Transactions of the Materials Research Society of Japan 28 [3] 545-548 (2003)

Self-assembling properties of mannosyl-erythritol lipids, yeast glycolipid biosurfactants

Dai Kitamoto* , Tomohiro Imura and Hiroshi Yanagishita
National Institute of Advanced Science and Technology, Central 5-2, Higashi 1-1, Tsukuba 305-8565, Japan.
Fax: 81-29-861-4660, e-mail: dai-kitamoto@aist.go.jp
Yu-ichiro Takeyama, Yasushi Idemoto and Koura Nobuyuki
Department of Industrial Engineering Chemistry, Tokyo University of Science, Yamazaki 2641, Noda 278-8510, Japan.

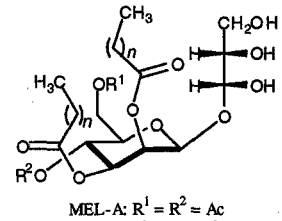
Mannosyl-erythritol lipids (MEL), which are abundantly produced by a yeast strain, are one of the most promising glycolipid biosurfactants. MEL exhibit not only excellent surface-active properties, but also versatile biological activities against mammalian tumor cells. We thus focused on applications of MEL to functional materials, and undertook the physico-chemical characterization of their self-assemblies such as monolayers and bilayers (vesicles). On AFM observation of LB monolayers prepared from MEL and phospholipids, the monolayers showed phase-separated structures, that is, glycolipid-rich microdomains. Interestingly, MEL efficiently self-assembled in water to form giant vesicles. The addition of MEL into phospholipid vesicles brought various effects on their properties: MEL weaken the intermolecular interaction between the lipids. On the other hand, MEL showed a potential binding affinity towards human immunoglobulin G (HIgG) on ELISA assay. Poly(2-hydroxyethyl methacylate) beads bearing MEL on the surface exhibited high binding affinity and capacity toward HIgG. The binding amount of HIgG towards the composite increased with increased applied concentration, reaching over 100 mg HIgG (per g of composite). The yeast biosurfactants may thus be functional devices such as affinity ligands or sensing units in nanobiotechnology, due to their excellent self-assembling properties.
Key words: self-assembling, glycolipid, biosurfactants, vesicle, glycoprotein binding.

1. INTRODUCTION

Biosurfactants have many unique properties (i.e., mild production conditions, biodegradability, wide range of biological activities) in comparison with their chemical counterparts. Their numerous advantages have prompted their application not only in the food, cosmetics and pharmaceutical industries, but also in environmental and energy-saving technology [1]. Mannosyl-erythritol lipids (MEL, Fig. 1), yeast glycolipids, are one of the most promising biosurfactants known and are abundantly produced from vegetable oils or n-alkanes by *Pseudozyma antarctica* T-34 up to 140 g/l [2]. MEL exhibits not only excellent surface-active properties [3], but also cell-differentiation inducing activities towards the human leukemia cells, rat pheochromocytoma cells and mouse melanoma cells [1]. In addition, we recently demonstrated that MEL efficiently self-assemble in water to form giant vesicles [4].

On the other hand, glycolipids have received much attention as leading materials for drug- and gene-carrying microcapsules and artificial cells, due to their specific effect on liposomes. In biomembranes, glycolipids such as glycosphingolipids also participate in vital functions, including signal transduction, cell recognition and cellular proliferation through protein-carbohydrate interactions [5]. Some of these glycolipids exhibit high affinity for immunoglobulins as a result of the "multivalent or cluster effect" [6]. The possibility of developing these membrane glycolipids into new functional materials, however, is far from straightforward due to their limited amounts and heterogeneity [7].

We thus focused on the feasible use of MEL for creating multifunctional materials, and undertook the physico-chemical characterization of monolayers and bilyaers (vesicles) prepared from MEL. In this paper, we describe for the first time various properties of vesicles comprising MEL and phosphatidylcholines. We also addressed the application of the yeast glycolipid assemblies to the affinity ligand for human immunoglobulin G (HIgG), which is the dominant immunoglobulin isotype in mammalian blood.



MEL-A: $R^1 = R^2 = Ac$
MEL-B: $R^1 = Ac$, $R^2 = H$
MEL-C: $R^1 = H$, $R^2 = Ac$
($n = 6 - 10$)

Fig. 1

2. MATERIALS AND METHODS

Materials : L-α-Dipalmitoylphosphatidylcholine (DPPC), L-α-egg york phosphatidylcholine (PC), HIgG (minimum purity 95%) and globulin-free human serum albumin (HSA) were purchased from Sigma Chemical Co. (St. Louis, USA). Other reagents were of biochemical grade and were commercially available.

