***Regular Article***

# Topology of Surface Ligands on Liposomes: Characterization Based on the Terms, Incorporation Ratio, Surface Anchor Density, and Reaction Yield

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**The surface topology of ligands on liposomes is an important factor in active targeting in drug delivery systems. Accurately evaluating the density of anchors and bioactive functional ligands on a liposomal surface is critical for ensuring the efficient delivery of liposomes. For evaluating surface ligand density, it is necessary to clarify that on the ligand-modified liposomal surfaces, some anchors are attached to ligands but some are not. To distinguish between these situations, a key parameter, surface anchor density, was introduced to specify amount of total anchors on the liposomal surface. Second, the parameter reaction yield was introduced to identify the amount of ligand-attached anchors among total anchors, since the conjugation efficiency is not always the same nor 100%. Combining these independent parameters, we derived: incorporation ratio=surface anchor density×reaction yield. The term incorporation ratio defines the surface ligand density. Since the surface anchor density represents the density of polyethylene glycol (PEG) on the surfaces in most cases, it also determines liposomal function. It is possible to accurately characterize various PEG and ligand densities and to define the surface topologies. In conclusion, this quantitative methodology can standardize the liposome preparation process and qualify the modified liposomal surfaces.**

**Key words** active targeting; surface ligand density; incorporation ratio; surface anchor density; reaction yield; surface topology

Nanotechnology has the capability to deliver drugs to specific cell types, but it is important to maximize therapeutic effects and minimize unexpected adverse effects, for this technology to be effectively used.[1–4] In using nanoparticles for specific drug delivery, they need to have both a stealth function to prevent non-specific recognition by the reticuloendothelial system (RES),[5,6] and an active targeting function to allow them to bind to the specific cell type of interest.[7–9] Polyethylene glycol (PEG) modification is frequently used to confer a stealth function to nanoparticles.[5,6,10] For active targeting, specific ligands for bioactive molecules such as nucleic acids, peptides, proteins, and antibodies are attached to the surface of nanoparticles *via* a PEG spacer.[11] Many attempts have been made to develop various types of specific ligands for use in active targeting, which is critical for targeting non-fenestrated tissues such as the brain, lung and related tissues.[12–14] The procedures used to prepare ligands that are used in modifying nanoparticles are relatively complicated because this increases the number of steps in the preparation process that can lead to conditions where ligand molecules can be unstable.[15] Therefore, optimal reaction (modification) conditions and methods for quantifying the density of the attached ligand that is attached to the nanoparticles need to be evaluated precisely.

Problems are usually encountered in evaluating both the density of the liposomal ligands and the PEG, since a variety of methods can be used to prepare such liposomes.[16–20] The issue of micelle contamination and controlling the orientation of the anchors are major impediments to such an evaluation. Currently, three methods are in widespread use for modifying the liposomal surface.[11,15,16] These methods include the pre-insertion method, the post-insertion method, and surface reaction modification. The first two methods result in quite different liposomal surface topologies, namely, the orientation of the ligand and its density. When ligand-PEG-lipids are incorporated into liposomes by the pre-insertion method, only about half of the ligands are facing outward from the membrane, and the rest are facing toward the inside of the liposomes and are therefore nonfunctional. In contrast, in the case of ligand-modified liposomes prepared by the post-insertion method, all of the ligands are facing outward from the membrane but ligand-modified micelles also remain in the reaction solution.[21–23] Because the modified micelles have the ability to bind to targeting receptors, it is important to determine the purity of the liposomes, and to evaluate the density of surface ligands,[24–28] which serve as an index of the quality of the liposomes.[29–32]

Surface reaction modification can efficiently modify the liposomal surface without micelle contamination, and ensures that the ligands are orientated facing outward from the membrane. Pan *et al.* applied both the post-insertion method and the surface reaction modification method to introduce cetuximab, a monoclonal antibody (mAb), against the human epidermal growth factor receptor (EGFR) on $F98_{EGFR}$ glioma cells as ligands on the liposomal surface for active targeting delivery.[33] The liposomes encapsulated the boron anion, dodecahydro-*closo*-dodecaborate (2-), to permit the preparation to actively and selectively deliver the boron derivative to selective tumors for boron neutron capture therapy. Based on thio-maleimide chemistry, the thiolated antibodies were covalently bound to the liposomal surface at room temperature (r.t.) for 4 h using maleimide-$PEG_{2000}$-distearoylphosphatidylethanolamine (DSPE) as anchors that had been previously incorporated into the liposomes. Nonetheless, attempts to evaluate the extent of binding to the cells using various liposomal surfaces became difficult because it was impossible

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Table 1. Reaction Yields and Incorporation Ratios Reported in the Literature Reports

| Ligands | Reaction types | Reaction conditions | Reaction yields (%) | Incorporation ratios (%) | References |
|---|---|---|---|---|---|
| Bovine γ-globulin | Thio-maleimide Michael addition | pH 7.5, r.t., 2 h | n.d. | (1.38) | 34) |
| mAb her2 Fab′ | Thio-maleimide Michael addition | pH >7.0 | n.d. | (1.08) | 35) |
| mAb her2 Fab′ | Thio-maleimide Michael addition | pH 7.3–7.4, r.t., o/n | n.d. | (0.65) | 36) |
| mAb CC52 | Thio-maleimide Michael addition | pH 6.7, r.t., 4 h | n.d. | (1.35/1.08) | 37) |
| mAb E-selectin | Thio-maleimide Michael addition | pH 6.7 | n.d. | (1.38) | 38) |
| GNGRGGVRSSSRTPSDKYC (peptide) | Thio-maleimide Michael addition | pH 7.4, 4°C, 16 h | n.d. | (0.35) | 39) |
| mAb E-selectin | Thio-maleimide Michael addition | pH 6.5, 4°C o/n | n.d. | (1.25) | 40) |
| mAb cetuximab | Thio-maleimide Michael addition | pH 7.4, r.t., 4 h | n.d. | (0.03) | 33) |
| cRGDFC (peptide) | Thio-maleimide Michael addition | pH 4.0–6.5, r.t., o/n | n.d. | (0.03) | 41) |
| GLase-ZZ-His (protein) | Thio-maleimide Michael addition | pH 7.4, r.t., o/n | n.d. | (0.03) | 42) |
| Transferrin (protein) | Staudinger ligation | pH 7.4, r.t., 6 h | n.d. | (0.10) | 43) |
| mAb 2C5 (anti-nucleosome) | Thio-maleimide Michael addition | pH 7.4, 4°C, o/n | n.d. | (0.05) | 44) |
| Hemoglobin | Thio-maleimide Michael addition | pH 7.4, 37°C, 1 h | n.d. | (2.27) | 45) |
| Octreotide (TATE, peptide) | Thio-maleimide Michael addition | pH 6.5, r.t., 10 min–24 h | 40 | 0.22 | 46) |
| p18-4 (peptide) | Carbodiimide mediate peptide coupling | pH 5.2–7.2, 4°C, 8 h | 35 | 0.30 | 47) |

Most reports assumed a reaction yield of 100%, ignored micelle contamination and implied that the bioactive results were affected by the original lipid composition. Only two prior studies reported such values but calculated the reaction yield using Eq. 1 in Chart 1, which is a conventional and inaccurate method. The values for incorporation ratios in parenthesis were calculated by us based on following equation: maleimide lipid molar ratio (of the total lipids in the liposomes)×0.55 (ratio facing outward from the membrane) (Eq. 1 in Chart 1) with the assumption of a 100% reaction yield, which means that all of the anchors contained an attached ligand[33–47]. mAb: monoclonal antibody, r.t.: room temperature, n.d.; no data.

to precisely calculate the reaction yield, and no information regarding the incorporation ratio (representing the surface ligand density) was available.[33] Most literature reports do not provide these two values or provide sufficiently accurate values to permit the surface topologies to be evaluated. Instead, bioactivities are evaluated with reference to differences in the composition of liposomal lipids[33–47] (Table 1).

