Immobilization of Giant Vesicles onto Hydrophobized Polymer Gel Particles

Yukihisa Okumura,* Satoshi Yabushita, and Aya Watanabe

Department of Chemistry and Material Engineering, Faculty of Engineering, Shinshu University, 4-17-1 Wakasato, Nagano 380-8553

Received March 17, 2011
E-mail: okumura@shinshu-u.ac.jp

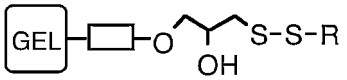
Giant lipid membrane vesicles (GVs) were immobilized onto the surface of Sephacryl S-1000 gel particles chemically modified to possess hydrophobic moieties. The immobilization was firm but not efficient. Strong preference of the immobilization for small lipid aggregates enabled their removal from GV preparation by repeated incubation with the gel.

Lipid membrane vesicles larger than 1 μm are known as giant vesicles (GVs).[1,2] The fundamental structure of a GV is the same as a biological cell, and GVs have been considered to be a structural framework of an artificial cell.[3] Various works recently reported herald the era of GV modification.[2]

Previously, liposomes smaller than 1 μm ("small liposomes") were successfully immobilized onto crosslinked polymer gel particles that are chemically modified to possess hydrophobic alkyl moieties.[4–8] Based on the immobilization, Lundahl developed liposome chromatography.[4] Khaleque and co-workers demonstrated detachment of liposomes immobilized on gel particles by reductive cleavage of disulfide linkages connecting alkyl moieties and the gel (Scheme 1).[5,6]

Although valid for small liposomes, it is not evident if similar immobilization could also work with GVs. In the present study, interaction of GVs with hydrophobized Sephacryl S-1000 gel particles was examined.

The procedure described by Moscho was modified to obtain GVs of 1–20 μm in diameter from a mixture of phosphatidylcholine, organic solvents, and water (see a microscopic picture in the Supporting Information).[9] Upon incubation of the hydrophobized gel particles with the GV suspension, firm adhesion of GVs onto the particles was observed (Figure 1).



□ = $-OCH_2CH(OH)CH_2O(CH_2)_4-$

Scheme 1. Hydrophobized Sephacryl S-1000 polymer gel. R = 1-octyl, 1-dodecyl, or 1-hexadecyl.

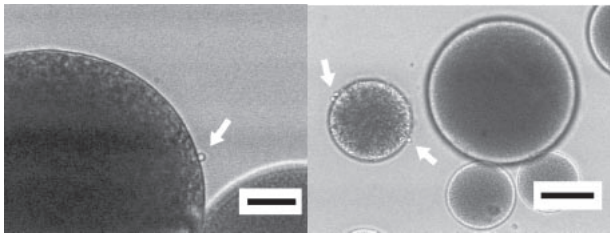


Figure 1. Hydrophobized polymer gel particles with GVs attached. Typical adhesion of a single GV to a particle (left; bar = 20 μm) and a particle with multiple GVs attached (right; bar = 40 μm).

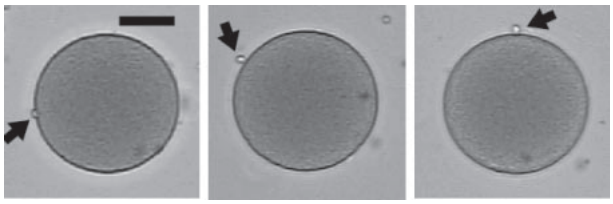


Figure 2. A gel particle moved with an immobilized GV under a gentle water flow. Bar = 20 μm.

Under the conditions, approximately 80% of the particles carrying GVs were found with one GV per particle, although some had multiple GVs attached. Gentle water flow moved the particle and the attached GV as one body (Figure 2). In fact, the adhesion was strong enough to tolerate washing of the particles using centrifugation. No significant deformation, such as rupture, of the GVs upon contact with the particles was observed.

Due to the nontransparency of the particles to light, only GVs that were at the circumference of the particle could be seen. There probably were more immobilized GVs hidden in the shadow of the particles. This makes the evaluation of the immobilization difficult. In the present study, the percentage of the particles carrying GVs was determined (GV immobilization index).

