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Lipid-Coating on Polyamide Microcapsules Having Long Alkyl Chains and the Characterization of the Lipid-Coated Surface

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Polyamide microcapsules with diameters in the range of 1—5 μm were prepared by interfacial polycondensation between terephthaloyl dichloride and a mixture of L-lysine and piperazine. The carboxyl groups of L-lysine residues involved in the polyamide microcapsules were reacted with alkylamine by using water-soluble carbodiimide as a coupling reagent. The alkylated polyamide microcapsules were incubated with liposome to have the surface of the polyamide microcapsules coated with lipid bilayer membrane. The amount of lipid assemblies adsorbed on the polyamide microcapsules with octyl chains increased up to 1.5 times as much as that on the polyamide microcapsules without octyl chains. The synthetic glycopeptides were introduced to the lipid membrane adsorbed on the surface of the polyamide microcapsules. Interactions of the glycopeptides with the lipid membrane on the polyamide microcapsules and of the glycopeptides in the lipid membrane on the polyamide microcapsules with lectin were investigated.

Liposome has been used as a model membrane in biophysical investigations of cell membrane. Furthermore, liposome can be a biocompatible carrier in drug-delivery systems.[1] However, the vesicular structure of liposome is not very stable and is easily breakable due to osmotic pressure or interactions with plasma proteins in vivo.

Generally, cell membrane is stabilized by skeletal proteins, such as microfilaments, spectrin and actin.[2] Polyamide microcapsules coated with lipid bilayer membrane should mimic the structure of cell membrane lined with proteins. Lipid-coating on capsules having diameters in the range of 1—3 mm has been already reported by Rosenthal and Chang[3] and Okahata et al.[4] It has been also shown that the large polyamide capsule membranes were modified by synthetic glycolipids and the permeation was regulated by lectin addition.[5] They incubated the prepared polyamide microcapsules with a lipid solution in alkane, and transferred them to water. However, this method of lipid-coating is not successfully applied to microcapsules having diameters in the range of 1—5 μm, because the small microcapsules suspended in alkane can not be transferred easily to water due to the low specific gravity. Since microcapsules having diameters in the range of 1—5 μm are a suitable model of the cell and a useful carrier in the drug-delivery system, we tried to synthesize small microcapsules coated with lipid membrane. In combination with biologically active peptides which are associated with lipid bilayer membrane, the lipid-coated microcapsules are expected to act as functional microcapsules.

In the previous report,[6] we have shown that small microcapsules can be coated with lipid bilayer membrane when they were incubated with liposome. However, the surface of poly(terephthaloylpiperazine) microcapsules was not completely coated with lipid bilayer membrane.

In the present investigation, polyamide microcapsules were surface-grafted with long alkyl chains and subjected to lipid-coating. The alkylated polyamide microcapsules are expected to be easily covered with lipid membrane due to the hydrophobic nature of the modified surface.

Experimental

Preparation of Polyamide Microcapsules (Uncoated/Unmodified Polyamide Microcapsules). Polyamide microcapsules were prepared in principally the same method as that reported by Muramatsu and Kondo.[7] An aqueous solution (5 ml) of piperazine (0.13 M; 1 M=1 mol dm^{-3}), L-lysine HCl (0.2 M), and Na_2CO_3 (0.47 M) was added to a $CHCl_3$/cyclohexane (1/3, v/v) solution (100 ml) containing sorbitan trioleate (5 vol%) at 0 °C, and the mixture was stirred for 10 min. To the water-in-oil (W/O) emulsion a $CHCl_3$/cyclohexane (1/3, v/v) solution (100 ml) of terephthaloyl dichloride (12.5 mM) containing sorbitan trioleate (5 vol%) was added, and the mixture was allowed to react for 30 min. The microcapsules prepared were washed succeedingly with cyclohexane (three times), an aqueous solution containing poly(oxyethylene) sorbitan monolaurate (5 vol%), and water. The molecular structure of polyamide is shown in Fig. 1.

