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Original Article

Liposome microencapsulation for the surface modification and improved entrapment of cytochrome c for targeted delivery

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Abstract

In this study, we established a procedure based on the microencapsulation vesicle (MCV) method for preparing surface-modified liposomes, using polyethylene glycol (PEG) and a site-directed ligand, with high entrapment efficiency of cytochrome c (CytC). For preparing a water-in-oil (W/O) emulsion, egg phosphatidylcholine and cholesterol were dissolved in organic solvents (O phase) and emulsified by sonication with aqueous solution of CytC (W_1). Although the dispersion stability of the W_1/O emulsions was low when *n*-hexane was used to dissolve the lipids in the O phase, it was substantially improved using mixed solvents consisting of *n*-hexane and other organic solvents such as ethanol and dichloromethane (DCM). The W_1/O emulsion was then added to another water phase (W_2) to prepare the W_1/O/W_2 emulsion. PEG- and/or ligand-modified lipids were introduced into the W_2 phase as external emulsifiers not only for stabilizing the W_1/O/W_2 emulsion but modifying the surface of liposomes obtained later. After solvent evaporation and extrusion for down-sizing liposomes, approximately 50% of CytC was encapsulated in the liposomes when the mixed solvent consisting of *n*-hexane and DCM at a volume ratio of 75/25 was used in the O phase. Finally, the fluorescence-labeled liposomes, with a peptide ligand having affinity to the vasculature in adipose tissue, were prepared by the MCV method and intravenously injected into mice. Confocal microscopy showed the substantial accumulation of these liposomes in the adipose tissue vessels. Taken together, the MCV technique, along with solvent optimization, could be useful for generating surface-modified liposomes with high drug entrapment efficiency for targeted delivery.

Keywords

Microencapsulation vesicle; water-in-oil-in-water emulsion; liposome; encapsulation efficiency; cytochrome c

Introduction

Liposomes, defined as spherical vesicles composed of one or more lipid bilayers, were first described in 1964 by Bangham *et al*. [1]. They were soon recognized as promising drug carriers because of their biocompatibility and their ability to incorporate both hydrophilic and hydrophobic compounds into their internal aqueous phase and lipid membrane, respectively. However, conventional liposomes, typically consisting of phospholipids and cholesterol, have been reported to be removed from circulation by the mononuclear phagocyte system (MPS) [2]. Surface modification of conventional liposomes with hydrophilic polymers such as polyethylene glycol (PEG) is one of the most popular ways to obtain the long-circulating liposomes, also called as “stealth” or “sterically stabilized” liposomes. The covalent attachment of PEG, referred to as PEGylation, to the outer surface of conventional liposomes creates a steric barrier against non-specific interactions between liposomes and biological components such as serum proteins and phagocytic cells, and is, hence, considered effective for escaping recognition from MPS and extending their circulation time [3]. Liposomes that circulate longer can passively accumulate in tumor tissues or inflammatory areas through the enhanced permeability and retention (EPR) effect [4]. However, despite the improved *in vivo* pharmacokinetics of the long-circulating liposomes, their therapeutic potential is still restricted owing to their inability to specifically and directly deliver the drugs to the target cells. Therefore, the attachment of site-directed ligands such as antibodies, peptides and small molecules on the surface of liposomes has been investigated to improve their *in vivo* performance of liposomes [5-7].

Apart from target specificity, improving drug entrapment efficiency is another major issue in the use of liposomal formulations. In general, liposomes show high efficiency in entrapping lipophilic drugs into the lipid bilayer, whereas the efficiency of entrapping hydrophilic agents into the aqueous core is quite low [8]. Therefore, preparing surface-modified liposomes with high drug entrapment efficiency will be useful for clinical/commercial application of liposomal formulations.

In a previous study, we had developed a ligand-modified liposomal carrier that actively recognized vascular endothelial cells in adipose tissues via a specific ligand (KGGRAKD) [9]. The KGGRAKD peptide motif, first identified by Kolonin *et al*. through an *in vivo* phage display [10], has been shown to have high affinity to prohibitin, which is expressed on the surface of white fat vasculature. Annexin A2 is an endogenous binding partner of prohibitin and is also located on the surface of adipose