With a GV suspension containing 1.0 mg lipid mL^{-1}, the index was 15% for gel bearing 1-dodecyl moieties. Higher lipid concentration (3.5 mg mL^{-1}) increased the value to 29%. In contrast, no GV was found adhered onto the unmodified gel. The chemical modification of the gel is essential for the immobilization. The presence of electrolytes (Hepes 7 mM + NaCl 110 mM, pH 7.5) had no significant effect on the immobilization (data not shown), suggesting little contribution of ionic interaction.

The gels with three different hydrophobic moieties, 1-octyl, 1-dodecyl, and 1-hexadecyl were tested, and they behaved similarly; the indexes were 22–29% ("First incubation" in Table 1). At the same time, 50–57% of the phospholipid in the system was immobilized to the hydrophobized gels but none to the unmodified. Similar indifference to the length of the alkyl moieties was also observed in the previous study of the immobilization of small liposomes (diameter 100–200 nm).[5] The study showed that the immobilization was caused by the penetration of the hydrophobic moieties into lipid bilayer membrane. Although a longer alkyl group is presumably thermodynamically favorable, the firm attachment of GVs

Published on the web October 15, 2011; doi:10.1246/bcsj.20110083

Table 1. Immobilization of Giant Vesicles (GVs) onto Hydrophobically Modified and Unmodified Sephacryl S-1000[a)]

Gels (moieties/g wet gel)	Immobilization on gel					
	First incubation		Second incubation		Third incubation[c)]	Fourth incubation[c)]
	Lipids/mg	GVs %[b)]	Lipids/mg	GVs %[b)]	GVs %[b)]	GVs %[b)]
Octyl (3.1 μmol)	1.7	28	1.2	40	37	45
Dodecyl (2.7 μmol)	1.5	29	1.3	32	37	44
Hexadecyl (2.5 μmol)	1.5	22	1.2	34	38	38

a) Conditions: Gel (1.0 g) was incubated with a GV suspension (1.0 mL, 3.5 mg lipid mL^{-1} in the first incubation) at 5 °C for 6 h. b) The percentage of the particles with an attached GV (GV immobilization index). c) Phospholipids left in the supernatant after the incubation was below the detection limit and not determined.

indicates that even the octyl moieties could work as sufficient anchors. Gels bearing 2–4 times more alkyl moieties than those used in the experiments for Table 1 were also tested but the immobilization remained similar. The immobilization indexes after incubation for 24 h were 23, 22, and 21% for the octyl (6.7 μmol), dodecyl (6.3 μmol), and hexadecyl (10.5 μmol) gels, respectively. The gels used in Table 1 should be sufficient for stable immobilization of a GV.

The gels have their alkyl moieties covalently connected via disulfide linkage (Scheme 1). In the previous studies, reduction with dithiothreitol (DTT) cleaved the linkage and detached immobilized small liposomes.[5,6] The scheme was also tested with the present case. After the first incubation with a GV suspension, the gel was treated with DTT (30 mM). It released approximately 65–70% of the phospholipid immobilized on the gels while a blank experiment using threitol in place of DTT caused no change (data not shown), indicating that the scheme worked for small lipid aggregates as was in the previous study (approximately 80% release).[5,6] Meanwhile, only a part of the immobilized GVs were detached from the gel particles. The GV immobilization indexes decreased slightly from the values in Table 1 (22–29%) to 25, 20, and 20% for the octyl, dodecyl, and hexadecyl gels, respectively. Even under higher DTT concentration (600 mM), most of the GVs stayed on the gel. For the detachment, enough disulfide linkages must be cleaved so that the number of the anchors reaches below the minimum required for the immobilization.[6] Once immobilized, GV membrane could gather additional anchors, and this extra-enforcement makes the release difficult. Also, adhered GV membrane could cover the gel surface and sterically hinder DTT molecules from approaching the disulfide linkages.

The GV preparation used in the present study seems to contain a sizable amount of small lipid aggregates that could not be seen with an optical microscope. The number of the GVs observed in the interacting GV suspension (determined as $2 \times 10^6\,mL^{-1}$ based on microscopic observation) was at least three orders smaller than that could be expected from the amount of the phospholipids used (estimated as $1.6 \times 10^{10}\,mL^{-1}$ by the calculation outlined in Supporting Information). Furthermore, to the amount of the immobilized phospholipids (1.5–1.7 mg), the number of the attached GVs was also clearly too small. In fact, an electron micrograph in Moscho's paper shows many vesicles of 100–200 nm present with a GV.[9] These small lipid aggregates might compete with GVs for the binding.

