pH-Responsive Release of Fluorophore from Homocysteine-Carrying Polymerized Liposomes†

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ABSTRACT: Polymerized liposomes consisting of anionic and neutral polymerizable lipids and *N*-methacryloly-D,L-homocysteine thiolactone (MHT) were prepared. The liposomes clearly showed a pH-dependent release of fluorescent dye from the inner water pool to the bulk phase. In contrast, when methacrylic acid was introduced instead of MHT, the polymerized liposome obtained showed no pH-dependent release. The homocysteine-containing polymerized liposomes might be useful as a device for a pH-responsive drug delivery system.

To develop polymer-containing systems that are simple but highly responsive to outer signals such as light, heat, electric field, pH, solvent content, and so on is very important in quite diverse fields such as communication technology, microelectronics, medical science, and so on. In this report we examined a pH-responsive release of a fluorophore, Eosin Y, from liposomes consisting of polymerized lipids and *N*-methacryloylhomocysteine thiolactone moiety (Figure 1).

Antitumor drugs are usually toxic for both tumor cells and normal cells. For an efficient targeting (localization) of drugs to tumor cells it is highly desirable to prepare drugs conjugated with tissue or tumor cell selective homing devices.[1] However, a tumor-specific antibody, which would be the most powerful homing device, cannot be readily obtained at the present time. Therefore, a second solution, a nonspecific targeting, has to be sought.

To utilize the difference in physical or chemical properties between tumor cells and normal cells is a hopeful nonspecific targeting method. One of the big differences between these cells is the pH of the microenvironment around the cells.[2] The pH around tumor cells is lower than that around normal cells due to a larger amount of neuramic acid derivatives on the surface of tumor cells.[3] In addition, tumor cells are very active in their metabolic functions to produce acidic compounds. For example, when glucose was added in the medium, a significantly lower pH value around tumor cells than in other regions was reported due to the production of lactic acid.[4]

Polymerized liposomes, which are spherical bilayers stabilized by polymer chains, are suitable analogues of biomembranes, which are stabilized by polymers such as polysaccharides and polypeptides.[5] Thiolactone derivatives have been reported to open their rings in higher pH regions and reclose in lower pH regions.[6] Using this concept, *N*-palmitoylhomocysteine thiolactone (PHC) is shown to be useful as a pH-sensitive moiety in a liposome system.[7] Here we have introduced a pH-sensitive moiety, a homocysteine thiolactone group, into the polymerized liposomes in order to examine the utility of the liposomes as a pH-responsive device.

Experimental Section

Materials. *N*-Palmitoyl-D,L-homocysteine thiolactone (PHC), *N*-methacryloyl-D,L-homocysteine thiolactone (MHT), itaconyldiglycyldioctadecylamide (IGGA), and methacryloyldiglycyldioctadecylamide (MGGA) were prepared as described below. L-α-Dipalmitoylphosphatidylcholine (DPPC) of the highest grade (>98.2% by HPLC) and dimethyl-2,2′-azobis(isobutyrate) (V-601) were donated from Wako Pure Chemicals, Osaka, Japan. Methacrylic acid (MA) was distilled in vacuo. 2,2′-Azobis(isobutyronitrile) (AIBN) was purified by recrystallization from methanol. Other materials were commercially available. Deionized water was distilled before use.

***N*-Palmitoyl-D,L-homocysteine Thiolactone (PHC).** PHC was prepared by the coupling of palmitoyl chloride with D,L-homocysteine thiolactone hydrochloride following the method of Yatvin et al.[7] (68% yield). Anal. Calcd for $C_{20}H_{37}O_2NS$: C, 67.55; H, 10.49; O, 9.00; S, 9.02. Found: C, 67.36; H, 10.63; O, 8.77; S, 8.87. IR: 2930 (ν_{as} of C–H), 2870 (ν_s of C–H), 1700 (C=O stretching of thiolactone), 1650 (C=O stretching of amide), 1540 cm^{-1} (N–H deformation of amide).