endothelial cells, and that the KGGRAKD peptide can bind to prohibitin by mimicking the amino acid sequence (KGRRAED) located in the connector loop region of annexin A2 protein [11]. Moreover, a chimeric peptide composed of the KGGRAKD peptide (hereinafter, referred to as "prohibitin-targeting peptide" [PTP]) and a proapoptotic peptide ($_D$(KLAKLAK)$_2$) have been reported to facilitate a significant weight loss in obese mice, rats, and monkeys via the destruction of the adipose vasculature as a result of targeted apoptosis [10,12,13]. Furthermore, we had also reported that selective delivery of a pro-apoptotic peptide ($_D$(KLAKLAK)$_2$) or protein (cytochrome c, CytC) through the prohibitin-targeting peptide (PTP)-modified PEGylated liposome, referred to as prohibitin-targeted nano particle (PTNP), to the adipose vasculature could effectively treat or prevent obesity in mice [14-16]. In these studies, the PTNP had been prepared by the reverse-phase evaporation (REV) method [17], which is a well-known method for preparing large unilamellar vesicles (LUVs) to improve the entrapment of water-soluble peptides ($_D$(KLAKLAK)$_2$) or proteins (CytC). However, the percentages of $_D$(KLAKLAK)$_2$ or CytC encapsulated in the PTNP prepared by the REV method were only 9% or 15%, respectively [14,16].

Various other methods for liposome preparation are also available today [18]. The microencapsulation vesicle (MCV) method, which utilizes a water-in-oil-in-water (W/O/W) emulsion, is one of the most promising strategies for achieving high entrapment of hydrophilic drugs into liposomes [19-21]. Briefly, lipids are dissolved in an organic solvent that is immiscible in water (O phase), and mixed with an aqueous solution (W_1 phase) containing hydrophilic agents to be encapsulated. The W_1/O emulsion is produced by mechanical agitation or sonication, and then added to another water phase (W_2) to prepare the W_1/O/W_2 emulsion. During continuous agitation, the excess amount of lipids in the O phase are transferred and oriented to the interface between the O and W_2 phases. After complete removal of the organic solvent by evaporation, lipid bilayers are formed and liposome suspension is obtained. In the MCV method, the aqueous solution containing hydrophilic drug compounds to be encapsulated first forms the W_1/O emulsion, and the emulsion is dispersed in the continuous water phase to produce the W_1/O/W_2 emulsion. As a result, the drug-containing W_1 phase is separated from the W_2 phase by the O phase containing the membrane-consisting lipids throughout liposome formation, which provides an advantage to prepare liposomes with a high encapsulation efficiency of a water-soluble agents. Recently, the combined use of a microchannel emulsification device and the MCV method was shown to have great potential for preparing size-controlled (approximately 1 μm) liposomes with high entrapment efficiency

of hydrophilic agents (> 80%) [22]. However, the potential of the MCV method for preparing sub-micron/nano-scale liposomes with surface modification and high entrapment efficiency has not yet been evaluated. Thus, the purpose of the current study was to establish an alternative protocol for preparing PTNP with high encapsulation efficiency of CytC by utilizing the MCV method.

Materials and Methods

Materials and animals

Egg yolk phosphatidylcholine (EPC), distearoylphoshatidylethanolamine- poly(ethylene glycol) 2000 (DSPE-PEG_{2k}), and DSPE-PEG_{5k}-Maleimide were obtained from NOF (Tokyo, Japan). Cholesterol (Chol) and CytC from bovine heart were purchased from SIGMA (St. Louis, MO). PEG monooleyl ether (n = 50 [approximately]) (PEG-MOE) was purchased from Tokyo Chemical Industry (Tokyo, Japan). The PTP (GKGGRAKDGGC-NH_2, >95% purity) was synthesized at Scrum Inc. (Tokyo, Japan). *n*-hexane, methanol (MeOH), ethanol (EtOH), methyl acetate (MeAc), ethyl acetate (EtAc), diethyl ether (DEE), diisopropyl ether (DIE), chloroform (CF), and dichloromethane (DCM) were obtained from Wako Pure Chemical Industries (Osaka, Japan), and were of extra pure grade.

C57BL/6J mice (8-weeks old, male) were purchased from SLC Japan (Shizuoka, Japan). All animals were acclimatized for one week prior to use. All animal experiments were approved by the research advisory committee of Hokkaido University, Sapporo, Japan and the National Institute of Advanced Industrial Science and Technology (AIST), Kagawa, Japan. All the experiments were performed in accordance with the guidelines of the Care and Use of Laboratory Animals.

Preparation of W_1/O emulsions

EPC and Chol were dissolved at a molar ratio of 2:1 (total lipid concentration: 100 mM) in *n*-hexane alone or one of the hexane-based solvent mixtures indicated below: *n*-hexane/MeOH (90:10 volume ratio), *n*-hexane/EeOH (90:10), *n*-hexane/MeAc (75:25), *n*-hexane/EtAc (70:30), *n*-hexane/DEE (50:50), *n*-hexane/DIE (50:50), *n*-hexane/CH (75:25), and *n*-hexane/DCM (75:25). These lipid solutions were used as the oil (O) phase. Phosphate-buffered saline (PBS) (pH 7.4) with 2 mM CytC was used as the internal water (W_1) phase. The O phase (500 μl) and the W_1 phase (100 μl) were poured into a glass tube and then sonicated with a probe-type sonicator (Misonix, Farmingdale, NY) at 10 W for 15 min at 25 °C.