To examine this point, the gel and the supernatant were separated after the first incubation, and the supernatant still containing phospholipid and GVs was again interacted with a fresh batch of gels. In this second incubation, the GV immobilization index increased to 32–40% for the newly interacted gels ("Second incubation" in Table 1). The process was further repeated, and after the third incubation, most of the phospholipid in the supernatant was removed (<0.1 mg, the detection limit). At this point, however, a number of GVs could still be seen. The process was repeated four times, and the indexes reached 38–45% ("Fourth incubation" in Table 1), which is 1.5–1.7 times higher than the first immobilization. This suggests that the small lipid aggregates in the GV preparation could be interfering with the immobilization of GVs.

This also demonstrated strong preference of the immobilization for smaller lipid aggregates over GVs. It is almost inevitable for a GV preparation to have coexisting small vesicles because the thermodynamic difference between the two types of the vesicles is small and in favor of the latter.[2] Though rarely mentioned, the contamination of small aggregates could cause problems in application of GVs. After the repetitive interaction of GV suspension with the dodecyl gel, the phospholipid was almost completely depleted while the suspension still contained approximately half of the GVs ($1 \times 10^6\,mL^{-1}$). The process may be used for removing small lipid contaminant from a GV preparation.

The previous study with liposomes less than 1 μm found that smaller vesicles are immobilized more efficiently because they require fewer anchors and may utilize even tinier binding sites.[7] In the present case, the advantage becomes more significant due to the larger difference in size. Unlike small vesicles or lipid aggregates that could permeate into and interact with the inner gel structure, contact between GVs and the gel should be only possible at the surface of the particles. This limits the number of the hydrophobic moieties that may work as the anchors and makes the immobilization of large GVs less favorable.

The number of GVs immobilized to the gels was rather smaller than one might expect from the strong adhesion. There were many GVs floating around and the vast surface on a particle but only a few GVs attached themselves to the particle. The results of the repetitive interaction indicate that the interference by the small aggregates exists but could not be the major cause of the inefficiency. There are many GVs available for the immobilization in the suspension. The inefficiency should be attributed to the gel. The firm adhesion indicates that the length and local number of the hydrophobic moieties on the gel are sufficient for the immobilization of a single GV. The present behavior suggests that the adhesion of GVs could occur

only at a limited number of binding sites on the gel surface. A steric factor on the surface might be crucial for the immobilization. Strong adhesion onto large planar smooth surface forces GVs to be deformed and burst.[10,11] In the present case, the immobilized GVs maintain near-spherical shapes. The Sephacryl gel particles possess many microscopic dents and pores on their surface (Figure 1). Only a pit that fits the size and shape of a GV could accommodate it by facilitating efficient contact of the gel surface with the GV, resulting in the penetration of a sufficiently large number of the hydrophobic anchors into the membrane.

In conclusion, the present study demonstrated firm immobilization of GVs onto hydrophobized gel particles. The process was not efficient, and this resulted in strong preference of the immobilization for smaller lipid aggregates over GVs. We are presently examining possible application of the behavior for removal of the aggregates contaminating a GV preparation. If applicable, the method is expected to have an advantage over high-speed centrifugation usually used for purification of GVs. The strong adsorption of small aggregates to the gel allows the interaction of the gel with a GV suspension in a batch mode as shown in the present study. The gel may be simply added to a GV suspension and, after incubation, removed by low-speed centrifugation. Also recently, electroformation has been frequently used for GV preparation.[2] When contamination of electroformed GVs, which are already attached on electrodes, with small aggregates is suspected, the gel could be conveniently used with no need of detaching the GVs.

Experimental

Hydrophobized Gels. Sephacryl S-1000HR gel (Amersham, Piscataway, NJ, U.S.A.) was chemically modified to possess hydrophobic moieties that were covalently bound by disulfide linkage to the gel particles as described elsewhere.[6] Three types of gels different in the length of the hydrophobic alkyl moiety (1-octyl, 1-dodecyl, and 1-hexadecyl) were prepared. The hydrophobized polymer gel particles thus obtained had the diameter of 40–200 μm, and, unless noted otherwise, the octyl, dodecyl, and hexadecyl gels possessed 3.1–3.2, 2.7–2.8, and 2.5–2.8 μmol of alkyl moieties per gram of a moist gel, respectively.