***N*-Methacryloyl-D,L-homocysteine Thiolactone (MHT).** D,L-Homocysteine thiolactone hydrochloride was dissolved in water–THF (1:1), and the necessary amount of triethylamine (TEA) was added to neutralize the hydrochloride. Into this solution succinimidyl methacrylate (prepared by the method of Pollak et al.[8] 50% excess to the thiolactone) dissolved in THF together with 2,6-di-*tert*-butylcresol (BHT, 10 mg, inhibitor) was added. The reaction mixture was stirred at room temperature while the solution was kept at about pH 9 by the addition of small portions of TEA. A total of 20 h later, the solution was acidified to pH 6 with 0.1 N HCl. THF and water were evaporated in vacuo (without heating to avoid polymerization of MHT). By the addition of more 0.1 N HCl (to keep the solution at about pH 2) and bubbling of nitrogen through the solution, the product precipitated (yield, 47%). Anal. Calcd for $C_8H_{11}NO_2S$: C, 51.87; H, 5.99; N, 7.56; S, 17.27. Found: C, 51.85; H, 5.75; N, 7.54; S, 17.31. IR: 1690 (C=O stretching of thiolactone), 1650 (C=O stretching of amide), 1615 (C=C stretching), 1520 cm^{-1} (N–H deformation of amide). The product sublimed at 125 °C.

Itaconyldiglycyldioctadecylamide (IGGA). Diglycyldioctadecylamide (GGA) was prepared by the reaction of *N*-carbobenzoxydiglycine *p*-nitrophenyl ester (Z–GlyGlyONP) and 50% excess amount of dioctadecylamine (Fluka, recrystallized from acetone) in THF for 5.5 h at room temperature. After evaporation of the solvent the product was precipitated by acetonitrile. The product (*N*-carbobenzoxydiglycyl dioctadecylamide, Z–GGA) was purified by column chromatography using diethyl ether (to remove *p*-nitrophenol) and then ethyl acetate–hexane (3:1) to remove the unreacted dioctadecylamine (yield of Z–GGA 71%).

The Z group was removed by the addition of 20% HBr–acetic acid to Z–GGA (10 mL to 3–4 mmol of Z–GGA). A total of 30 min later HBr and acetic acid were removed by an aspirator. Diethyl ether–ethanol (4:1) was added to precipitate the product. The product filtrated was dissolved in chloroform, and the remaining HBr was removed by washing with 5% $NaHCO_3$ aqueous solution. The purified product (GGA) was obtained

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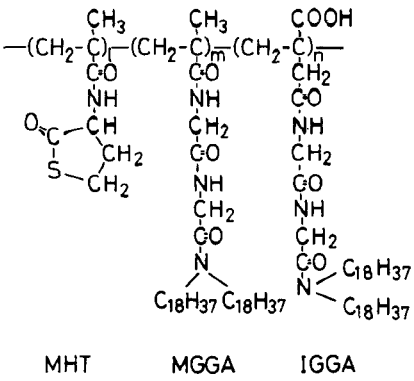

Figure 1. Chemical structures of MHT-containing polymerized lipids.

by the precipitation from acetonitrile (yield 90%). GGA and itaconic acid anhydride (40% excess to GGA) were dissolved in chloroform containing BHT (10 mg). Anhydrous TEA was added dropwise until everything was dissolved, and the mixture was stirred at room temperature. A total of 3 days later acetic acid (5 mL) and water (50 mL) were added to the reaction mixture and stirred for a few minutes. After evaporation for a short time, the precipitated product was collected on a glass filter and washed with cold water. IGGA was further purified by silica gel column chromatography using $CHCl_3$–acetone–acetic acid (40:10:3) (yield 80%). Anal. Calcd for $C_{44}H_{85}O_3N_3$: C, 72.22; H, 11.47; N, 5.62. Found: C, 70.85; H, 11.34; N, 5.56. IR: 2926 (ν_{as} of C–H), 2853 (ν_s of C–H), 1705 (C=O stretching of carboxyl group), 1675 cm^{-1} (C=O stretching of tertiary amide), 1650 (C=O stretching of secondary amide), 1610 (C=C stretching), 1535 cm^{-1} (N–H deformation of amide). An amide bond at the β-carboxyl group of itaconic acid was confirmed by a ^{13}C NMR spectrum using a JEOL JSX 270. Mp: 64 °C.