When the fluorescence-labeled liposomes were prepared for confocal imaging analysis, described later, PBS (pH 7.4) with 1 mM sulforhodamin B (Thermo Fisher Scientific, Carlsbad, CA) was used as the W_1 phase.

Preparation of $W_1/O/W_2$ emulsions

Membrane emulsification with the shirase-porous-glass (SPG) membrane module (pore size: 10 μm, Direct connector, SPG Techno, Miyazaki, Japan) was used for preparing $W_1/O/W_2$ emulsions. PBS with 25 μM DSPE-PEG_{2k}, 31.25 μM DSPE-PEG_{5k}, and 75 μM PEG-MOE was used as the external water (W_2) phase to obtain the PEGylated liposomes without any ligand modification. Ten milliliters of the W_2 phase was poured into a 20-ml glass beaker and stirred at 800 rpm using a magnetic stirrer. The W_1/O emulsion (dispersed phase) was slowly added into the W_2 phase through the SPG membrane, which was dipped into the continuous phase. To obtain the PTNP, DSPE-PEG_{5k} was replaced with DSPE-PEG_{5k}-PTP, which was synthesized by conjugating DSPE-PEG_{5k}-Maleimide with PTP, as described previously [9].

Preparation of liposomes by solvent evaporation from $W_1/O/W_2$ emulsions

To obtain liposomes from the $W_1/O/W_2$ emulsion, the organic solvents were evaporated by continuous stirring at 800 rpm for 24 h using a magnetic stirrer under atmospheric pressure and room temperature. The lipid vesicles were then extruded thrice through two stacked polycarbonate membranes (pore size: 200 nm) using a Mini-Extruder (Avanti Polar Lipids, Alabaster, AL). Finally, the non-encapsulated CytC or sulforhodamine B were removed by dialysis (Biotech cellulose ester (CE) membrane, 300K MWCO, Spectrum, Rancho Dominguez, CA) against PBS for 24 h at room temperature.

The schematic diagram of liposome formation by the MCV method is illustrated in **Fig. 1**.

Characterization of lipid particles

The particle size, polydispersity index (PdI), and ζ-potential of the lipid particles were measured using a Malvern Zetasizer Nano ZS (Malvern, UK).

The encapsulation ratio of CytC into liposomes was calculated as the ratio between the encapsulated CytC and the total amount of CytC. Liposome samples, before and after dialysis, were diluted with DMSO, and the CytC was quantified by measuring the absorbance at 408 nm using a spectrophotometer (Beckman Coulter, Brea, CA). For determining the encapsulation ratio of sulforhodamine B into liposomes, the amount of sulforhodamine B was determined based on its

fluorescence intensity at λEm/λEx = 550/580 nm. Empty liposomes were prepared in parallel to the loaded ones and used as blanks.

In this study, all preparations and evaluations of the PEGylated liposomes and PTNPs were performed independently three times.

Confocal observation of adipose tissues

C57BL/6J mice (9-week old; male) were intravenously injected with sulforhodamine B-loaded PTNP or PEGylated liposomes prepared by the MCV method at a dose of 0.125 mmol/kg. To visualize the blood vessels, FITC-conjugated *Griffonia simplicifolia* isolectin B4 (GSIB4) (Vector Lab, Burlingame, CA) was also injected intravenously (50 μg/mice), 30 min prior to tissue collection. At 24 h after injection of the liposomes, the inguinal adipose tissue was collected and cut into small pieces. The tissue pieces were transferred onto a glass-base dish and observed using a confocal laser-scanning microscopy (CLSM) (model A1, Nikon, Tokyo, Japan).

Statistical analysis

All statistical analyses were performed using the SigmaPlot software (Version 13.0, Systat Software, San Jose, CA). One-way ANOVA, followed by Tukey's test, was used to evaluate the statistical significance. A p-value < 0.05 was considered indicative of statistical significance.