Preparation of GVs. GVs were prepared by modification of the method of Moscho.[9] Typically, 3.0 mg of phosphatidylcholine extracted and purified from egg yolk (EggPC; Avanti Polar Lipids, Alabaster, AL, U.S.A.) dissolved in 0.04 mL of chloroform was added to a mixture of 0.96 mL of chloroform and 0.20 mL of methanol in a 50 mL round bottom flask. To the solution was gently added 1.0 mL of pure water. Then the flask was attached to a rotary evaporator, and the evaporation started at 40 °C. The pressure was reduced gradually during the evaporation and kept at a final value of 14 mmHg. The amount of phospholipid was determined using a commercially available assay kit (Wako Pure Chemical Industries, Ltd., Osaka).

Interaction of GVs and Gels. Gel particles (1.0 g) was mixed with a GV suspension (1.0 mL; phospholipid concentration 3.5 mg mL^{-1}) and gently incubated at 5 °C for 6 h. Then the supernatant was removed by gentle pipetting and centrifugation (1200 × *g*, 20 min). The residual gel was washed with water three times (1.0 mL each) by centrifuge. The final residue was gently suspended in water (1.0 mL), and a drop of the suspension was taken into the space between two pieces of glass coverslip and observed on an inverted optical microscope (Olympus IX-50; Tokyo) equipped with a phase contrast and digital image enhancement system. To calculate the GV immobilization index, typically, 130 particles were randomly chosen and examined. A difference in the index larger than 3% could be accepted as significant. The amount of phospholipid immobilized on the gel was estimated by subtracting the lipid in the separated supernatant from the total lipid in the interacted GV suspension. When the lipid assay was not necessary, the incubation was carried out on one-tenth scale.

For the repetitive interaction, the separated supernatant and the washings were combined (approximately 3 mL) and concentrated to 1.0 mL by gentle evaporation under reduced pressure. The obtained GV suspension had approximately the same GV concentration as the one used in the first incubation and was used in the interaction with a fresh batch of gels.

For interaction with DTT, the gel particles separated and washed after the immobilization (1.0 g) was incubated with an aqueous solution containing DTT (10 equivalents to the disulfide linkage, approximately 30 mM, 1.0 mL) at 5 °C for 12 h. A part of the sample was taken and inspected under a microscope.

Supporting Information

A microscopic image of GVs in a typical preparation and an estimation of the number of GVs in the preparation. This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

References

1 *Giant Vesicles* in *Perspectives in Supramolecular Chemistry*, ed. by P. L. Luisi, P. Walde, John Wiley & Sons, Chichester, U.K., **2000**.

2 P. Walde, K. Cosentino, H. Engel, P. Stano, *ChemBioChem* **2010**, *11*, 848.

3 P. L. Luisi, F. Ferri, P. Stano, *Naturwissenschaften* **2006**, *93*, 1.

4 P. Lundahl, F. Beigi, *Adv. Drug Delivery Rev.* **1997**, *23*, 221.

5 Md. A. Khaleque, Y. Okumura, S. Yabushita, M. Mitani, *Colloids Surf., B* **2004**, *37*, 35.

6 Md. A. Khaleque, Y. Okumura, M. Mitani, *J. Bioact. Compat. Polym.* **2006**, *21*, 539.

7 Y. Okumura, H. Mizushima, K. Fujinaga, J. Sunamoto, *Colloids Surf., B* **2007**, *55*, 235.

8 Y. Okumura, H. Mizushima, J. Sunamoto, *J. Colloid Interface Sci.* **2007**, *307*, 296.

9 A. Moscho, O. Orwar, D. T. Chiu, B. P. Modi, R. N. Zare, *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11443.

10 T. J. Feder, G. Weissmüller, B. Žekš, E. Sackmann, *Phys. Rev. E: Stat., Nonlinear, Soft Matter Phys.* **1995**, *51*, 3427.

11 F. Quemeneur, A. Rammal, M. Rinaudo, B. Pépin-Donat, *Biomacromolecules* **2007**, *8*, 2512.