Methacryloyldiglycyldioctadecylamide (MGGA). GGA, succinimidyl methacrylate (30% excess to GGA), and BHT (10 mg) were dissolved in chloroform. TEA was added to the suspension until everything was dissolved. The mixture was stirred at room temperature for about 2 days. After evaporation of the solvent, the solid product obtained was washed with acetonitrile. The product was further purified by silica gel column chromatography (ethyl acetate–hexane (3:1)) (yield 71%). Anal. Calcd for $C_{45}H_{85}O_5N_3$: C, 74.71; H, 11.98; N, 4.05. Found: C, 73.73; H, 11.94; N, 4.06. IR: 2926 (ν_{as} of C–H), 2853 (ν_s of C–H), 1680 (C=O stretching of tertiary amide), 1655 (C=O stretching of secondary amide), 1620 (C=C stretching), 1545 cm^{-1} (N–H deformation of amide). Mp: 95–97 °C.

Preparation of Liposomes. Lipids were dissolved in THF in a round-bottomed flask, and the solvent was evaporated. By this procedure a thin film of lipids was formed on the inner wall of the flask. Buffer solution (HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; pH 8.0, 5 mM) was added. For fluorescence measurements a fluorescent indicator, Eosin Y, was dissolved in the buffer solution in high concentration (0.1 M). Eosin Y shows a self-quenching phenomenon at high concentration,[5c] and upon dilution it shows an intense fluorescence.

The lipid mixtures were dispersed first by shaking by hand at 60 °C and then by sonication for 30 min at 60 °C using an ultrasonicator (Astrason W-385, Heat Systems–Ultrasonics Inc., NY). The fluorophore-containing liposomes obtained were purified and separated from the fluorescent solution by gel permeation chromatography (Sephacryl S-1000 Superfine column 2 × 25 cm eluent, HEPES buffer).

Polymerization of Lipids. Polymerization of lipids was carried out both in liposome and homogeneous systems. For the liposome system, suspensions containing MHT, IGGA, and MGGA were deoxygenated by bubbling N_2 for 10 min; potassium peroxydisulfate (KPS; 1 mg) was then added as an initiator. The suspension was incubated at 60 °C for 24 h.

In the homogeneous system, all the lipids were dissolved in an organic solvent (mostly $CHCl_3$–MeOH (1:1), 0.1 g monomer/15 mL). After bubbling N_2 the initiator was added, and the mixture was incubated at 60 °C overnight. After evaporation of the solvent in vacuo, the polymers were recovered almost quantitatively by precipitation in acetonitrile. The absence of nonpolymerized monomer in the precipitates was confirmed by thin-layer chromatography [ethyl acetate–hexane (3:1) and $CHCl_3$–acetone–acetic acid (40:10:3)].

Table I
Preparation Conditions of Polymerized Lipids

name	contents of lipids, %	polymerizn conditions
L-9	IGGA, 40	in $CHCl_3$–MeOH (1:1)
	MGGA, 10	60 °C, 17 h
	MHT, 50	V-601, 15 mol %
L-11	IGGA, 40	in $CHCl_3$–MeOH (1:1)
	MGGA, 10	60 °C, 17 h
	MA, 50	V-601, 15 mol %
L-12	IGGA, 35	in DMF
	MGGA, 5	60 °C, 22 h
	MHT, 60	AIBN, 5 mol %

Measurements of pH Sensitivity. A release of the fluorescent dye from the liposome was observed using a fluorescent stopped-flow spectrophotometer (RA-401, Otsuka Electronics, Hirakata, Japan) at 35 °C. The fluorescent dye-carrying liposome suspension (pH 8.0, 5 mM HEPES) was mixed with an equal volume of HEPES solution (5 mM, acidified with 0.1 N HCl beforehand to attain various pH values after mixing with a pH 8.0 HEPES buffer). The increase in fluorescence intensity was followed for 10 s (excitation at 305 nm). For detection a Hoya Y46 filter was used to allow emitted light with wavelength longer than 460 nm to enter the detector). A 100% permeation was attained by the sonication of the suspension at 60 °C for 3 min before the measurements. Thus the percent of fluorescence increase in 6 s was evaluated. The pH of the liposome suspension after mixing with an equal volume of acidic HEPES solution was also measured using a high-sensitivity pH meter (F-7ss, Horiba, Kyoto, Japan).