Results and Discussion

Preparation of W_1/O emulsion using EPC and Chol as lipid emulsifiers

In this study, a mixture of EPC and Chol at a molar ratio of 2:1 was used as the constituent lipids of the liposome. Moreover, CytC was used as the test drug compound, as it has been successfully encapsulated into PTNPs previously by the REV method, with an encapsulation ratio of 15% [16]. To obtain liposomes using the MCV method, it is essential to prepare the stable primary W_1/O emulsion. However, the W_1/O emulsion droplets, prepared using *n*-hexane as the organic phase with EPC/Chol (2/1), immediately coalesced when the sonication was stopped, indicating that EPC and Chol had a weak emulsifying ability in *n*-hexane, which was consistent with the previous study [22]. It has been previously shown that the dispersion stability of W_1/O emulsions could be dramatically improved using a mixture of *n*-hexane and other organic solvents, including DCM, as the O phase for dissolving the phospholipids [23]. Thus, it was investigated whether the stability of W_1/O emulsions was improved by preparing them by using mixtures of *n*-hexane with different organic solvents as the O phase containing EPC/Chol (2/1). The results showed that the clear or opalescent W_1/O emulsions were obtained in all 8 types of organic solvent mixtures used in this study. The average diameter of these W_1/O emulsion droplets was below 80 nm (**Table 1**), and no coalescence was observed for at least 3 h after preparation (data not shown). Mixing other solvents with *n*-hexane would affect the density, viscosity, and interfacial properties of the W_1 and O phases, as well as the solubility of the O phase in water, which are known as determinant factors for the droplet size and emulsion stability [24,25]. However, the mechanism of emulsion stabilization and creation of smaller droplets in the presence of other solvents was not fully elucidated. Further investigations are required to resolve this issue.

Preparation of PEGylated liposomes using the MCV method.

To formulate PEGylated liposomes via the MCV method, we subjected the prepared W_1/O emulsions to secondary emulsification in the external aqueous (W_2) phase containing DSPE-PEG_{2k}, DSPE-PEG_{5k}, and PEG_{2k}-MOE (0.5, 0.65, and 3 mol% of total lipids, respectively) as external emulsifiers, using an SPG membrane (pore size: 10 μm). After removing the organic solvents from the $W_1/O/W_2$ emulsion by evaporation, lipid vesicles with average diameter of around 200 nm were observed (**Table 2**). As the mean size of the W_1/O emulsion droplets was substantially smaller than the pore size of the SPG

membrane, large oil droplets (O) containing multiple small water droplets (W_1) would be dispersed in the W_2 phase at immediately after preparation of the $W_1/O/W_2$ emulsion (**Fig.1**). The lipid vesicles were then formed by solvent evaporation from the $W_1/O/W_2$ emulsion by continuous agitation under atmospheric pressure. Therefore, it is possible that some W_1/O droplets would fuse and become larger during solvent evaporation. These liposome suspensions were extruded to ensure uniform size distribution, followed by dialysis to remove non-encapsulated CytC. The average diameter of the liposomes after extrusion and dialysis was approximately 140 nm (PdI < 0.2), and the ζ-potential was around -10 mV (**Table 2**). There were no remarkable differences in particle size or electrostatic charge between liposomes prepared using the 8 different mixed organic solvents; however, the encapsulation ratio of CytC in the liposomes was significantly different. When *n*-hexane was mixed with chlorinated solvents such as CF and DCM, the encapsulation ratio of CytC was higher than when *n*-hexane was mixed with other types of solvents (**Fig. 2**). In particular, approximately 50% of CytC was incorporated into liposomes when the O phase was an organic solvent mixture of *n*-hexane and DCM at a volume ratio of 75 : 25. This was approximately 3-fold higher than the encapsulation ratio previously achieved by the REV method [16]. In the case of microspheres of biocompatible polymers, it has been reported that the encapsulation efficiency could be affected by the hydrophobicity of the materials (polymer, solvent, and additive) used for microsphere preparation [26]. The 8 solvents used in this study can be arranged based on their water solubility as follows: CF, DIE, DCM < DEE, EtAc < MeAc << MeOH, EtOH [27]. Our observations revealed that the use of *n*-hexane mixed with sparingly or slightly water soluble solvents (DIE, CF, and DCM) tended to generate liposomes with higher encapsulation efficiency of CytC than the use of *n*-hexane mixed with more water miscible or soluble solvents (MeOH, EtOH, MeAc, EtAc, and DEE). This observation suggested that the hydrophobicity of the solvent used for liposome preparation by the MCV method would be an important determinant of the encapsulation ratio of water-soluble proteins such as CytC. However, although DCM has higher solubility in water than CF [27], its mixture with *n*-hexane resulted in the highest encapsulation efficiency (**Fig. 2**), indicating that the hydrophobicity of the solvent was not the only factor that determined the encapsulation efficiency. The rate of solvent removal and the solubility of lipids in the solvent have also been known to affect the encapsulation efficiency in the microencapsulation technique [28-30]. In case of the MCV method, solvent removal can occur in the following two steps; gradual solvent diffusion form the O to W_2 phase and evaporation of solvents from

the surface of the W_2 phase. Accordingly, organic solvents having higher solubility in water and lower boiling point would favor their fast removal from the W/O/W emulsion and facilitate the formation of lipid vesicles with high encapsulation efficiency. This consideration is consistent with our findings that higher encapsulation of CytC was achieved when the mixed solvent of *n*-hexane/dichloromethane (DCM) (75/25) rather than *n*-hexane/chloroform (CF) (75/25) was used. However, although diethyl ether (DEE) has higher solubility in water and lower boiling point than DCM does [27], the improvement of CytC encapsulation by DEE was significantly lower than that by DCM. These findings suggest that other unknown factors also affect the encapsulation efficiency. Although the exact molecular mechanisms behind the improvement of encapsulation efficiency using organic solvents might be very complex, the selection and optimization of the organic solvent is expected to be critical in this process. Systematic investigations are required to resolve this issue.