The affinity of a liposome toward binding and its pharmacokinetics are related to its surface topology,[29,30,48–50] which is defined as the ratio of the density of the surface ligand and PEG that are located on the liposomal surface. An accurate value for incorporation ratio reflects the surface ligand density. To obtain an accurate figure for this ratio, it is necessary to indicate that not all the anchors have ligands attached to them. Hence, we introduced 2 accurate and precise independent parameters, namely the surface anchor (anchor-PEG lipid) density and the reaction yield to verify the difference between the anchors, *i.e.*, one with no ligand attached, and the other with a ligand attached. First, an analysis for the density of the total amount of anchor on the surface of the liposome is needed. This critical parameter, surface anchor density, represents both the amount of ligand acceptor that present on the liposome surface and, in the most cases, the amount of PEG present on the liposomal surface. It is therefore necessary to determine the purity of the liposome by removing contaminating micelles from the anchor grafted liposomes solution. Secondly, the reaction yield was determined by determining the amount of ligand-conjugated anchors that are present, because the conjugation efficiency is not always 100%. The surface topology therefore, was derived using the following mathematical relationship: incorporation ratio=surface anchor density×reaction yield. In the past, such an evaluation was based on using the original lipid constituents, which is implied the surface composition to predict bioactive results but without having an accurate value for the incorporation ratio. Such a procedure fails to correctly predict and explain bioactivities (Table 1). Furthermore, such an inaccuracy can lead to large deviations in the bioactivities of a liposomal preparation.

## MATERIALS AND METHODS

**Materials** Non-hydrogenated egg phosphatidylcholine (EPC) and *N*-[(3-maleimide-1-oxopropyl)aminopropyl polyethyleneglycol-carbamyl]distearoylphosphatidyl-ethanolamine (maleimide-$PEG_{2000}$-DSPE, MA-PEG lipid) were purchased from NOF Corp. (Tokyo, Japan). Cholesterol (chol) and Sepharose® CL-4B was purchased from Sigma (St. Louis, MO, U.S.A.). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (rhodamine-DOPE, rho lipid) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.). 5-Carboxyfluorescein (5-FAM) was purchased from TCI Corp. (Tokyo, Japan). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sephadex® G-25 was purchased from GE Healthcare Corp. (Sweden). All reagents were without further purification before use.

**Preparation of Capped Liposomes by Pre-insertion Method** The anchor grafted liposomes were composed of EPC : chol : rho lipid : MA-PEG lipid (60 : 33 : 2 : 5). The lipids were dissolved in ethanol, and chloroform was added and the solution was mixed well. The solution was evaporated to form a lipid film, and hydrated in HEPES–NaOH (pH 7.4) for 10 min at r.t. The mixture was sonicated for 2 min, yielding the anchor grafted liposomes.

For analyzing conventional surface anchor density, three individual 100 μL, 5 mM (ensured by 2% rho lipid UV absorbance) liposomal solutions were treated with 10, 0.9, and 0.5 eq 5-FAM-SH to MA-PEG lipid (surface molar amount, calculated by MA-PEG lipid amount ×0.55, outside surface ratio)

Table 2. Calculations of Lipids Amount and Liposomal Surface Anchor Density

| Tube No. | Capped anchor abs. | Capped anchor amount (nmol)[a)] | Rho abs. | Total lipids amount (nmol)[a)] | Surface anchor density (%) | Contribution value[b)] |
|---|---|---|---|---|---|---|
| 5 | 0.240 | 0.489 | 0.744 | 18.338 (36.47%) | 2.600 | 0.0095 |
| 6 | 0.264 | 0.513 | 0.767 | 18.905 (37.60%) | 2.644 | 0.0099 |
| 7 | 0.132 | 0.288 | 0.353 | 8.701 (17.30%) | 3.206 | 0.0055 |
| 8 | 0.069 | 0.171 | 0.176 | 4.338 (8.63%) | 3.794 | 0.0033 |

*a*) These values were obtained by HPLC analysis. *b*) The values were calculated by surface anchor density (%)×(total lipids amount)/(sum of total lipids amounts (tube No. 5 to 8)), *i.e.*, in tube No. 5 the value of normalized by total lipids concentrations=2.600%×(18.338/(18.338+18.905+8.7010+4.3380))=0.0095. The absorbance of surface capped anchor and liposomes (rhodamine-DOPE) were measured, respectively. Based on each calibration curves, the molar amount of surface capped anchor (5-FAM conj.) and total lipids were calculated by HPLC analysis. Using the formula (Eq. 5) in Chart 3, the surface anchor density of each fraction was calculated. Furthermore, each surface anchor density was normalized by total lipids amounts in order to get an average surface anchor density (0.0095+0.0099+0.0055+0.0033=2.82%) of these 4 fractions.

to incubate at 60°C for 1 h. The excess unreacted 5-FAM-SH was removed by passage through a Sephadex® G-25 column ($\phi$12 mm×40 mm), eluted with HEPES–NaOH (pH=7) to get pure capped liposomes. Particle size, size distribution, and zeta potential of anchor grafted liposomes, capped liposomes, and capped liposomes after purification were determined by dynamic laser light scattering using a Malvern Zetasizer Spectrometer (see Supplementary data Table S1).

**Preparation of Capped Liposomes by Post-inserted Method** The plain liposomes were composed of EPC : chol : rho lipid (60 : 38 : 2). The hydration method followed by sonication was performed, as described above. While the 250 $\mu$L, 3.83 mM (ensured by 2% rho lipid UV absorbance: 959.5 nmole total lipids) plain liposomes were produced, the post-insertion manner by adding MA-PEG lipid (MA-PEG lipid : plain liposomes lipids=1 : 20) to incubate at 60°C for 30 min was performed to offer the anchor grafted liposomes solution, mixed with the anchor micelles.

In order to cap the entire anchors of the liposomes and micelles in the solution, the mixture was treated with 3 eq 5-FAM-SH to the added MA-PEG lipid and allowed to react under 60°C for 1 h. The reaction mixture was purified by passing through the same Sephadex® G-25 column to remove excess unreacted 5-FAM-SH. Further purification was performed by means of a Sepharose® CL-4B column ($\phi$15 mm×350 mm), eluted with HEPES–NaOH (pH=7) to produce the pure capped liposomes. The average surface anchor density (2.82%, normalized by concentrations) was calculated as described in Table 2. Particle size, size distribution, and zeta potential of plain liposomes, anchor grafted liposomes, capped liposomes, and capped liposomes after purification were determined by the same instrument as above (see Supplementary data Table S2).

**Preparation of Conjugated Liposomes by Surface Reaction Modification** The plain liposomes were composed of EPC : chol : rho lipid (60 : 38 : 2). The anchor grafted liposomes mixed with a solution of anchor micelles (MA-PEG lipid) were treated exactly the same conditions as described above (parallel). The 250 $\mu$L 3.83 mM (calculated by a 2% rho lipid UV absorbance) liposomal solution was purified by means of a Sepharose® CL-4B column, as described above, resulting in the production of pure anchor grafted liposomes with a known surface anchor density, 2.82%.