Reaction of Polyamide Microcapsules with Alkylamine. An aqueous solution of octylamine or decylamine (0.5 M) was adjusted to pH 4.75 with HCl, and added to a dispersion of the uncoated/unmodified polyamide microcapsules. Condensation reaction of carboxyl groups of microcapsules with alkylamine was carried out by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide as a coupling reagent. After standing at room temperature for 12 h, the dispersion was washed several times with water until washing solution became neutral. Although free alkylamines might be adsorbed on polyamide microcapsules, they should be desorbed at the following treatment with the excess amount

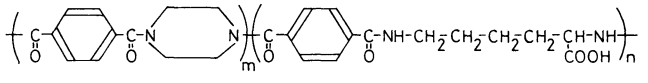

Fig. 1. Chemical structure of uncoated/unmodified polyamide microcapsules.

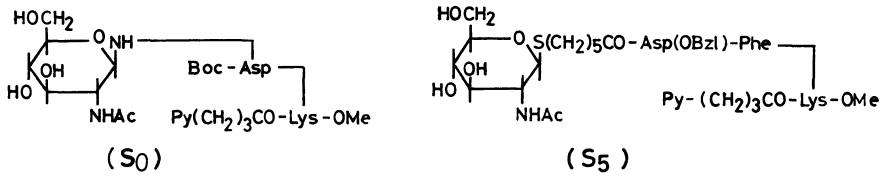

Fig. 2. Structure of synthetic glycopeptides.

of liposome.

Lipid-Coating on Microcapsules. Small unilamellar vesicles (SUV) composed of dimyristoylphosphatidylcholine (DMPC) were prepared by the sonication method. The prepared uncoated/unmodified polyamide microcapsules were incubated with DMPC SUV at 24 °C for 24 h. The dispersion was washed seven times with buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES); 10 mM, NaCl; 0.1 M, pH 7.4) to remove SUV not in use for coating. Other types of microcapsules were also prepared according to the similar procedures.

Synthetic Glycopeptides. The molecular structure of synthesized glycopeptides is shown in Fig. 2. These glycopeptides carry a 1-pyrenyl group in the hydrophobic peptide moiety. The synthetic procedure has been reported previously.[8]

Wheat germ agglutinin (WGA) and lipids were purchased from Sigma. The available agents of the highest grade were used.

Measurements. ζ-Potential of the microcapsules was measured in an aqueous solution buffered with Na_2CO_3/$NaHCO_3$ (pH 10.2, ionic strength 1.8×10^{-4}) using Lazer Zee Meter Model 501 (Ten Kem Inc., New York). The concentration of lipid was determined by colorimetric method using phospholipase D (Diacolor PL, Toyobo, Japan).

The effect of lectin addition on the distribution of glycopeptides in membrane was examined by measuring monomer and excimer emissions of 1-pyrenyl group introduced to the peptide moiety.

Aggregation of polyamide microcapsules upon the addition of lectin was detected by measuring the absorbance at 800 nm.

Fluorescence was measured on an MPF-4 fluorescence spectrophotometer (Hitachi, Japan). 1-Pyrenyl group in the glycopeptides was excited at 345 nm, and monomer and excimer emissions were monitored at 378 and 480 nm, respectively. Absorption spectra were measured on an UVIDEC-1 spectrophotometer (JASCO, Japan).

Results and Discussion

Characterization of the Polyamide Microcapsules. Polyamide microcapsules were prepared by the interfacial polycondensation of terephthaloyl dichloride with L-lysine and piperazine. The size distribution of polyamide microcapsules was examined with a photomicroscope (Fig. 3). The most popular diameter was found to be in the range of 1—2 μm.

The presence of L-lysine as the component of the polyamide microcapsules was confirmed by the existence of free carboxyl groups of L-lysine in microcapsules. That is, ζ-potential of the polyamide microcapsules prepared in the presence of L-lysine was evidently lower than that of poly(terephthaloylpiperazine) microcapsules (Table 1).