Dynamic Light-Scattering Measurements. To evaluate the diameter of the liposomes, a dynamic light-scattering (DLS) apparatus (BI 2230, Brookhaven, NY, He–Ne laser (6328 Å, NEO-15 MS, Japan Science Engineering, Osaka, Japan) was used.

Gel Permeation Chromatography. To estimate the molecular weight of the polymerized liposomes, gel permeation chromatography was used (Waters Model 440 HPLC system; columns Cosmosil 5GPC-100 (4.6 × 250 mm), 5GPC-300 (4.6 × 250 mm), Nacalai Tesque, Kyoto, Japan; mobile phase, chloroform–methanol (1:1); flow rate, 0.5 mL·min^{-1}). As standard samples poly(ethylene glycol)s with various molecular weights [M_w 4250, 22 000 (Polysciences, Inc., Warrington, PA), M_w 6000, 20 000 (Nacalai Tesque, Kyoto, Japan) were used.

Spectrophotometric Measurements. A reduction of 5,5′-dithiobis(2-nitrobenzoic) acid by various kinds of homocysteine derivatives was followed by the change in absorbance at 435 nm using a high-sensitivity spectrophotometer (SM-401, Union Engineering, Hirakata, Japan). The observation cell was thermostated at 30 ± 0.05 °C using a Neslab RTE-8 waterbath.

Differential Scanning Calorimetry. The temperature of the midpoint of phase transition (T_m) was determined by a differential scanning calorimetry (DSC) using a SSC580 (Daini-Seikosha, Tokyo). The raising rate of temperature was 2 °C/min.

Results and Discussion

A. Preparation of Liposomes. The introduction of MHT into the liposome by polymerization in the liposome phase was not successful, probably due to a large difference in the lipophilicity of MHT compared to IGGA and MGGA. Therefore, we tried to introduce MHT into the liposome by the polymerization of three kinds of monomers (MHT, IGGA, and MGGA) in homogeneous solution such as chloroform–methanol or DMF (Table I). In order to obtain polymerized lipids with a good dispersibility into water, the amount of initiator added was larger than that of ordinary radical polymerization. Polymerization of MHT with other monomers by UV light was not successful due to decomposition of MHT.

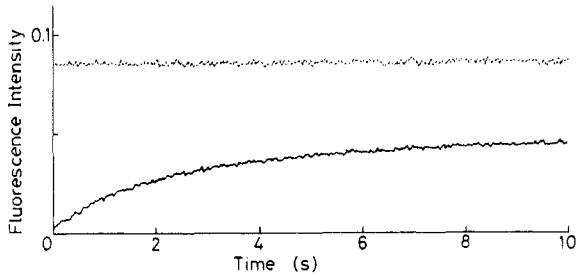

Figure 2. Release profiles of Eosin Y from the polymerized liposome at 35 °C. L-12 liposome: (—) at pH 5.9 after mixing; (···) sonicated at 60 ° for 3 min before mixing (100% release).

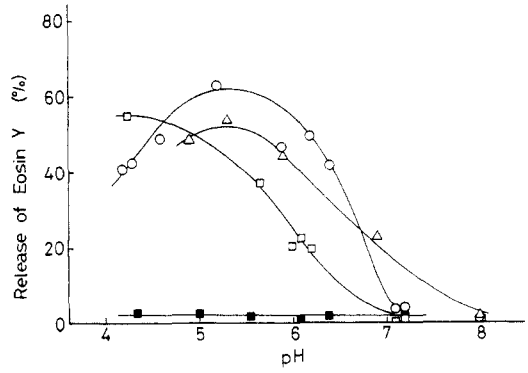

Figure 3. pH-dependent release of Eosin Y from various liposomes in 6 s at 35 °C: (○) L-9 liposome; (△) L-12 liposome; (□) PHC-DPPC liposome (■) DPPC liposome (in 50 s).[8]

By dispersing the polymerized MHT–MGGA–IGGA into the 5 mM HEPES buffer using vortexing and ultrasonication, we could obtain a stable polymerized liposome. The presence of liposomes was confirmed by electron microscopy, dynamic light scattering, and fluorescent dye inclusion methods. The diameters of the liposomes were evaluated to be 2200–2700 Å by the DLS technique.