Preparation of MEL: The mixture of MEL was produced from soybean oil with the yeast strain of *Candida antarctica* T-34. MEL-A was always the major component of the yeast product, and was purified from

the mixture by silica-gel column chromatography as previously reported [2]. MEL-A (mean Mw: 676), has decanoic acids as their major fatty acids, and is sparingly soluble in water [3].

Preparation of vesicles and their characterization: MEL-A and DPPC were dissolved in chloroform and dried to a lipid film by nitrogen flushing and rotary evaporation. The dried lipid film was swollen in 10 mM Tris-HCl buffer (pH 7.2) and vortexed to form multilammellar vesicles. The trap volume of the multilammellar vesicle was determined by the method of Oku *et al* [8] using aqueous 3,3'-Bis[*N*,*N*-di(carbonxymethyl)-aminomethyl] fluorescein (calcein) solution. The multilammellar vesicle suspension was subjected to 5 freeze-thaw cycles, and then extruded 10 times with polycarbonate filters of 200 nm pore size. The extruded vesicles were then used to determine the membrane permeability by the method Hara *et al.* using the calsein solution [9]. The phase transition of the multilammellar vesicles was estimated by differential scanning calorimetry (DSC). The vesicle suspension was loaded into a calorimeter (Seiko Instruments, DSC 6100), and a thermogram was obtained at a scan rate of 0.5°C per min.

Atomic force microscopy (AFM): The monolayers comprising MEL-A and DPPC were deposited onto mica (1 cm square) by Langmuir-Blodgett method. The Langmuir-Blodgett monolayers were imaged using tapping mode by AFM (Seiko Instruments, SPA400/SPI3800A) equipped with a silicon nitride probe.

Preparation of MEL-PHEMA composite: poly(2-hydroxyethyl methacrylate) (PHEMA) beads (diameter: 50 to 150 μm) were employed as a supporting material for MEL. MEL-A was mixed with PHEMA in methanol, and then the solvent was slowly evaporated under a nitrogen stream. The obtained residue was washed with water and filtrated to give MEL-PHEMA composite. The weight of MEL-PHEMA is expressed in terms of dry weight.

Protein binding to MEL-PHEMA composite: The binding studies between HIgG and the composites were conducted according to the general methods reported by Teng *et al* [10]. HIgG or HSA (1.0 mg) was added to the mixture of MEL-PHEMA composite (0.30 ± 0.05 g) bearing different amounts of MEL-A and 3 ml of 50 mM phosphate buffer (pH 7.0) in a polypropylene tube (15-mL). The tube was incubated for 1 hr, and then centrifuged. The binding amount of protein to the composite was estimated by measuring the UV absorbance at 280 nm of the supernatant.

Binding capacity of MEL-PHEMA composite for HIgG: Preliminarily experiments showed that HIgG binding to the composite reaches a maximum in 50 mM phosphate buffer of pH 4.6 including 1M of Na_2SO_4. This experiment was thus carried out using the Na_2SO_4 phosphate buffer. The composite (0.33 ± 0.08 g) bearing 7.1 μmol MEL-A (per g of composite) was suspended in 3 ml of the phosphate buffer in the tube. Different amounts of HIgG (1 to 36.0 mg) were added to the tube, and the tube was treated as above. The binding yield of HIgG to the composite was expressed in terms of the weight percentage of the bound protein to the applied protein.

Determination of the binding constant between HIgG and MEL-PHEMA composite: The binding constant was determined from the equation for the Langmuir adsorption isotherm, $q = (Q_{max} K_a C) / (1+K_a C)$, where q is the binding amount of HIgG, C is the unbound concentration of HIgG, K_a is the binding constant and Q_{max} is the binding capacity.

3. RESULTS

In our previous study, all the MEL were found to efficiently self-assemble in water to form giant vesicles over 10 μm of diameter [4]. MEL-A was the major component of the yeast product. Phosphatidylcholine is one of the representative vesicle matrixes to be used for drug- and gene-delivery systems. The vesicles comprising MEL-A and different phosphatidylcholines were thus prepared and subjected to various physic-chemical characterizations.