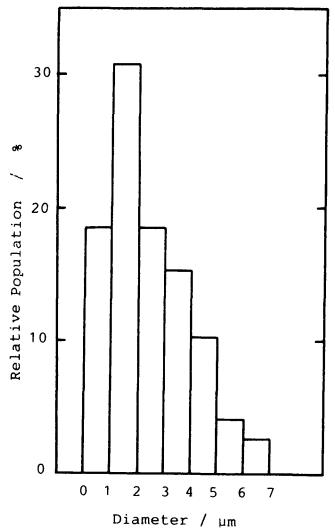

Fig. 3. Size distribution of uncoated/unmodified polyamide microcapsules determined with optical microscope.

Table 1. Zeta-Potential of Polyamide Microcapsules Containing Different Diamine Components

Diamine	Zeta-potential/mV
Piperazine	−20.9±1.6
L-Lysine/piperazine (0.20/0.13 mol/mol)	−42.1±2.0

Potentiometric titration of the polyamide microcapsules revealed that the diamine component of polyamide is composed of 70% of piperazine and 30% of L-lysine units. These values were calculated on the basis of the determination of negative charges on the surface of polyamide microcapsules, in which the contribution from the terminal carboxyl groups was ignored, because it was reported that the interfacial polycondensation of terephthaloyl dichloride and diamines gave polyamides with average molecular weight of about 20000.[9]

The polyamide microcapsules were reacted with

alkylamine to make the surface more hydrophobic. The hydrophobic surface should be favorable for lipid-coating. It was estimated that more than 80% of carboxyl groups of L-lysine units reacted with alkylamine, which was deduced from the titration of the reaction product of poly(terephthaloyllysine) with alkylamine under the same conditions.

Lipid-Coating. The polyamide microcapsules were incubated with DMPC liposome, and washed several times with buffer. It has been reported that lipid molecules not in use for coating are completely removed after seven times of washing, and that the lipid molecules adsorbed on the polyamide microcapsules take a bilayer structure, which was confirmed by the observation of phase transition of membrane by differential scanning calorimetry and measurement of membrane fluidity.[6] The amount of lipid molecules adsorbed on the polyamide microcapsules was determined and is summarized in Table 2. It is clearly shown that the adsorption of lipid molecules on microcapsules is enhanced by the modification of the polyamide microcapsules with alkylamine. The amount of lipid molecules adsorbed on the polyamide microcapsules grafted with octyl chains is 1.5-fold of that on unmodified polyamide microcapsules. Assuming 1.37 g cm^{-3} for the density of polyamide membrane,[10] 13.3 nm for the thickness of polyamide membrane,[10] and 70 $Å^2$ for the area occupied by a DMPC molecule,[11] this amount corresponds to 2.4 bilayers of lipid assemblies. On the other hand, 1.6 bilayers of lipid assemblies were formed on the surface of poly(terephthaloylpiperazine) microcapsules.[6]

Interaction of Glycopeptides with the Polypeptide Microcapsules. The synthetic glycopeptides consist of a hydrophilic glucosamine moiety and a hydrophobic peptide chain. These glycopeptides were shown to be distributed to lipid membrane with the peptide chain buried in the hydrophobic region of lipid membrane.[8] Since the pyrenyl group is connected to the peptide moiety, the interaction of glycopeptides with lipid membrane can be monitored by the fluorescence behavior of the pyrenyl group.

For example, the extent of the distribution of S_0 to lipid membrane can be determined by measuring the quenching rate of pyrenyl fluorescence with acrylamide, because the pyrenyl group is shielded from the aqueous phase upon incorporation into the lipid membrane. S_0 was incubated with various kinds of polyamide microcapsules and the rate of quenching by acrylamide was measured (Table 3). The fluorescence intensity from S_0 was reduced to different extents with the addition of acrylamide in all cases. The fraction of unquenchable S_0 increased in the order of uncoated/unmodified polyamide microcapsules<DMPC-coated/unmodified polyamide microcapsules<DMPC-coated/decyl-grafted polyamide microcapsules<DMPC-coated/octyl-grafted polyamide microcapsules. This order coincides with that of the amount of lipid molecules adsorbed on microcapsules. Therefore, it can be concluded that most of S_0 molecules adsorbed were incorporated into the lipid bilayer membrane formed on the surface of polyamide microcapsules.