Preparation of ligand-modified PEGylated liposomes using the MCV method

Next, the CytC-loaded PTNPs, a peptide ligand-modified PEGylated liposome, were prepared using the MCV method with the mixed organic solvent (*n*-hexane/DCM = 75/25). As shown in **Table 3**, approximately 50% encapsulation of CytC was achieved, even when DSPE-PEG_{5k}-PTP, instead of the DSPE-PEG_{5k}, was employed as one of the external emulsifiers in the W_2 phase to prepare the PTNP. The particle size of the PTNP was similar to that of PEGylated liposomes; the ζ-potential of the PTNP was almost neutral, whereas that of PEGylated liposomes was around −10 mV, indicating that the cationic peptide ligand (GKGGRAKDGG) might be grafted onto the surface of the lipid vesicles after the organic solvent of the $W_1/O/W_2$ emulsion was evaporated. In general, a lack of surface charge of liposomes increases their aggregation and reduces their storage stability. However, surface modification of liposomes with PEG provides strong steric repulsion, thus stabilizing the liposomes by avoiding aggregation [31]. Despite the neutrality of the ζ-potential of the PTNP prepared by the MCV method, it was sufficiently stable on storage at 4°C for longer periods (data not shown), suggesting that the surface of the PTNP was also modified with PEGs.

Furthermore, it was examined whether the encapsulation of sulforhodamine B, a water-soluble small molecule, instead of CytC into the PTNP and PEGylated liposomes was also ameliorated by the MCV method. The result showed that the encapsulation ratio of sulforhodamine B into the PTNP was

approximately 20% (**Table 3**). The entrapment efficiency of sulforhodamine B into the PTNP by the MCV method was approximately 7-fold higher than that achieved by the REV method (2.8%) in our previous study [16]. The encapsulation ratio of CytC into PTNP was higher than that of sulforhodamine B, although both are water-soluble molecules. A similar phenomenon was also observed in our previous study [16], suggesting that the incorporation of CytC into the liposomes involved not only physical entrapment to the internal aqueous solution, but also interaction with the liposome-constituting lipids such as EPC and Chol [32,33].

***In vivo* delivery of PTNP prepared by the MCV method to adipose tissue**

In our previous study, it was found that the exposure of PTP to organic solvents such as CF during the preparation of the PTNP by thin film hydration method [34,35] caused an irreversible alteration of ligand conformation, resulting in the failure of the targeting activity of the PTP to prohibitin receptor [36]. To evaluate the functional ability of the peptide ligands of the PTNP prepared by the MCV method, sulforhodamine B-loaded PNTPs were intravenously administered to mice. Confocal microscopic observation of adipose tissues isolated from the treated mice clearly showed that the PTNPs were accumulated in the blood vessels of the adipose tissues, whereas the PEGylated liposomes were not (**Fig. 3**). From this result, we assumed that, in case of the MCV method, the peptide ligand was not exposed to the O phase, as the DSPE-PEG_{5k}-PTP was one of external emulsifiers in the W_2 phase.

Conclusion

The findings of this study showed that the organic solvent chosen for dissolving the liposome-constituting lipids was an important determinant of the entrapment efficiency (approximately 50%) of water-soluble proteins such as CytC, when the liposomes are prepared by the MCV method. Apart from the high CytC entrapment achieved, this method also enables the preparation of surface-modified liposomes with PEGs or site-directed ligands simply by adding them into the external aqueous phase as a secondary emulsifier during the preparation of the $W_1/O/W_2$ emulsion. Based on our findings, the MCV method should be useful for preparing nanosized liposomes modified with a functionally active peptide ligand.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Tables

Table 1. Diameters of the W_1/O emulsion droplets prepared with various compositions of organic solvent mixtures