For ligand conjugation, the anchor grafted liposomes, *e.g.*, the surface MA-PEG lipids, were treated with 1.1 eq 5-FAM-SH (the liposome concentration was calculated based on a 2% rho lipid UV absorbance, and the amount of surface MA-PEG lipids was calculated based on the surface anchor density, 2.82%) as four groups at different temperatures, 4, 25, 37, and 60°C for 1 h. The reaction solutions were quenched by adding mercaptoethanol (107 eq of MA-PEG lipids in each group). One additional anchor grafted liposome group was treated with 3 eq 5-FAM-SH at 60°C for 1 h to completely consume the surface MA-PEG lipid. All reaction mixtures that were the five groups were purified using the same Sephadex® G-25 column to remove excess unreacted 5-FAM-SH and mercaptoethanol, respectively, to produce the different pure conjugated liposomes. Particle size, size distribution, and zeta potential of the plain liposomes, the anchor grafted liposomes, conjugated liposomes, and the conjugated liposomes after purification were determined using the same instrument as above (see Supplementary Tables S3 and S4).

**Liposome Separation by Sepharose® CL-4B** To ensure that contaminating micelles were completely separated from liposomes (as shown in the separation profile in Fig. 1), separation conditions were optimized as follows: a 45 mL bed volume of Sepharose® CL-4B in a $\phi$15 mm×350 mm column. Sample mounting amount was within 250 $\mu$L and eluted by HEPES–NaOH (pH=7) buffer. Before each separation, the column was washed with 200 mL of buffer. While the sample mounted, it was eluted with HEPES–NaOH (pH=7) buffer, and the 1 mL fractions were collected by the autocollector for 40s tubes after void volumes were eluted.

**UV-Vis Spectrophotometric and Fluorescence Spectrophotometric Analysis of Liposomes** Firstly, the calibration curves were drawn as UV absorbance *versus* molar amount (nmole) by two series of four different concentrations of rhodamine-DOPE solutions and 5-FAM-$PEG_{2000}$-DSPE conjugate solutions, respectively. To calculate total lipids amount of a liposomal sample is basing on 2% rho molar amount contained in the liposomes, and the rhodamine-DOPE absorbance of each tube were measured by UV-Vis spectrometer. The value in $\lambda_{ex}$=570 nm, and $\varepsilon$=142000 were utilized to calculate (in HEPES–NaOH pH=7.4). To calculate the molar amount of 5-FAM-$PEG_{2000}$-DSPE conjugate in the liposomes, its absorbance of each tube was measured by a UV-Vis spectrometer as well. The value in $\lambda_{ex}$=495 nm, and $\varepsilon$=31000 were utilized in the calculation (in HEPES–NaOH pH=7.4). To prepare the data in Fig. 1, 40s sample fractions were pipetted into 96 well plates within 100 $\mu$L and the fluorescence intensities were recorded by means of a fluorescent spectrophotometer. For 5-FAM-$PEG_{2000}$-DSPE, the excitation wavelength was $\lambda_{ex}$=495 nm, the emission wavelength was $\lambda_{em}$=545 nm, for the rhodamine-DOPE, the excitation wavelength was $\lambda_{ex}$=575 nm,

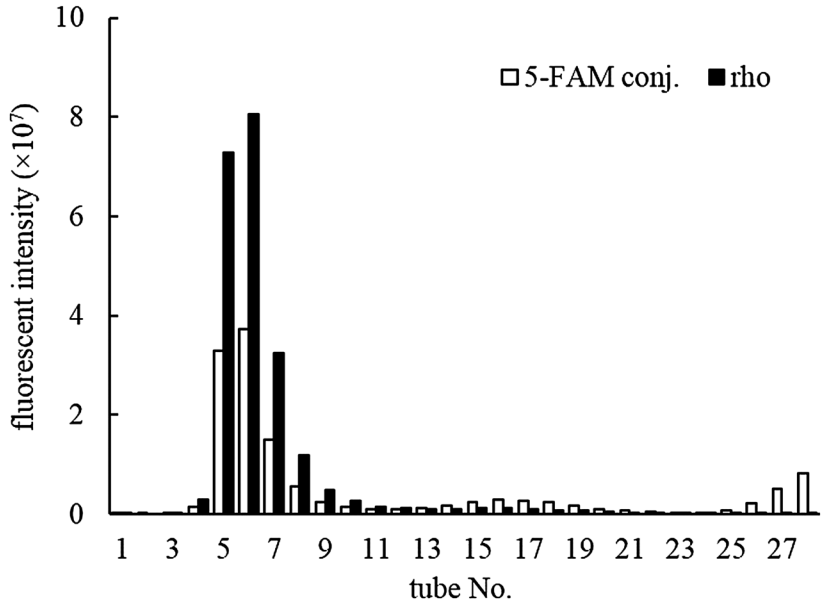



Fig. 1. Sepharose® CL-4B Fluorescent Chromatogram Shows That Micelles Were Removed from Liposomes

Capped liposomal solution mixed with capped micelles was separated by Sepharose® CL-4B. The two fluorescents, rhodamine-DOPE (rho) and 5-FAM-$PEG_{2000}$-DSPE conjugate (5-FAM conj.) were shown as red and green bars, respectively. The red bars represent different amount of liposomes in different fractions with various intensity. The green bars represent capped MA-PEG lipid (5-FAM conj.) in liposomes and micelles. The tubes from Nos. 5 to 8 were collected as pure capped liposomes due to most reliable fractions. The tubes from Nos. 14 to 19 contained aggregated micelles. The tubes from Nos. 26 to 28 contained micelles.

and the emission wavelength was $\lambda_{em}$=650 nm.

**HPLC Analysis of Liposomes** For preparing a HPLC standard analytical curve (area under the curve (*AUC*) *vs.* UV absorbance), covering the concentration range of all samples, a series of concentrations (2–50 $\mu$M, 100 $\mu$L) of 5-FAM-$PEG_{2000}$-DSPE conjugate solutions (HEPES–NaOH, pH=7.4) were prepared (see Supplementary Fig. S2). The analytical curve of rhodamine-DOPE also prepared as the same way above. Each sample (70–99 $\mu$L) then analyzed by injecting into HPLC to calculate molar amount of rhodamine-DOPE and 5-FAM-$PEG_{2000}$-DSPE by the following conditions: C4 column, eluting by triethylammonium acetate (TEAA) buffer (100 mM, pH=7.4)/acetonitrile and gradient elution of 0–90% (acetonitrile) in 25 min at 40°C, $\lambda_{ex}$=495 nm (see Supplementary Fig. S3).