The ratio of excimer emission and monomer emission intensities (I_D/I_M) represents the extent of the distribution of glycopeptides in the lipid membrane. Figure 4 shows the dependence of I_D/I_M of S_0 on the temperature in the presence of various kinds of

Table 2. The Amount of Lipid Molecules Adsorbed on Polyamide Microcapsules

Polyamide microcapsule	DMPC adsorbed onto 1 mg microcapsule
Unmodified	280 μg
Octyl-grafted	415 μg
Decyl-grafted	382 μg

Table 3. Quenching of Fluorescence of S_0 Distributed to the Surface of Various Kinds of Polyamide Microcapsules

Polyamide microcapsule	Rate of quenching (%)
Uncoated/unmodified	52.5
DMPC-coated/unmodified	29.9
DMPC-coated/octyl-grafted	22.6
DMPC-coated/decyl-grafted	26.5

[microcapsules]=0.64 mg ml^{-1}. [S_0]=2.2 μM. [acrylamide]=50 mM.

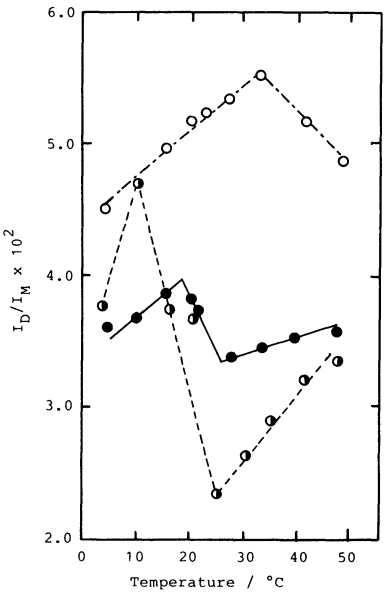

Fig. 4. The temperature dependence of I_D/I_M of S_0 in the presence of uncoated/unmodified polyamide microcapsules (○), DMPC-coated/octyl-grafted polyamide microcapsules (●), and DMPC SUV ([DMPC]= 0.44 mg ml^{-1}) (○). [S_0]=4.3 μM, [microcapsules]= 1.1 mg ml^{-1}.

polyamide microcapsules or DMPC SUV. I_D/I_M of S_0 in the presence of DMPC SUV decreased linearly with descending temperature with a break in the region of the phase-transition temperature of the DMPC bilayer membrane. The monotonous decrease of I_D/I_M should be ascribed to the different temperature dependence of the quantum yields of the excimer and the monomer emission. On the other hand, the abrupt increase of I_D/I_M occurring in the region between 24 and 10 °C can be ascribed to the increasing local concentration of S_0 in the DMPC bilayer membrane due to the phase separation below the phase transition temperature. It is well-known that the phase separation of lipid membrane produces domains of pure lipid molecules in a gel state as well as domains containing high concentrations of solute.[8]

The temperature dependence of I_D/I_M of S_0 in the presence of uncoated/unmodified polyamide microcapsules showed the break at 33 °C. The reason for this phenomenon is two fold. Increasing adsorption of the S_0 to the uncoated/unmodified polyamide microcapsules with descending temperature leads to increasing I_D/I_M in the region of higher temperatures, whilst I_D/I_M by nature decreases with descending temperature as it does in the region of lower temperatures.