Compositions of mixed organic solvents (volume ratio)	Average particle diameters (nm)	PdI
n-Hexane/MeOH (90/10)	79.9 ± 4.5	0.172 ± 0.003
n-Hexane/EtOH (90/10)	56.5 ± 4.3	0.213 ± 0.029
n-Hexane/MeAc (75/25)	40.5 ± 1.7	0.189 ± 0.026
n-Hexane/EtAc (70/30)	36.5 ± 0.7	0.180 ± 0.007
n-Hexane/DEE (50/50)	61.6 ± 5.9	0.159 ± 0.023
n-Hexane/DIE (50/50)	66.9 ± 5.2	0.221 ± 0.052
n-Hexane/CF (75/25)	54.5 ± 2.5	0.194 ± 0.026
n-Hexane/DCM (75/25)	67.2 ± 2.4	0.164 ± 0.008

The O phase containing EPC/Chol (2/1) as emulsifiers and the W_1 phase (2 mM CytC in PBS) were poured into a glass tube and then sonicated using a probe-type sonicator at 10W for 15 min at 25 ^{o}C. Then, average diameters of the W_1/O emulsions were measured. Data are represented as the mean ± SD (n=3).

Table 2. Physicochemical characteristics of the PEGylated liposomes prepared by the MCV method using various mixtures of organic solvents

Compositions of organic phase (volume ratio)	After solvent evaporation			After extrusion and dialysis		
	Average particle diameters (nm)	PdI	ζ-potentials (mV)	Average particle diameters (nm)	PdI	ζ-potentials (mV)
n-Hexane/MeOH (90/10)	214.8 ± 28.1	0.475 ± 0.099	-4.7 ± 1.3	143.8 ± 5.1	0.132 ± 0.007	-8.9 ± 0.9
n-Hexane/EtOH (90/10)	191.0 ± 4.0	0.384 ± 0.045	-4.7 ± 2.1	141.5 ± 6.2	0.124 ± 0.011	-9.9 ± 3.0
n-Hexane/MeAc (75/25)	230.9 ± 24.1	0.466 ± 0.088	-2.5 ± 1.0	148.9 ± 3.8	0.151 ± 0.022	-7.3 ± 0.3
n-Hexane/EtAc (70/30)	225.2 ± 51.0	0.412 ± 0.061	-3.6 ± 0.6	146.1 ± 2.3	0.142 ± 0.023	-8.3 ± 0.4
n-Hexane/DEE (50/50)	226.4 ± 76.5	0.425 ± 0.020	-2.0 ± 0.6	142.4 ± 10.7	0.150 ± 0.004	-9.9 ± 2.0
n-Hexane/DIE (50/50)	200.0 ± 33.5	0.474 ± 0.102	-1.4 ± 0.3	138.4 ± 3.5	0.148 ± 0.016	-3.9 ± 1.2
n-Hexane/CF (75/25)	166.8 ± 3.6	0.378 ± 0.076	-1.0 ± 0.2	130.5 ± 6.2	0.170 ± 0.024	-9.8 ± 1.7
n-Hexane/DCM (75/25)	158.7 ± 7.2	0.248 ± 0.052	-2.2 ± 1.0	138.0 ± 1.6	0.117 ± 0.004	-11.0 ± 0.6

After preparation of the $W_1/O/W_2$ emulsions, the organic solvents were evaporated by continuous stirring for 24 h under atmospheric pressure and room temperature. The obtained lipid vesicles were then extruded and dialyzed. Physicochemical characteristics of the crude liposomes and the extruded/dialyzed ones were determined. Data are represented as the mean ± SD (n=3).

Table 3. Physicochemical properties of the CytC- or sulforhodamine B-loaded PTNP prepared by the MCV method using *n*-Hexane / DCM (75 / 25) as an oil phase

Parameters	CytC-loaded PTNP	sulforhodamine B-loaded PTNP
Average particle diameter (nm)	141.7 ± 23.9	124.1 ± 1.2
PdI	0.238 ± 0.131	0.277 ± 0.005
ζ-potential (mV)	1.9 ± 0.3	3.6 ± 2.5
% encapsulation of CytC	50.4 ± 9.8	20.7 ± 1.6

The physicochemical properties of CytC- or sulforhodamine B-loaded PTNP, prepared via the MCV method using the mixed organic solvent (*n*-hexane/DCM = 75/25), were measured. Data are represented as the mean ± SD (n=3).