## RESULTS AND DISCUSSION

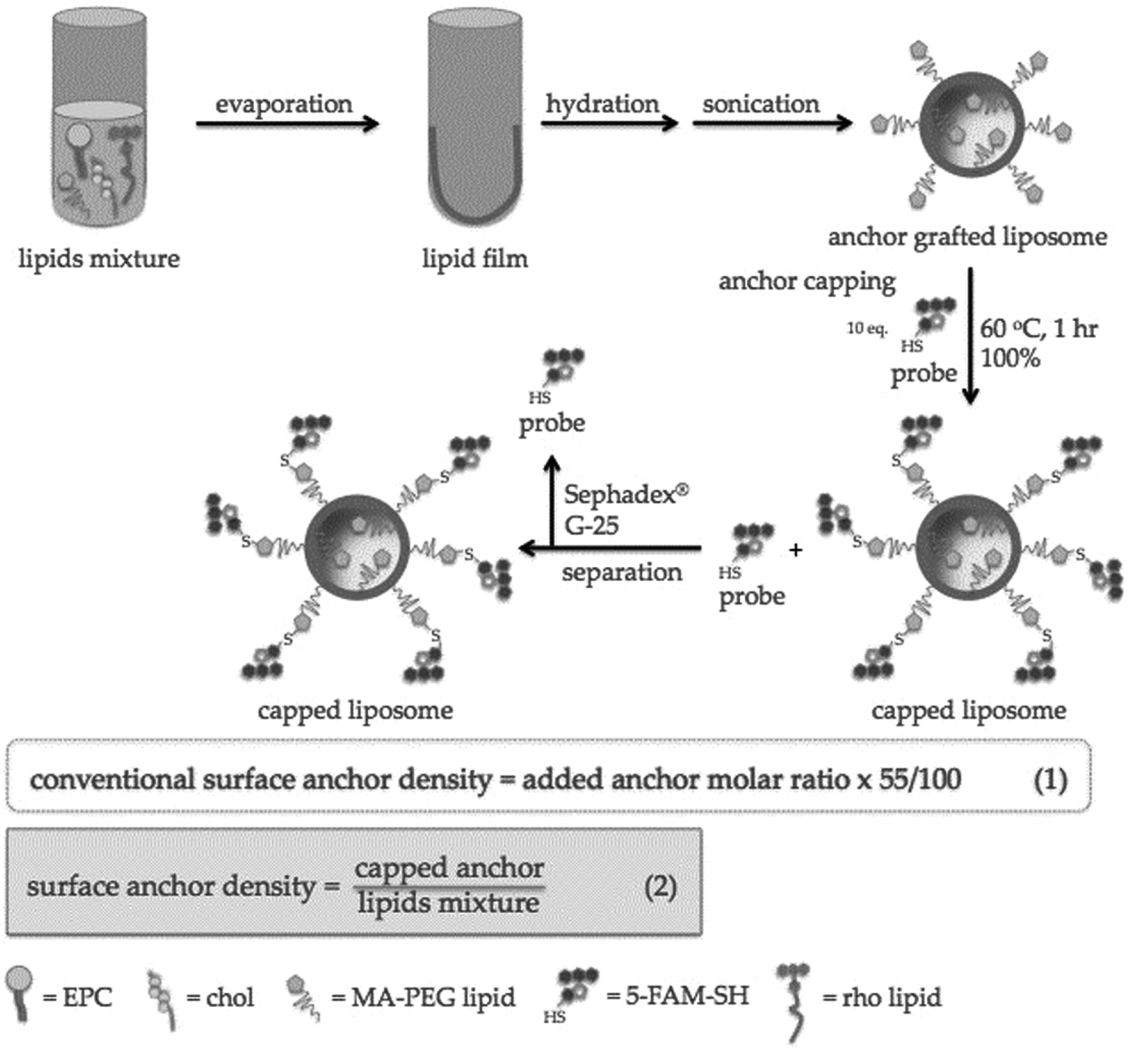
**Determination of the Conventional Surface Anchor Density of Pre-inserted Liposomes** The anchors of anchor grafted liposomes prepared by the pre-insertion method (Chart 1) are generally assumed to be facing both outward and inward from the membrane surface in a ratio of 55 : 45.

The density of surface anchors that are facing outward, which is referred to as the conventional surface anchor density (shown in Eq. 1), was determined by means of a theoretical calculation. Thiolated 5-carboxyfluorescein (5-FAM-SH) was employed as a probe to cap the outward facing anchors on the liposomes. The reaction conditions for conjugation between the probes and anchors were adjusted to ensure that all of the anchors had completely reacted in advance (see Supplementary for the synthetic route in Chart S1 and the mass spectroscopy in Fig. S1). Unreacted probes were removed by Sephadex® G-25 to result in pure capped liposomes that were used in the surface anchor density calculations. The conventional surface anchor density, calculated as 5%×(55/100)=2.75% (=added anchor molar ratio×(55/100)) was inconsistent with the surface anchor density (=(capped anchor)/(lipids mixture)), 3.04%, that was calculated by the probe capping method (Eq. 1) (see the physical data of the liposomes in Table S1, and HPLC chromatogram of the liposomes analysis in Fig. S3). This result indicates that the ratio of outward facing to inward facing ligands was 61 : 39. Possible reasons for this are that the ratio of the outward facing surface anchors is higher than 55/100, that anchor micelles exist in the liposomal solution, or that the accuracy and reproducibility of the theoretical ratio is low. However, the density of anchors facing the two sides was difficult to control. It is important to know the surface anchor density before surface reaction modification. While the calculation of surface anchor density of pre-inserted liposomes was performed, it is not possible to estimate the amount of inward facing anchors using the above probes. The uncertainty associated with the ratio of inward *versus* outward facing anchors indicates that pre-inserted liposomes are not acceptable as candidates for calculating surface anchor density.

**Analysis of the Conventional Surface Anchor Density of Post-inserted Liposomes** The conventional surface anchor density is considered by the added molar ratio of anchors in the total lipids. While anchor grafted liposomes are prepared, which are referred to as post-inserted liposomes by the anchor insertion process, micelles may present as contaminants of the anchor grafted liposomes (Chart 2).

As micelles result in an inaccurate liposomal solution, applying a size exclusion column, Sepharose® CL-4B, to remove micelles from liposomes is necessary.[22,23] Since the anchors in post-inserted liposomes are only outward facing, it is easy to control and calculate the surface anchor density. Therefore, the post-insertion method is a viable candidate for use in evaluating surface anchor density.

To evaluate the surface anchor density of anchor grafted




To analyze surface anchor density, the conventional calculation method (1) was examined with 5-FAM-SH as a probe based on a thio-maleimide Michael addition reaction at the surface of the pre-prepared liposomes. It should be noted that, in the pre-prepared liposomes, maleimide anchoring groups were exposed and facing both inward and outward with a theoretical ratio 45/55, and the probes became attached only to the outward facing moieties. However, our result (2) was not consistent with the surface anchor density calculated using conventional assumptions (1) (liposomes containing EPC : chol : rho lipid : MA-PEG lipid=60 : 33 : 2 : 5). All equations are shown in detail in Supplementary materials.

Chart 1. Surface Anchor Density on Pre-inserted Liposomes

liposomes prepared by the post-insertion method, the probes were introduced again to cap the anchors on both the micelles and the outer membrane of the liposomal surface (Chart 3).

