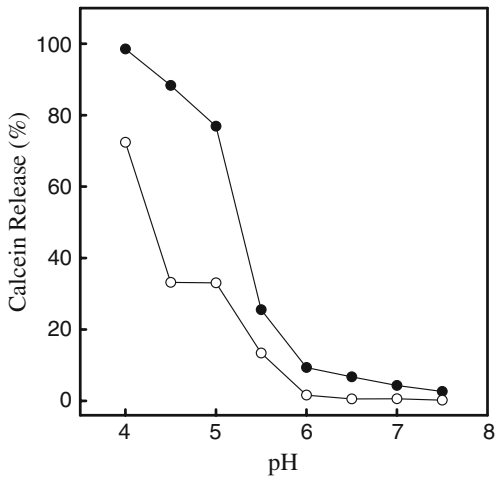

Fig. 2 Release of calcein from egg PC (○) and DOPE (●) liposomes at various pHs

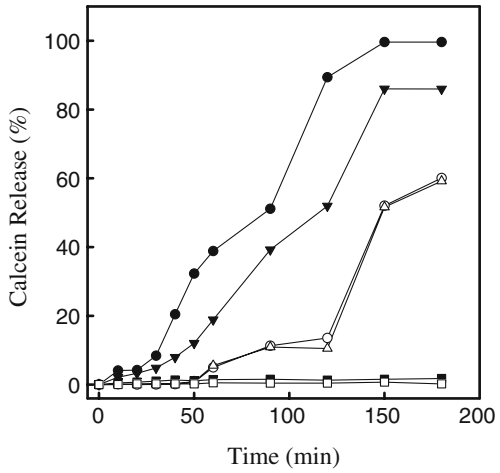

Fig. 3 Release of calcein from egg PC (*blank symbols*) and DOPE (*filled symbols*) liposomes with time in the presence of glucose. The glucose concentrations were 0 mg/l (*square*), 50 mg/l (*triangle*), and 200 mg/l (*circle*)

range of 6.0–7.5. The degree of release became significant at pH 5.5 and the value was about 75% at pH 4.0. The p*K* value of carboxylic group of MMA residue is between pH 5.0 and 6.0. Thus, the carboxylic group tends to be ionized in the pH range of 6.0–7.5 and the copolymer will take an expanded form. An electrostatic intramolecular repulsion force would prevail over a hydrophobic interaction between ODA residues. On the contrary, the carboxylic group tends to be unionized in the pH range of 4.0–5.0 and the copolymer will take a contracted form due to a hydrophobic interaction between ODA residues [23]. Since the copolymers are anchored to the liposomal membrane through the intercalation of ODA residues into the membrane, the contraction of the copolymer would give a stress to liposomal membrane, leading to the formation of packing defects in the membrane [29, 30]. This may account for why the degree of release was extensive in the acidic conditions. The pH-dependent release pattern of DOPE liposome was similar to that of egg PC liposomes. The release was less than 10% in the pH range of 6.0–7.5. The release was accelerated at pH 5.5 and almost 100% of release was observed around pH 4.0. Obviously, the pH sensitivity of DOPE liposome was much higher than that of egg PC liposome in terms of calcein release. DOPE hardly forms bilayer vesicles due to its small head group [31]. In order to constitute the vesicle, it needs complementary molecules which fill the space between the head groups [31]. In this study, P(NIPAM-co-MAA-co-ODA) was used as a complementary molecule for the stabilization of DOPE liposomal bilayers. Therefore, DOPE liposomes are likely to be disintegrated under acidic conditions because the copolymers undergo an acidification-induced contraction. According to a previous report, when the polymer-to-phospholipid ratio was less than 1/10 (*w*/*w*), almost 100% of polymers turned out to be immobilized on the surface of liposomes [20, 32]. Thus, most of polymers used in the preparation of liposomes might be incorporated into the liposomal membrane and cause a stress on the membrane. Several researches have been done on the liposomes coated with hydrophobically modified water soluble polymers. Unfortunately, no information about the distribution of polymers was reported yet. In our opinion, the polymers would more favorably rest on the outside surfaces of liposomes. The liposomal membrane is asymmetric and the curvature of the inside surface is higher than that of the outside. Thus, the resting of macromolecules on the inside surface would be unfavorable due to steric hindrances among polymer chains. And it seems to be more unfavorable that macromolecules were located between bilayers due to the limited space.

Glucose-triggered calcein release

Figure 3 shows glucose-triggered calcein release from liposomes incorporating HmGOD and P(NIPAM-co-MAA-co-ODA). In the absence of glucose, no significant releases were observed for 180 min with both egg PC and DOPE liposomes. At the glucose concentration of 50 mg/dl, the release profile of egg PC liposomes was almost the same as that of DOPE liposomes. No appreciable amount of calcein was released for the first 50 min. And then the degrees of release increased with time and the values were about 60% at time elapse of 180 min. When glucose concentration was 200 mg/dl, the degree of release steadily increased with time. At time elapse of 180 min, the releases from egg PC and DOPE liposomes were about 80% and 100%, respectively. Glucose could be converted to gluconic acid and hydrogen peroxide by the enzymatic reaction of GOD. As a result, the pH of liposomal suspension would decrease with time. In this circumstance, the copolymers anchored to the liposomal membrane would become contracted, giving a mechanical stress to the bilayers. This mechanism may explain the glucose-triggered release. Following the results of calcein release at 200 mg/dl, the glucose sensitivity of DOPE liposomes was found to be higher than that of egg PC liposomes. As described in the previous section, the higher sensitivity of DOPE may be explained by the disintegration of DOPE bilayers.