Trap volume of MEL-phospholipid vesicle

The trap volume of the vesicle prepared from the mixture of MEL-A and DPPC increased with increases in the molar concentration of the glycolipid. With 40 mol% of MEL, the vesicle showed an approximately 4-fold higher trap volume (2.4 liter water/ mol lipid, 25°C) compared to its control (100 mol% of DPPC). The further increase in the MEL concentration, however, resulted in the decrease of the trap volume.

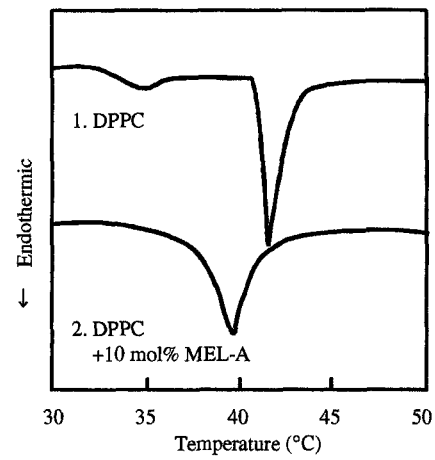


Fig. 2 DSC curves of DPPC multilamellar vesicles in the absence (1) and presence (2) of 10 mol% MEL-A.

Membrane permeability of MEL-phospholipid vesicle

Some glycolipids give a stabilizing effect on phospholipid-based vesicles, owning to their exclusive volume effect of the extending and rotating oligosaccharide chains, and repress the vesicle permeability [11]. The effect of MEL-A on the permeability of the vesicles prepared from PC was thus examined. The addition of small amounts of MEL-A enhanced the permeability of calcein across the membrane. No repressing effect of MEL was observed for the vesicle permeability under the conditions employed. With 5 mol% of MEL, the permeability became 1.5-fold higher compared to its control (100 mol% of PC).

Phase transition of MEL-phospholipid vesicle

The vesicle prepared from only MEL-A did not show any phase transition between 0 and 90°C on the DSC study. This lipid seems to be in a liquid-crystalline state over a wide temperature range. The effect of MEL-A on the gel to liquid-crystalline phase transition of the vesicle prepared from DPPC was then examined (Fig. 2). In the absence of MEL-A, a sharp onset of the main transition appeared at 41.9°C with a pre-transition (35°C) for DPPC pure vesicles. The addition of 10 mol% of MEL-A abolished the pre-transition and somewhat broadened the main transition, and lowered the temperature.

Topology of MEL-phospholipid monolayers

DPPC and DPPC-MEL-A (90 : 10) monolayers were transferred to mica at a surface pressure of 25 mN/m, respectively, and were imaged by AFM (Fig. 4). DPPC monolayers in the absence of MEL-A gave flat uniform monolayers (Fig. 3a). However, DPPC containing 10 mol% of MEL-A showed network-like glycolipid-rich microdomains that are 0.7 to 0.8 nm lower than surrounding matrix (Fig. 3b).

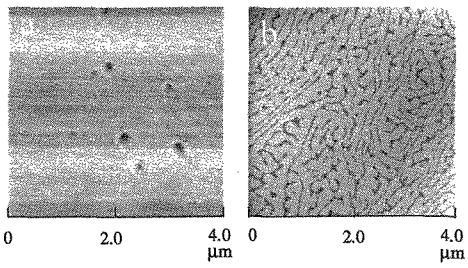


Fig. 3. AFM images of DPPC (a) and DPPC-MEL (b) monolayers.

Protein binding to MEL

Enzyme-linked immunosorbent assay (ELISA) indicated that MEL-A exhibit nearly the same binding affinity towards HIgG as that of bovine ganglioside GM1, which is known to directly bind to the glycoprotein [7]. In order to characterize the binding between MEL and HIgG, the polymer composites were thus prepared from MEL-A and PHEMA beads.

Binding selectivity of MEL-PHEMA for HIgG and HSA

HSA is the most dominant protein in serum that is the essential source of HIgG, and its binding to the composite was thus examined (Fig. 4). The composite bearing no glycolipid showed no selective binding for HIgG and HAS. However, the binding amount of HIgG to the composite increased depending on the attached amount of MEL-A, whereas the amount of HSA slightly decreased. The binding amount of HIgG was 2.7-fold higher than that of HSA with the composite bearing 4.4 μmol MEL (per g of composite). During the binding experiments, no leakage of MEL from the composite was observed.