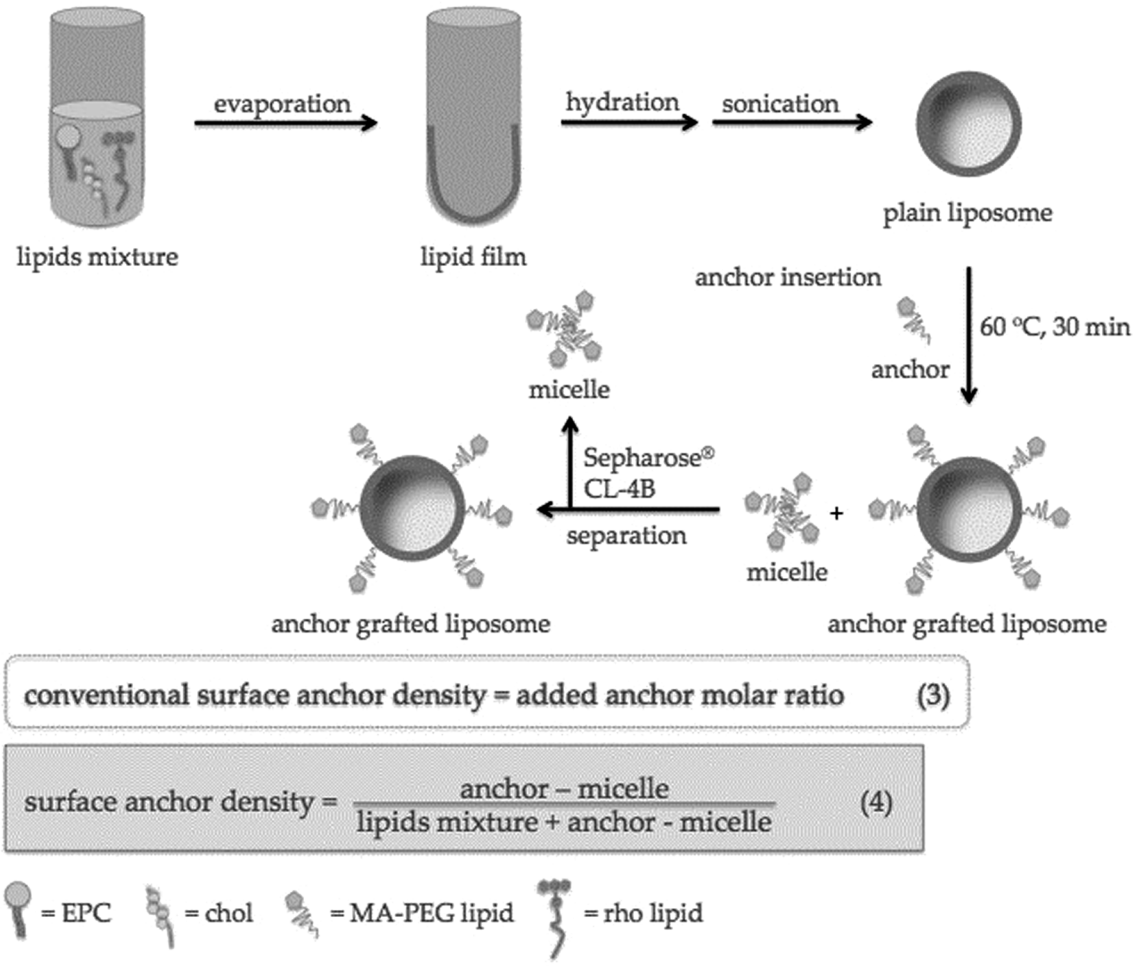
All of the anchors are consumed because all are outward facing. Next, Sephadex® G-25 was utilized to remove unreacted probes (see Supplementary for the physical data of the liposomes of Chart 3 in Table S2), and further capped micelles were removed by Sepharose® CL-4B, resulting in the production of pure capped liposomes for surface anchor density calculation (see Supplementary for the physical data of the liposomes of Chart 3 in Table S3). By doing this, we were able to verify that there were micelles in the liposomal solutions, and that the micelles could be completely removed from the liposome suspension. Sepharose® CL-4B separation is critical in evaluating the surface anchor density of intact liposomes. The size of micelles and liposomes are in the resolution range of the Sepharose® CL-4B column. Some studies have used this approach to show that micelles can actually be removed.[22,23] The separation results (Fig. 1) show three peaks with two kinds of fluorescent intensity along the tube number sequence.

Three peaks, tubes 5–8, 14–19, and 26–28, containing capped liposomes, aggregating capped micelles, and capped micelles, respectively, were well separated. The ratio of the intensity of these three peaks of 5-FAM conj. was 63 : 14 : 23. This shows that the amount of contaminating micelles is significant.

We defined the surface anchor density as:

$$\frac{\text{surface capped anchor}}{\text{lipids mixture} + \text{surface capped anchor}} \quad (5)$$

as shown in Chart 3. The surface anchor density of pure liposomes in tube No. 5 (Fig. 1) of the purified liposomal fractions was 0.489/(0.489+18.338)=2.600% (Table 2). Furthermore, when normalized by the concentrations of total lipids in tubes 5–8, the contribution value was=36.47×2.600%=0.0095. By summing up the four contribution values, the average surface anchor density of post-inserted liposomes was 2.82%, as explained in Table 2.




Using the post-inserted method to determine the conventional surface anchor density. Although the anchors of these liposomes are only outward facing, micelles that contaminate liposomal solutions are usually not considered after the post-insertion process. Ignoring the existence of micelles leads the inaccurate estimation of surface anchor density. Therefore, to remove micelles Sepharose® CL-4B is recommended for producing pure anchor grafted liposomes. All equations are shown in detail in Supplementary materials.

Chart 2. Surface Anchor Density on Post-inserted Liposomes

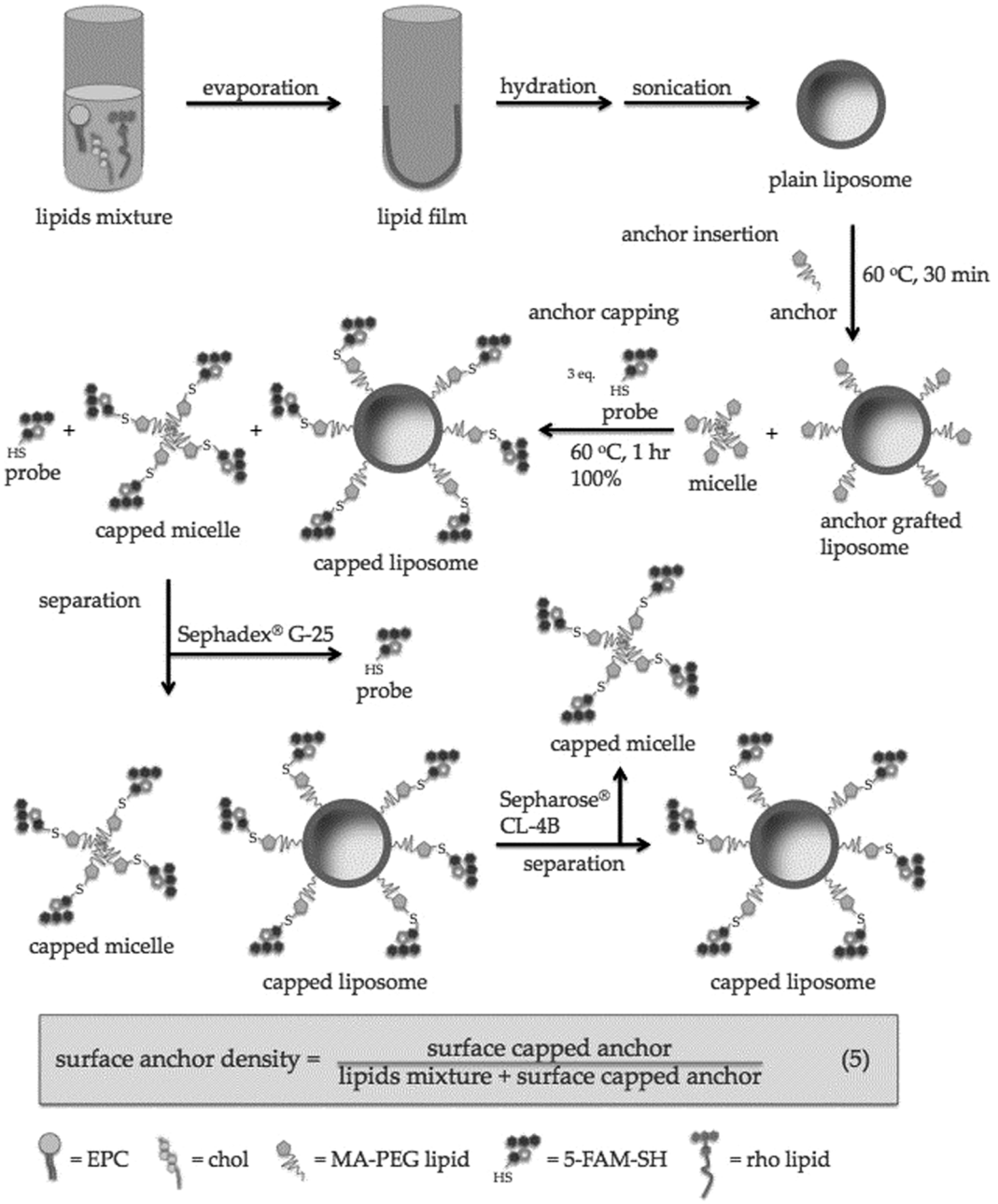
However, the 2.82% also represents a discordant result in comparison with the conventional surface anchor density, which is defined as the added anchor molar ratio in the total lipids=1/(20+1)=4.76% (Eq. 3). 4.76% also represents the maximum value for the surface anchor density. Therefore, the inconsistent results indicate that the surface anchor density, as calculated by conventional methods, overestimates the actual density. Only about 60% of the added anchors are actually inserted into the anchor grafted liposomes (Chart 3).