Size of liposomes

Figure 4 shows the mean diameters of liposomes incorporating HmGOD and P(NIPAM-co-MAA-co-ODA) at various pH. The diameters of egg PC liposomes were 300–350 nm and it was almost constant with respect to pH. According to the result of pH-triggered calcein release, the release from egg PC liposomes at pH 4.0 was about 75% in 120 s (Fig. 2). Despite the high percentage of release during the short period, the size at pH 4.0 was almost the same as that of

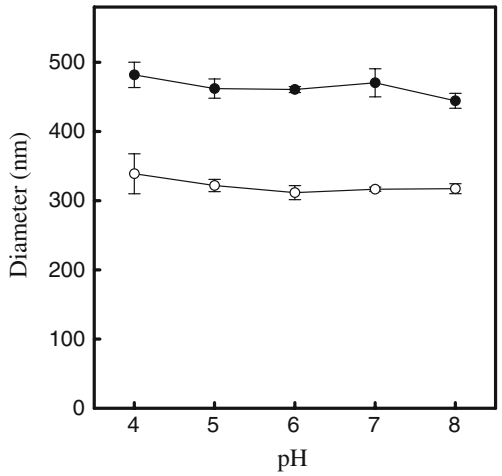

Fig. 4 Size of egg PC (○) and DOPE (●) liposomes at various pH

liposomes at pH 8.0 (pH of liposome preparation). This means that egg PC liposomes changed their membrane permeability significantly, while maintaining their integrities. In fact, the packing parameter of egg PC is almost 1 and it forms bilayers spontaneously. Thus, even though significant packing defects may be formed in the bilayers, the bilayer can be maintained [30]. On the other hand, the diameters of DOPE liposomes were 450–500 nm and also no significant change in size was observed with pH. Following the result of pH-triggered calcein release, the release from DOPE liposomes at pH 4.0 was almost 100% in 120 s (Fig. 2). One hundred percent of release during the short period indicates the disintegration of the DOPE bilayers. Nevertheless, the size at pH 4.0 was insignificantly different from that of liposomes at pH 8.0 (pH of liposome preparation). In general, when DOPE liposomes at neutral pH are disintegrated due to the depletion or the lateral diffusion of complementary molecules, large aggregates composed of hexagonal phases are formed [31]. However, according to the size data, there was no evidence for the formation of large aggregates. In fact, HmGOD exhibited a high surface activity at air/water interface due to the hydrophobic palmitic acid residues [33]. Hence, it was thought to be a dispersant, preventing the hexagonal phases from clustering each other. Another reason is that, under acidic conditions, the amino group in the head group of DOPE tends to be protonated and the effective size of the head increases. Accordingly, DOPE might constitute another kind of self-assembly rather than hexagonal phase at acidic pH. This may account for why the size at acidic pH was not much bigger than the size at neutral pHs. But the reason is not clear yet.

Enzyme activity

In order to anchor GOD into the liposomal membranes, GOD was hydrophobically modified according to TNBS method [25, 26, 34]. Six palmitic acids were found to be covalently attached to one GOD molecule by amide bond formation with reactive lysine residues. The covalent attachment of alkyl chains to lysine residues deteriorates the enzymatic activity, possibly because of the deformation of pockets and the formation of enzyme micelles. When HmGOD was immobilized in liposomal membrane, the activity was further reduced. The mobility of immobilized HmGOD would be restricted, leading to a reduced activity [33]. According to the results of enzyme activity analysis, the activity of HmGOD fell within 55–65% of that of native GOD, whichever it was free or immobilized. Despite a significant reduction in the activity, immobilized HmGOD can acidify the liposomal suspension. To address this question, pH change of liposomal suspension was investigated with time (Fig. 5). When no glucose was contained in the suspension, the pH was constant with time. In the presence of glucose (50 mg/dl), the pH linearly decreased with time for 90 min. And thereafter, the rate of pH decrease was slackened down and finally the pH reached around 4.0. Therefore, it is believed that the acidification induced by enzymatic reaction is responsible for the glucose-triggered release shown in Fig. 3.

Conclusions

Glucose-sensitive liposomes were developed by modifying the surface of liposomes with poly(*N*-isopropylacrylamide-co-methacrylic acid-co-octadecylacrylate) and hydrophobically modified glucose oxidase. Due to the acidification by enzymatic reaction, the copolymers alter their conformation in response to pH change, leading to the release from

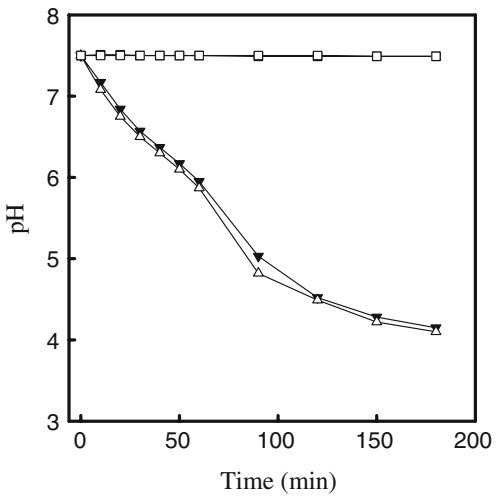

Fig. 5 Change in pH of egg PC (*blank symbols*) and DOPE (*filled symbols*) liposomal suspension with time elapse. The concentrations of glucose in liposomal suspensions were 0 mg/dl (*square*) and 50 mg/dl (*triangle*)

liposomes. The liposomes proposed in this study could be one of model carriers for the treatment of type I diabetes.

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