To estimate the molecular weights of these terpolymers, a gel permeation chromatography was used. Due to the disadvantageous adsorption of these amphiphilic polymers to the columns, however, the polymers could not be recovered from the column completely. From the vague peak in the chart of GPC, therefore, we could only say that the molecular weights of these polymers were at least 10 000. From elemental analyses and conductometric titration the contents of three kinds of monomers in the polymers obtained were similar to those of monomers dissolved in the preparation solution.

The temperatures of the midpoint of the phase transition (T_m) were evaluated to be 38, 37, and 38 °C for L-9, L-11, and L-12 liposomes, respectively, using the DSC method. Since the pH-responsive release of the inner content of the liposome was rapid in the phase-transition regions,[9] the DSC data mentioned above suggest that liposomes prepared here are suitable as a pH-responsive device.

B. pH Sensitivity of the Liposomes. Using a fluorescence stopped-flow method, we could follow the releasing process of Eosin Y from the inner water pool of the liposomes to the bulk phase as exemplified in Figure 2.

Figure 3 shows the pH profile of the percent release of Eosin Y from the polymerized liposome in 6 s. In the figure the pH profiles of the *N*-palmitoylhomocysteine thiolactone–DPPC liposome (PHC:DPPC = 3:7) and DPPC liposome were also shown for comparison. It is clear that the polymerized liposome composed of MHT, IGGA, and MGGA shows a larger release at weakly acidic

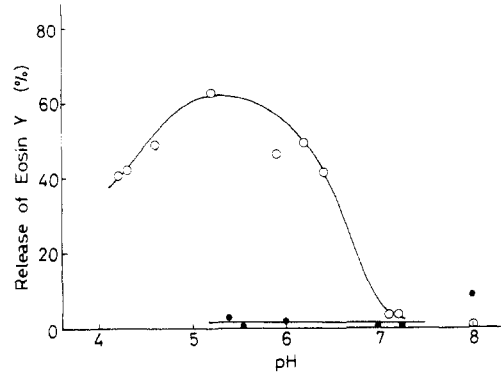

Figure 4. pH-dependent release of Eosin Y from various liposomes in 6 s at 35 °C: (○) L-9 liposome; (●) L-11 liposome.

conditions than the PHC–DPPC and DPPC liposome systems.

As for nonpolymerized liposomes a purturbation in the packing of lipids by the pH change would be smaller than that of polymerized liposomes, because the nonpolymerized lipid molecules are mobile, and the collapse of packing can be weakened by the neighboring lipid molecules. In the case of polymerized lipids, in contrast, the lipids are immobilized and the catastrophe in part of the liposome surface induced by the reduction of pH cannot by quickly buffered by the neighboring block of polymerized lipids. Therefore, the pH-responsive release in the polymerized liposome system became larger than that in the nonpolymerized liposome system.

We further examined the release of Eosin Y from the methacrylic acid containing liposome (Figure 4). The pH sensitivity of the methacrylic acid (MA)–IGGA–MGGA liposome, however, was not so large, probably due to the much smaller change in the space volume of MA residues by the protonation–deprotonation processes than that of MHT residues. These results, however, do not exclude the possibility of using the MA-carrying polymer amphiphiles as a tool to *cover* liposomes composed of natural phospholipids (lecithin, for example) to attain pH sensitivities.[10,11]

C. Cause of pH Sensitivity. In order to check whether the ring opening–closure phenomenon is the real reason for the pH sensitivity of the MHT-containing liposome, we examined the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of various kinds of homocysteine derivatives (when a thiol group exists in a reaction suspension, DTNB is reduced quickly).[12]

Table II shows that, in the presence of thiolactone derivatives, the reduction of DTNB is much slower than in the presence of cysteine or homocysteine. Furthermore, an incubation of homocysteine in acidic solution for 1 h (prior to the mixing with DTNB solution at pH 7.7) did not change the reaction rate. These results showed that the ring opening–closure phenomenon is very slow.