Surface anchor density is a key parameter, as it accurately represents the amount of PEG in most cases. It also represents the amount of active anchors in determining the liposomal functions, and provides a more precise evaluation of the ligand conjugation efficiency of a liposomal preparation. After ligand conjugation, the evaluations of the processes are used in calculating the surface ligand density.

**Characterizing the Liposomal Surface Topology Functionalized by the Surface Reaction Method** To develop a model to explain the surface topology, 5-FAM-SH was used as a ligand to modify the liposomal surface. As the surface anchor density (2.82%) is already known by a previous analysis, the same anchor grafted liposomes were used in the next surface functionalization (Chart 4). A thio-maleimide Michael addition reaction was performed at different temperatures from 4 to 60°C in order to produce different liposomal surface conditions. After removing all of the unreacted ligands by passage through a Sephadex® G-25 column, the qualities of different liposomal surfaces of conjugated liposomes are ready for further surface examination (see Supplementary for the physical data of the liposomes of Chart 4 in Table S4).

To describe the surface topology, it is necessary to have information regarding the conjugation efficiency of the anchors and ligands. Therefore, the reaction yield was introduced as a primary parameter. Here, we define the reaction yield of the surface reaction as (surface 5-FAM conj)/(surface anchor) shown in Chart 4. In the case of 4°C (Table 3), which is usually used to prevent proteins as ligands from denaturing, the reaction yield was 0.109/0.248=44.6% (by Eq. 6 in Chart 4).

This result suggests that the assumption of a reaction yield of 100%, which is usually assumed in most studies, is not valid, but less than half of the total ligands on the surface undergo conjugation. It is reasonable that the reaction yields increase from 44.6 to 87.8% showing a trend alone the temperatures from 4 to 60°C. Surprisingly, the findings also indicate that a reaction at 37°C gave a yield similar to that at 60°C, with a yield in excess of 86%. Therefore, in the case of

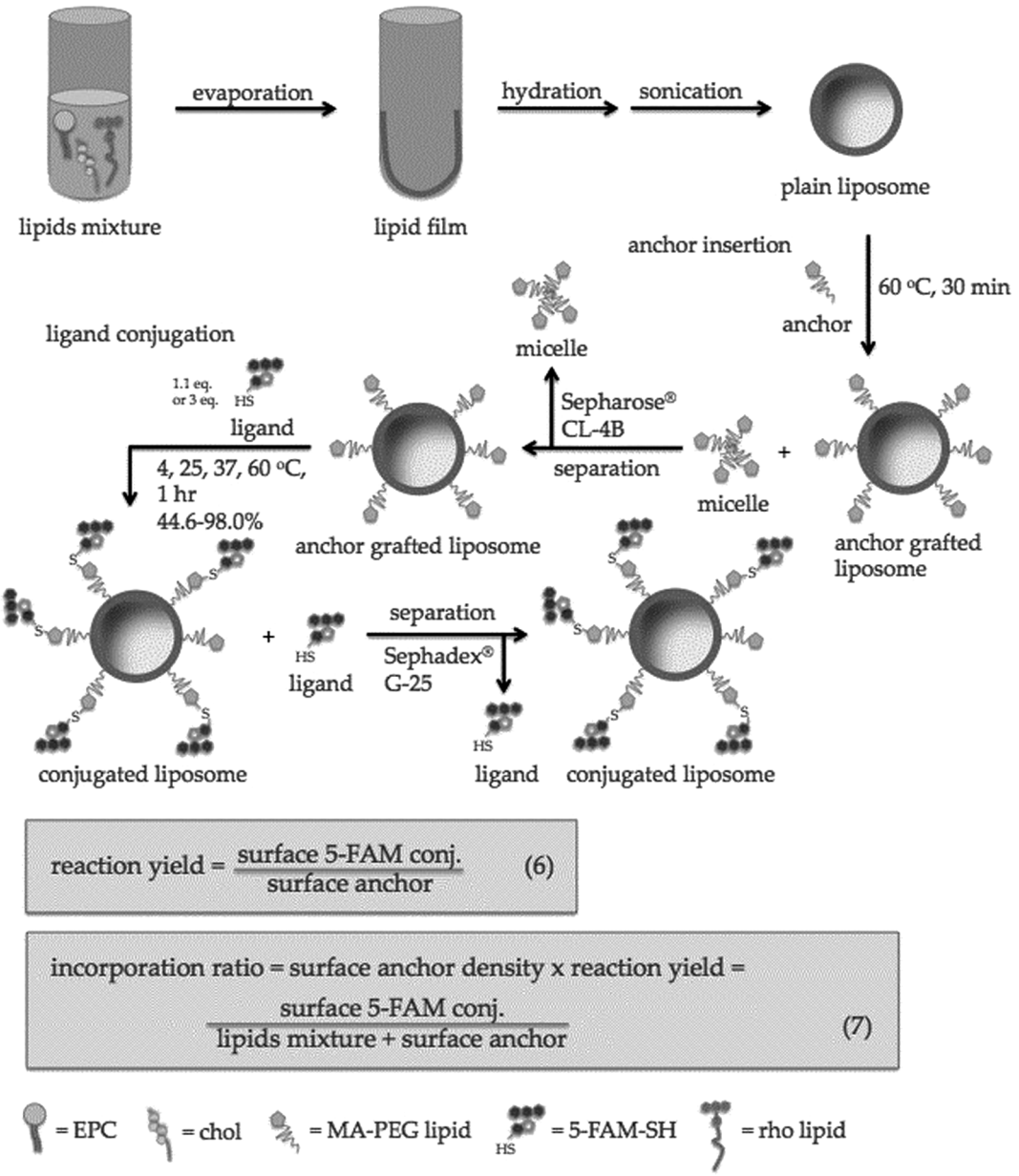



The flowchart was designed to precisely estimate the surface anchor density of post-inserted liposomes. A 5-FAM-SH was introduced as a probe to visualize micelles that are removed and to quantitatively determine the surface anchor density. The result proved that micelles were presented after the post-insertion process and showed that Sepharose® CL-4B can be used to efficiently remove micelles to produce pure and homogeneous capped liposomes (liposomes containing EPC : chol : rho lipid=60 : 38 : 2; liposome : anchor=20 : 1). All equations are shown in detail in Supplementary materials.