In the presence of DMPC-coated/octyl-grafted polyamide microcapsules, the abrupt increase of I_D/I_M of S_0 with descending temperature was observed in the region of the phase transition temperature, indicating that S_0 should be distributed to lipid assemblies, which take the structure of lipid bilayer membrane on the polyamide microcapsules. Although the change observed in the presence of DMPC-coated/octyl-grafted polyamide microcapsules was less marked than that in the presence of DMPC SUV, the former change was more significant than that observed in the presence of DMPC-coated poly(terephthaloylpiperazine) microcapsules.[6] Therefore, the surface of polyamide microcapsules uncoated with lipid membrane can be reduced considerably by grafting octyl groups into microcapsules, resulted in the increase of the amount of lipid assemblies coated on microcapsules.

Interactions of Lectin with Glycopeptides on the Polyamide Microcapsules. The glycopeptide S_5 was used for the investigation on the interaction of lectin and the glycopeptide on the polyamide microcapsules. It has been reported that S_5 in lipid bilayer membrane interacts with lectin without being pulled out of the membrane.[8] WGA is known to possess four binding sites with a high affinity to *N*-acetylglucosamine and to crosslink two glycoproteins embedded in membrane.[12] Interaction of WGA with S_5 on the polyamide microcapsules was investigated by measuring the effect of the lectin addition on I_D/I_M ratio (Fig. 5). I_D/I_M of S_5 increased with the addition of WGA. The relative increase of I_D/I_M was the largest in the presence of DMPC-coated/octyl-grafted polyamide microcapsules, and the second largest in the presence of DMPC liposome. The increase of I_D/I_M with the addition of WGA was not remarkable in the presence of uncoated/unmodified polyamide microcapsules. The interaction of WGA with S_5 on the surface of uncoated/unmodified polyamide microcapsules should have been suppressed by negative charges on the surface of the microcapsules, because the crosslinking of S_5 with the addition of WGA occurred to a higher extent in the presence of uncoated poly(terephthaloylpiperazine) microcapsules.

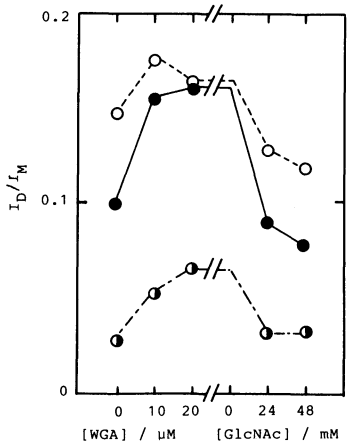

Fig. 5. The change of I_D/I_M of S_5 with the addition of WGA and the dissociation of crosslinked S_5 with the addition of GlcNAc in the presence of uncoated/unmodified polyamide microcapsules (○), DMPC-coated/octyl-grafted polyamide microcapsules (●), and DMPC SUV ([DMPC]=0.44 mg ml^{-1}) (◑). [S_5]=4.5 μM, [microcapsules]=1.1 mg ml^{-1}.

The crosslinking of S_5 molecules by WGA is facilitated on the surface of DMPC-coated/octyl-grafted polyamide microcapsules. The larger change of I_D/I_M in the presence of DMPC-coated/octyl-grafted polyamide microcapsules than that in the presence of DMPC SUV is ascribed to the high local concentration of S_5 on the surface of the polyamide microcapsules. That is, although 2.4 bilayers of lipid membrane are accumulated on DMPC-coated/octyl-grafted polyamide microcapsules, S_5 molecules can not be distributed to the inner bilayers due to the inability of S_5 to permeate through the hydrophobic core of lipid bilayer membrane. Therefore, the concentration of S_5 molecules dispersed in the lipid assembly is higher on the surface of the polyamide microcapsules than that in DMPC SUV for the same total amount of lipid molecules. This explanation is consistent with the higher value of I_D/I_M of S_5 in the presence of DMPC-coated/octyl-grafted polyamide microcapsules than that in the presence of DMPC SUV without the addition of WGA.