Figure legends

Figure 1. Schematic diagram of the preparation of surface-modified liposomes via the MCV method

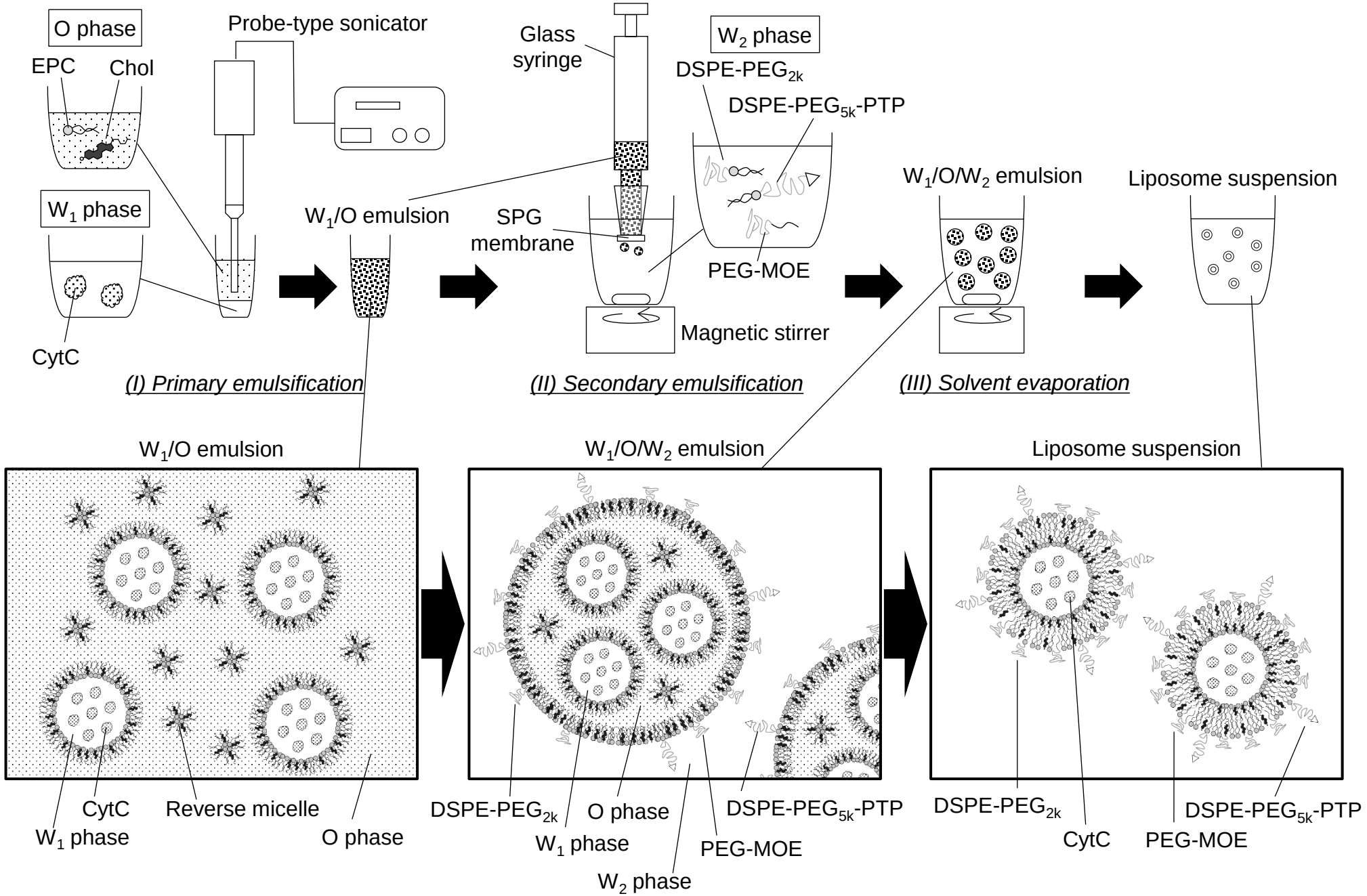
The MCV method for liposome preparation consists of the following steps: (I) dispersion of the internal water (W_1) phase containing CytC to be encapsulated into an organic solvent containing liposome-consisting lipids, *i.e.* EPC and Chol, to yield an W_1/O emulsion; (II) dispersion of the W_1/O emulsion into the outer aqueous (W_2) phase containing PEG- and ligand-modified lipids as external emulsifiers to prepare the $W_1/O/W_2$ emulsion; (III) evaporation of the organic solvent from the O phase to form lipid bilayers. The PEG- and ligand-modified lipids used to stabilize the W/O/W emulsion in the secondary emulsification step would be retained on the surface of liposomes even after solvent evaporation.

Figure 2. Encapsulation ratio of CytC in PEGylated liposomes prepared by the MCV method using various mixtures of organic solvents

Encapsulation ratio of CytC into the PEGylated liposomes was calculated as the ratio of encapsulated CytC to the total amount of initial CytC. Data are represented as the mean ± SD (n=3). *$p<0.05$, **$p<0.01$, one-way ANOVA, followed by Tukey tests.