Chart 3. Quantification of Surface Anchor Density on the Post-inserted Liposome by Probe Introduction

37°C, the surface topology can be described as follows: the PEG density is 2.825% (surface anchor density), and ligands are attached to 86.8% of the anchors (Table 3). In order to conjugate efficiently, we conclude that in contrast to r.t., heating at 37°C will provide efficient activating energy for surface reaction modification by a thio-maleimide Michael addition. Again, applying excess ligands to conjugate all the anchors on the surface verified the accuracy of the surface anchor density determined in this study, *i.e.*, surface ligand density=surface anchor density=0.316/(11.092+0.316)=2.770%, which was very close to the previous value (2.82%).

The binding affinity of ligand-modified liposomes to target receptors is usually attributed to the surface ligand density, *i.e.*, the incorporation ratio. The incorporation ratio was introduced as a second parameter to describe surface topology. For an accurate incorporation ratio, the following two steps are required: removal of micelles to obtain the surface anchor density, and removal of unreacted ligands to calculate the reaction yield. Therefore, we proposed a definition for the incorporation ratio as:




To evaluate surface topology, 5-FAM-SH was introduced as a ligand. Surface conjugation was performed to analyze the ligand density and surface conditions based on a thio-maleimide Michael addition reaction at different temperatures. The surface reaction functionalizing method takes account of micelles remaining, ligand remaining and insufficient reaction problems, which result in the failure to understand the active targeting capability of liposomes. In order to determine the actual topology, Sepharose® CL-4B and Sephadex® G-25 was used to remove contaminating micelles and unreacted ligands, respectively. Two factors, reaction yield and incorporation ratio were introduced to describe the actual surface topology. Based on these two factors, the qualities of ligand modified liposomal surfaces reacted at different temperature are accurately described (liposomes containing EPC : chol : rho lipid=60 : 38 : 2; liposome : anchor=20 : 1). All equations are shown in detail in Supplementary materials.

Chart 4. Characterization of Functionalized Liposomal Surface Topology by Reaction Yield and Incorporation Ratio

$$\frac{\text{surface 5-FAM conj.}}{\text{lipids mixture}+\text{surface anchor}} = \text{surface anchor density} \times \text{reaction yield} \left( \frac{\text{surface capped anchor}}{\text{lipids mixture}+\text{surface capped anchor}} \times \frac{\text{surface 5-FAM conj.}}{\text{surface anchor}} \right) \quad (7)$$

Therefore, the incorporation ratio at 4°C can be calculated as 2.825×44.620%=1.261%. The increasing trend of incorporation ratios from 4 to 60°C was governed only by reaction yields because the surface anchor density was fixed in this model (Fig. 2). However, during the anchor insertion processes, the following three factors will mainly affect the surface anchor density: ratio between anchors and lipids during the insertion process, the PEG density on the liposomes, and the temperature.

It is definitely possible to determine the incorporation ratio directly using the following formula,

$$\text{incorporation ratio} = \frac{\text{surface 5-FAM conj.}}{\text{lipids mixtures}+\text{surface 5-FAM conj.}}$$

without knowing the surface anchor density and the reaction yield only when unreacted ligands and conjugated micelles are removed, and importantly, the reaction yield is assumed to be

Table 3. Calculations of Lipids Amount and Liposomal Surface Reaction Yield

| Temperature (°C) | 5-FAM conj. (abs.) | 5-FAM conj. amount (nmol)[a)] | Rho (abs.) | Total lipids amount (nmol)[a)] | Surface anchor density (%) | Surface anchor amount (nmol) | Reaction yield (%) |
|---|---|---|---|---|---|---|---|
| 4 | 0.052 | 0.109 | 0.242 | 8.521 | 2.825 | 0.248 | 44.620 |
| 25 | 0.058 | 0.122 | 0.249 | 8.768 | 2.825 | 0.248 | 48.512 |
| 37 | 0.099 | 0.277 | 0.313 | 11.021 | 2.825 | 0.312 | 86.799 |
| 60 | 0.089 | 0.287 | 0.320 | 11.268 | 2.825 | 0.319 | 87.830 |
| 60[b)] | 0.186 | 0.316 | 0.315 | 11.092 | 2.825 | 0.314 | 98.024 |

The absorbance of surface 5-FAM conj. and rho (rhodamine-DOPE) were measured. The molar amount of the surface 5-FAM conj. and total lipids were calculated by HPLC and calibration curves as well as Table 1. Following the formula (6) in Chart 4, the reaction yields on different conditions were obtained. *a*) These values were obtained by HPLC analysis. *b*) This sample was treated with excess amount of 5-FAM-SH (3 eq) to ensure complete reaction, confirming surface maleimide molar ratio under 60°C (surface maleimide molar ratio=2.77%, very close to the average surface anchor density, 2.82%).

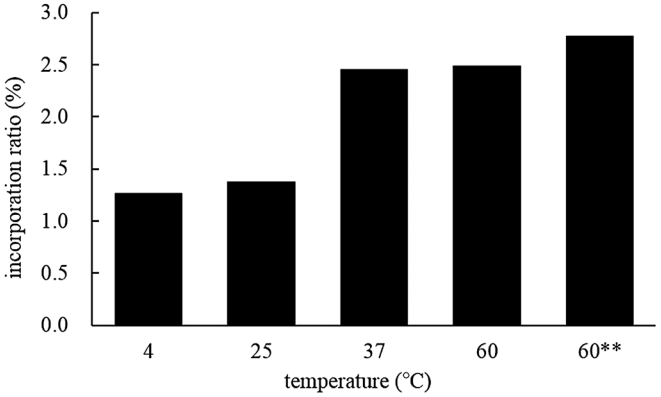



Fig. 2. Incorporation Ratios at Different Liposomal Surface Modifying Conditions

The incorporation ratio was calculated. Liposomal incorporation ratios in ideal reaction environment with different temperatures were well differentiated. Thirty-seven degree offered enough activation energy of thio-maleimide Michael addition reaction on liposomal surface. **This is described in the legend of Table 3.