The crosslinking of S_5 by WGA was shown to be reversible on the surface of DMPC-coated/octyl-

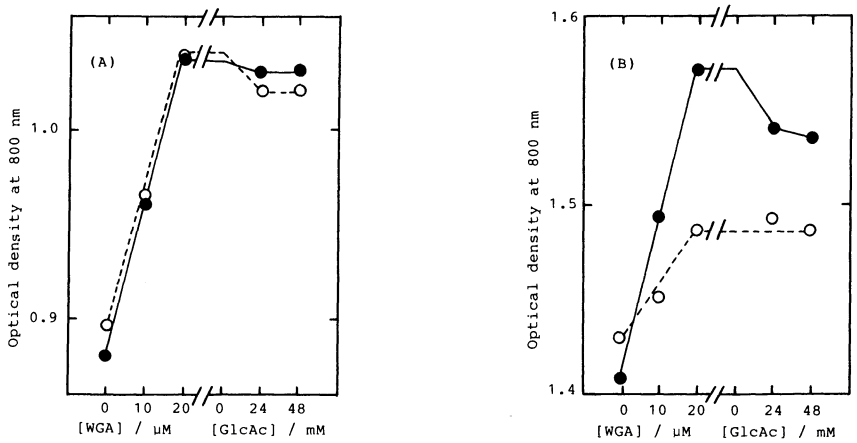

Fig. 6. Aggregation of microcapsules with the addition of WGA and dissociation of the aggregates with the addition of GlCNAc. [microcapsules]=0.64 mg ml^{-1}. [S_5]=0 M (○) and [S_5]=4.5 μM (●). In the presence of (A); uncoated/unmodified microcapsules, and (B), DMPC coated/octyl-grafted polyamide microcapsules.

grafted polyamide microcapsules, since the addition of inhibitor, *N*-acetylglucosamine, restored the original value of I_D/I_M in the absence of WGA.

As shown in Fig. 6A, aggregation of microcapsules was also observed with the addition of WGA, which might have been brought about by crosslinking of microcapsules by WGA. The absorbance of the suspension of uncoated/unmodified polyamide microcapsules increased with the addition of WGA either in the presence or in the absence of S_5. The aggregation of polyamide microcapsules was not dissociated by the addition of *N*-acetylglucose. Therefore, the aggregation should be nonspecific one between WGA and the surfaces of polyamide microcapsules. This type of aggregation was suppressed by coating the microcapsules with lipid membrane, as shown in Fig. 6B. The aggregation of DMPC-coated/octyl-grafted polyamide microcapsules by WGA should occur in the uncoated part of the polyamide microcapsules. The aggregation was enhanced in the presence of S_5 and suppressed partly by the addition of *N*-acetylglucose. Therefore, a part of the aggregation was caused by the specific interaction of WGA with S_5 in lipid membrane on the polyamide microcapsules.

To conclude, it was shown that the surface of polyamide microcapsules having diameters of 1—5 μm can be coated with lipid assemblies, and the efficiency of the lipid coating increased by the introduction of alkyl chains to the surface. The lipid assemblies adsorbed on polyamide microcapsules take the structure of lipid bilayer membrane, and mimics the cellular surface, in which glycopeptides interact with lectin. Therefore, lipid-coated microcapsules provide lipid bilayer membrane stabilized by polyamide microcapsules, and can be utilized as functional microcapsules by modification with biologically active peptides. However, polyamide surface can not be completely covered with lipid membrane. This imperfectness causes some problems in analyzing interactions of glycopeptides in the lipid membrane on the polyamide microcapsules. In addition, the encapsulation of small molecules into the obtained lipid-coated microcapsules will not be possible, which becomes a problem for utilization of microcapsules to the drug-delivery system. Three possibilities are considered for perfect covering the surface with lipid membrane. The first is to introduce more L-lysine residues as a basic component and increase the amount of alkyl chains grafted to the polyamide microcapsules. However, increasing content of L-lysine residues decreases the mechanical strength of the polyamide microcapsules. The second is to use longer alkyl chains to modify the surface. However, the alkylation reaction is very difficult because of the low solubility of long chain alkylamine in water. The third is to use amphiphilic polypeptides to increase the affinity of the polyamide microcapsules to lipid membrane. The last possibility is under investigation.

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