Binding capacity of MEL-PHEMA composite for HIgG

The binding capacity of the composite for HIgG was then evaluated over a wide rage of HIgG concentrations

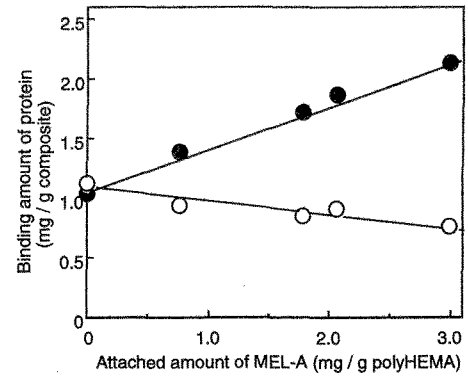


Fig. 4. Binding of HIgG and HSA to the MEL-PHEMA composite. HIgG, closed circle; HAS, open circle.

(Fig. 5). The amount of HIgG bound to the composite increased in proportion with the applied concentration of the protein. Interestingly, the binding fraction drastically increased with applied concentrations above 4 mg/ml. The binding amount of HIgG was 105.8 mg (per g of composite) at an applied concentration of 12 mg/ml, which corresponds to the typical concentration of HIgG in serum. The binding yield of HIgG was below 50% at an applied concentration of 4 mg/ml, and then, with increased applied concentration, rose sharply to over 80%. The highest binding yield (82%) was attained at an applied concentration of 9 mg/ml.

At applied concentrations of below 4 mg/ml, HIgG binding to the composite was observed to follow a Langmuir type. However, at higher concentrations, it increased linearly and showed no binding plateau under the conditions employed. This HIgG binding was assumed to follow a Freundlich type [12], which does not exhibit a saturation or limiting binding value.

On the other hand, HIgG bound to the composite was efficiently recovered by using 50 mM phosphate buffer (pH 7.0) including no Na_2SO_4. With the composite bearing 77.8 mg HIgG (per g of composite), the neutral buffer gave the highest recovery yield of 87%. After the elution of HIgG, the composite was again subjected to the binding experiment. Interestingly, the composite showed nearly the same binding yield towards HIgG compared with the first experiments, indicating that no MEL-A is eluted from the composite thorough the binding with HIgG.

Binding constant between HIgG and MEL-PHEMA composite

The binding constant between HIgG and the composite was estimated using the observed Langmuir isotherm. From the equation for the Langmuir isotherm, the binding constant was calculated to be $1.53 \times 10^6\ M^{-1}$.

4. DISCUSSION

The addition of MEL-A to phosphatidylcholine vesicles brought various effects on their physico-chemical properties. From the DSC experiment on DPPC-MEL vesicles, MEL-A is likely to destabilize the gel phase of DPPC and weaken the molecular interaction between the phospholipid molecules. It thus seems reasonable that the addition of MEL-A to phospholipis

vesicles resulted in the increase of their membrane permeability.

DPPC-MEL monolayers exhibited network-like glycolipid-rich microdomains as reported on DPPC-ganglioside GM1 monolayers [14]. To our knowledge, this is the first observation on the microdomains prepared from yeast glycolipids. This means that MEL-A molecules are not homogeneously distributed into the phospholipid monolayers. The glycolipids are thus likely to efficiently self-assemble and make microdomains even in the mixed vesicle system with phosphatidylcholines.

The binding amount of HIgG to the composite increased with increases in the attached amount of MEL-A, and the potential selectivity was obtained for HIgG and HSA. The increase of MEL on the composite probably enhance the density of a self-ordered conformation needed for the interaction with HIgG; this finally might impede the binding of HSA. Therefore, the binding selectivity of the composite would be improved with a greater attached amount of MEL or with a better-ordered orientation of MEL.

The composite bearing MEL-A showed a high binding capacity of 105 mg HIgG (per g of composite bearing 7.1 mmol of MEL-A) with a binding yield of 81%. Teng *et al.* recently reported that a synthetic triazine ligand on agarose beads exhibits an apparent binding capacity of 51.9 mg HIgG (per g of gel bearing 90 mmol of the ligand) [10]. Although the binding conditions were different from those in the present study, the capacity of the MEL-A composite appears to be superior to that of the synthetic ligand. In addition, the composite exhibits a high binding affinity of 1.5×10^6 (M^{-1}) for HIgG, which is approximately 4-fold greater than that reported for immobilized protein A [10].