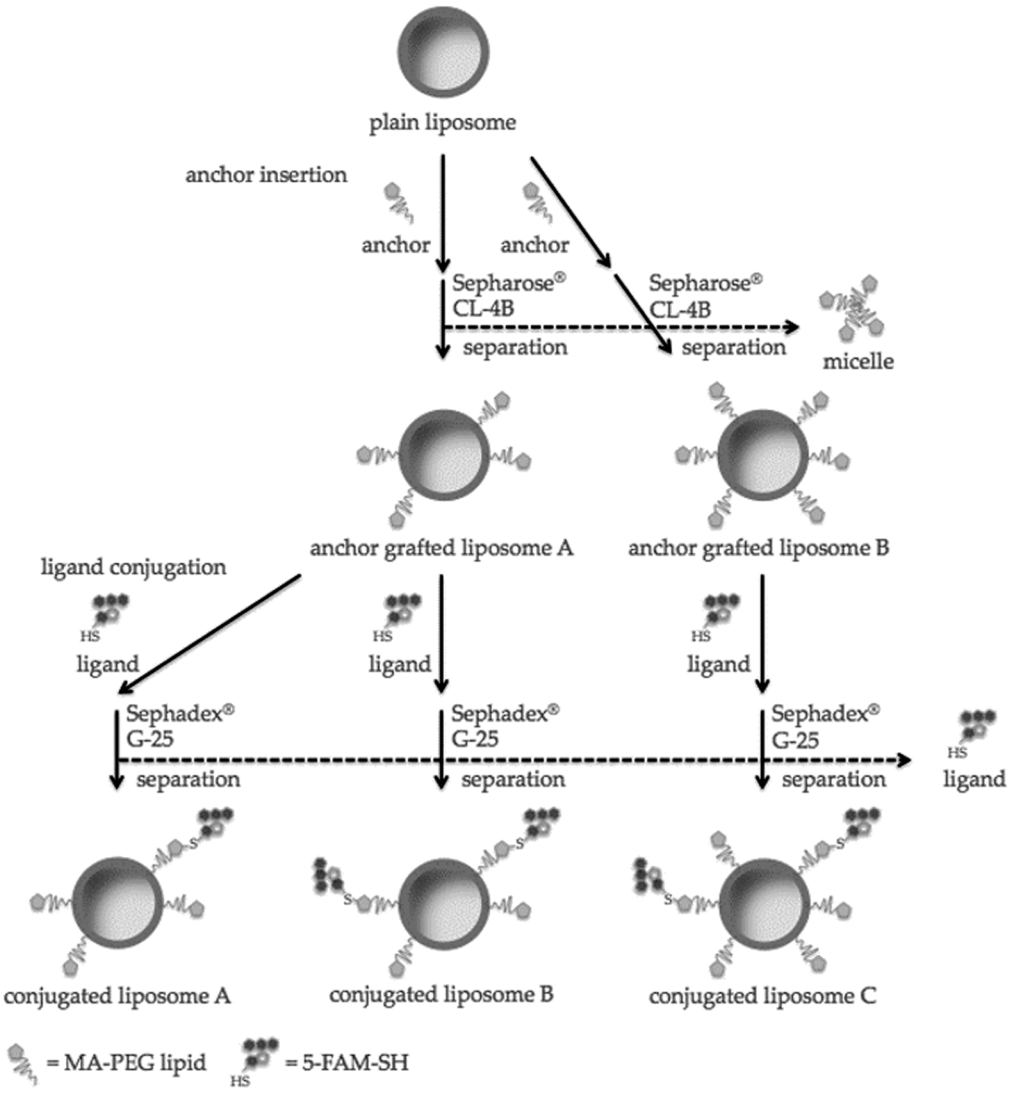
100%. However, in the denominator, the surface 5-FAM conj. is not always equal to the surface anchor as the reaction yield is not 100%. This means while the reaction yield is lower than 100%, the surface PEG density remains unknown, which influences the pharmacokinetics, and makes the incorporation ratio vague. In Chart 5, a practical case, two kinds of anchor grafted liposomes with different surface anchor densities may be obtained, while the plain liposomes repeat the anchor insertion process (if the process is not exactly identical). After this, through the surface reaction respectively (if the reaction condition is not exactly identical), three kinds of conjugated liposomes may be produced.

All of the parameters on the table in Chart 5 can only be calculated by the method outlined herein. Different liposomal surface topologies can be distinguished clearly. On the other hand, if the incorporation ratio was calculated directly (by Eq. 5), conjugated liposome B can be distinguished from conjugated liposome A but the result is quite different from that for conjugated liposome C. The similarity of conjugated liposomes B and C do not adequately reflect the liposomal binding affinity and pharmacokinetic properties, even though the ligand density is very similar in each case. Various PEG densities (*e.g.*, surface anchor density) have an effect on the liposomal pharmacokinetics in the delivery process. In the circulating blood, PEG confers different degrees of stability, hindrance, and endurance time. When liposomes reach the target cells, the surface ligand confers binding affinity, thus facilitating receptor-mediated endocytosis. In the case of intracellular trafficking, however, a higher PEG density impedes liposomal endosome release, which can influence therapeutic efficacy. Therefore, the density is indicative of the degree that PEG influences the pharmacokinetics and intracellular pharmacokinetics of the process, thus further determining the efficiency of drug delivery. Collectively, to our knowledge, this is first report to demonstrate that three parameters (surface anchor density, reaction yield and incorporation ratio), and Eq. 7 are essential to precisely depict the topology of a liposomal surface for explaining and predicting bioactivities.

This precise methodology standardizes the surface ligand topology for active targeting. Purity, accuracy and reproducibility for Good Laboratory Practice (GLP) and Good Manufacture Practice (GMP) level, and for proceeding to clinical trials of the designed liposomes are required. Precise values draw an accurate profile of surface topology to explain or predict the bioactivities of such preparations. The incorporation ratio represents the surface ligand density, which is mainly referred to as the binding affinity and this leads directly to active targeting capability. Regarding the binding affinity evaluation assay, an inaccurate incorporation ratio misleads the results, and further affects $EC_{50}$ values. Besides, the standard deviation (S.D.) of bioactivities is also enlarged due to this type of inaccuracy. In this study, we offer a methodology to assess the quality control of liposomes with reliable parameters for further applications.

## CONCLUSION

The methodology described herein provides a precise method for calculating the parameters needed to accurately characterize different liposomal surfaces. During the process of modifying a liposomal surface by a surface reaction, the micelles and excess reactants are removed to produce pure and high quality liposomes for further study. Also, the ligand orientation is fixed so as to be facing outward. Surface anchor density is a critical parameter for representing PEG density and to calculate the amount of surface anchor present. Further, while proceeding through the surface reaction modification, the reaction yield, another parameter, of ligands and anchors is calculated based on the amount of surface anchor as the denominator. These two independent parameters can be estimated separately and precisely. Consequently, we are the first to propose an equation capable of describing liposomal surface topology: incorporation ratio=surface anchor density×reaction yield. By the incorporation ratio, the ligand density on the li-




| Conjugated liposome | Surface anchor density* | Reaction yield* | Incorporation ratio |
|---|---|---|---|
| A | $\frac{4}{100+4} = 3.85\%$ | $\frac{1}{4} = 25.0\%$ | $3.85\% \times 25.0\% = 0.963\%$ |
| B | | $\frac{2}{4} = 50.0\%$ | $3.85\% \times 50.0\% = 1.925\%$ |
| C | $\frac{6}{100+6} = 5.66\%$ | $\frac{2}{6} = 33.3\%$ | $5.66\% \times 33.3\% = 1.885\%$ |

On the preparation of conjugated liposomes by surface reaction procedure, following conventional calculation, incorporation ratio=(surface 5-FAM conj.)/(lipids mixtures+surface 5-FAM conj.), results in an overestimated result. It is particularly difficult to distinguish between conjugated liposomes B and C. However, in this Chart taking an account of a given practice assumption, simulating calculation of this methodology gives three parameters to precisely describe the surface topology of different liposomal surfaces on the table. *We assume these values as lipids molar amount of each part of liposomes (liposomes molar amount=100 nmol, surface anchor molar amount=4 or 6 nmol, and surface ligand molar amount=1 or 2 nmol).

Chart 5. Differentiation of Various Anchor Grafted Liposomes and Conjugated Liposomes

posomal surface is well recognized, and surface anchor density represents the density of the PEG on the liposomal surface as well. Using this methodology it is possible to characterize liposomal surfaces accurately by surface anchor density and these two parameters can be used for evaluating liposome delivery capabilities.

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**Conflict of Interest** The authors declare no conflict of interest.

**Supplementary Materials** The online version of this article contains supplementary materials.

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