It was also shown that about 75% of thiolactone groups were hydrolyzed to thiol and carboxyl groups by the ultrasonication at pH 8.0 and 60 °C for 30 min. Therefore, only the deprotonation and protonation of thiol and carboxyl groups in the MHT residues occurred quickly upon the change in pH of the liposome suspension (pK_1 and pK_2 of *N*-acetyl-D,L-homocysteine and homocysteine were 3.4, 10.2, 2.2, and 8.9, respectively. Dissociation of polyacids occurs over a much wider pH range than the corresponding low molecular weight acids), which disagrees with the hypothesis proposed by Yatvin et al.[7]

Under acidic conditions these two kinds of ionizable groups in each vinyl unit of the polymer protonate, and

Table II
Rate Constants for Reduction of DTNB at 30 °C[a]

reducing reagent	reaction rate constant, M^{-1} s^{-1}
L-cysteine	1.6×10^3
D,L-homocysteine	1.3×10^3
D,L-homocysteine	1.5×10^3 [b]
D,L-homocysteine thiolactone	0.29
N-acetyl-D,L-homocysteine thiolactone (*N*-Ac-D,L-HCTL)	0.13
N-Ac-D,L-HCTL	6.3×10^2 [c]
N-Ac-D,L-HCTL	6.2×10^2 [d]

[a] pH 7.7, [HEPES] = 5 mM. [b] A homocysteine solution was acidified to pH 1.3, and the solution was incubated at 30 °C. A total of 1 h later the solution was neutralized and mixed with DTNB solution quickly. [c] A *N*-Ac-D,L-HCTL solution was alkalinized to pH 12.7, and the solution was incubated at 30 °C. A total of 1 h later the solution was neutralized and mixed with DTNB solution quickly. [d] A *N*-Ac-D,L-HCTL solution, alkalinized to pH 12.7, incubated at 30 °Cfor 1 h, and neutralized, was further acidified to pH 1.5, and the solution was incubated at 30 °C. A total of 1 h later the solution was neutralized again and mixed with DTNB solution quickly.

the hydrophobicity of the *N*-methylacryloylhomocysteine residues is much higher than under basic conditions. Consequently, the blocks of *N*-methacryloylhomocysteine residues penetrate into the lipid bilayers quickly and deeply upon reduction of pH and as a result perturb the packing of the lipids substantially, thereby inducing the permeation of Eosin Y.

These results show that, though the thiolactone formation is not the real reason for the high pH sensitivity observed here, polymerized liposomes containing the homocysteine moiety are useful for a construction of pH-responsive devices.

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Absolute Free Energies in Biomolecular Systems

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ABSTRACT: We investigate a technique for convenient, accurate, and numerically efficient determination of absolute free energies of biomolecular conformations, regardless of mechanical stability and applicable to systems in vacuo or in solution, under any conditions of temperature and volume. It is anticipated that this free energy approach will aid elucidation of free energy surfaces in phase space and thermodynamic consequences to changes in structure and environment.

Introduction

Polypeptides and proteins can exist in a variety of metastable conformational states, corresponding to minima in some postulated potential energy function.[1,2] Determination of native conformations of these systems and investigation of kinetic processes responsible for conformational transitions between nearby free energy minima are important goals. In addition to these small structural fluctuations, the problem of large structural rearrangement in computer modeling needs to be solved.[3] This involves, ideally, elucidation of the entire free energy surface for the system of interest. Consequently, evaluating absolute free energies for particular configurations is an important theoretical goal allowing, in principle, construction of free energy surfaces in these systems. Additionally, relative values of absolute free energies from different thermodynamic states describe their relative stability.

The search for the optimum configuration has had a long history and still receives considerable experimental

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