Another interesting feature on HIgG binding behavior to the present composite is that the binding mode varies according to the applied concentration. At high concentrations, the binding mode was found to switch from the Langmuir type to the Freundlich type. These two combined binding modes were recently reported by Jenney and Anderson in connection with the binding of IgG to a hydrophilic polystyrene surface: it follows a Langmuir isotherm at low protein concentrations but a Freundlich isotherm at high concentrations [12]. The observed linear increase in the binding amount of HIgG may thus be due to multilayer or aggregate formation, which are considered to be characteristics of the Freundlich binding mode [13].

Some of the bindings between gangliosides and glycoproteins like HIgG are enhanced by a "multivalent or cluster effect"; a simultaneous association of two or more ligands and receptors. More importantly, the effect is considerably dependent on the density, orientation and conformation of the saccharide moieties of gangliosides [6]. As mentioned above, MEL-A efficiently self-organize to form molecular assembles in aqueous systems. This instantly means that the glycolipid has a superior property on the molecular orientation and packing. Therefore, MEL-A is likely to position densely on the polymer surface so as to generate a "multivalent domain" leading to the interaction with HIgG. This may compensate for the

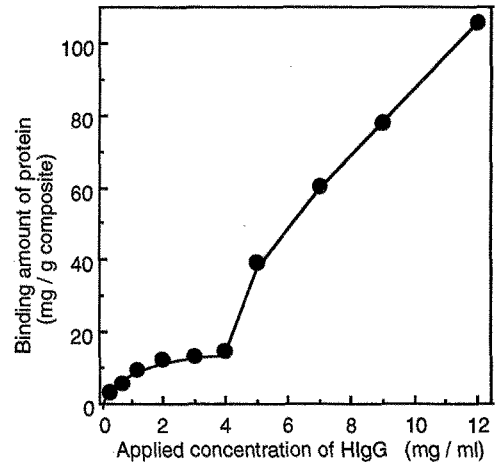


Fig. 5. Binding capacity of the MEL-PHEMA composite for HIgG.

small saccharide moiety and provide MEL-A with a similar binding affinity to that of gangliosides. Based on the observed binding capacity (Langmuir binding region), the binding molar ratio between HIgG and MEL-A is approximately 1 : 80, supporting the binding is attributed to the "multivalent effect."

These results clearly indicated that the yeast glycolipids may thus open new avenues for the development of advanced functional devices and units using their excellent self-organizing properties.

5. ACKNOWLEDGEMENTS

We are grateful to Dr. A. Endo of our institute for the experiments of AFM.

6. REFERENCES

[1] D. Kitamoto, H. Isoda, T. Nakahara, *J. Biosci.*
[2] D. Kitamoto, T. Ikegami, T. Suzuki, A. Sasaki, Y. Takeyama, Y. Idemoto, Y. Koura, H. Yanagishita, *Biotechnol. Lett.*, 23 (20), 1709-1714 (2001).
[3] D. Kitamoto, H. Yanagishita, T. Shinbo, T. Nakane, T Nakahara, *J. Biotechnol*, **29**, 91-96 (1993).
[4] D. Kitamoto, G. Sangita, G. Ourisson, Y. Nakatani, *Chem. Commun.*, (11), 861-862 (2000).
[5] K. Iwabuchi, S. Yamanura, A. Prinetti, K. Hand, SI. Hakomori, *J. Biol. Chem.*, **273**, 9130-9138 (1998).
[6] S. V. Evans, C. R. MacKenzie, *J. Mol. Recognit.*, **12**, 155-168 (1999).
[7] T. Feizi, *Immunol. Rev.*, **173**, 79-88 (2000).
[8] N. Oku, D. A. Kendall, R. C. MacDonald, *Biochim. Biophys. Acta.*, **691**, 332-340 (1982).
[9] M. Hara, H. Yuan, Q. Yang, T. Hoshino, A. Yokoyama, J. Miyake, *Biochim. Biophys. Acta.*, **1461**, 147-154 (1999).
[10] S. F. Teng, K. Sproule, C. R. Lowe , *J. Chromatogr.* B , **740**, 1-15 (2000).
[11] S. Takeoka, K. Sou, T. Ohgushi, E. Tsuchida, *Supramolec. Sci.*, **5**, 159-162 (1998). 1
[12] C. R. Jenney, J. M. Anderson, *J. Biomed. Mater. Res.*, **50**, 281-290 (2000).
[13] I. Lundström, *Prog. Colloid. Polym. Sci.*, **70**, 76-82 (1985).
[14] Y. Chunbo, L. J. Johnson, *Biochemical J.*, **79**, 2768-2781 (2000).

(Received December 21, 2002; Accepted April 28, 2003)