Figure 3. In vivo targeted delivery of PTNP to adipose vasculature prepared by the MCV method

The sulforhodamine B-loaded PTNP and PEGylated liposomes (red) prepared by the MCV method were intravenously injected into C57BL/6J mice at a lipid dose of 0.125 mmol/kg. To visualize blood vessels, FITC-GSIB4 (green) was also intravenously administered at 30 min prior to tissue collection (50 μg/mice). At 24 h after administration of PTNP or PEGylated liposomes, the inguinal adipose tissue was removed from each mouse, fluorescently stained with Hoechst33342, and then observed by a CLSM. Bars represent 100 μm.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W1 phase
W1/O emulsion
SPG membrane
PEG-MOE
W1/O2 emulsion
Liposome suspension
CytC
Magnetic stirrer
(I) Primary emulsification
(II) Secondary emulsification
(III) Solvent evaporation
W1/O emulsion
W1/O/W2 emulsion
Liposome suspension
CytC
Reverse micelle
W1 phase
O phase
DSPE-PEG2k
O phase
W1 phase
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CytC
PEG-MOE
DSPE-PEG5k-PTP

Fig.1

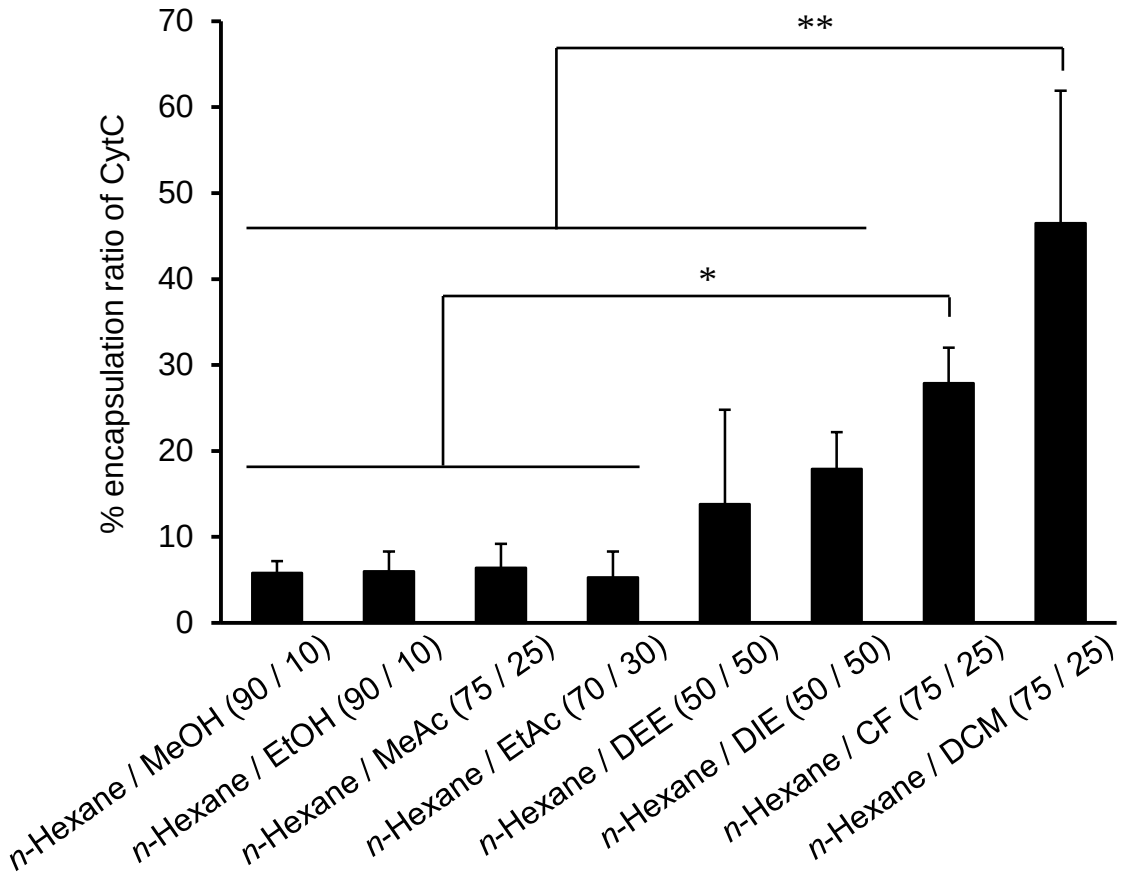
% encapsulation ratio of CytC
70
60
50
40
30
20
10
0
**
*
n-Hexane / MeOH (90 / 10)
n-Hexane / EtOH (90 / 10)
n-Hexane / MeAc (75 / 25)
n-Hexane / EtAc (70 / 30)
n-Hexane / DEE (50 / 50)
n-Hexane / DIE (50 / 50)
n-Hexane / CF (75 / 25)
n-Hexane / DCM (75 / 25)

Fig.2

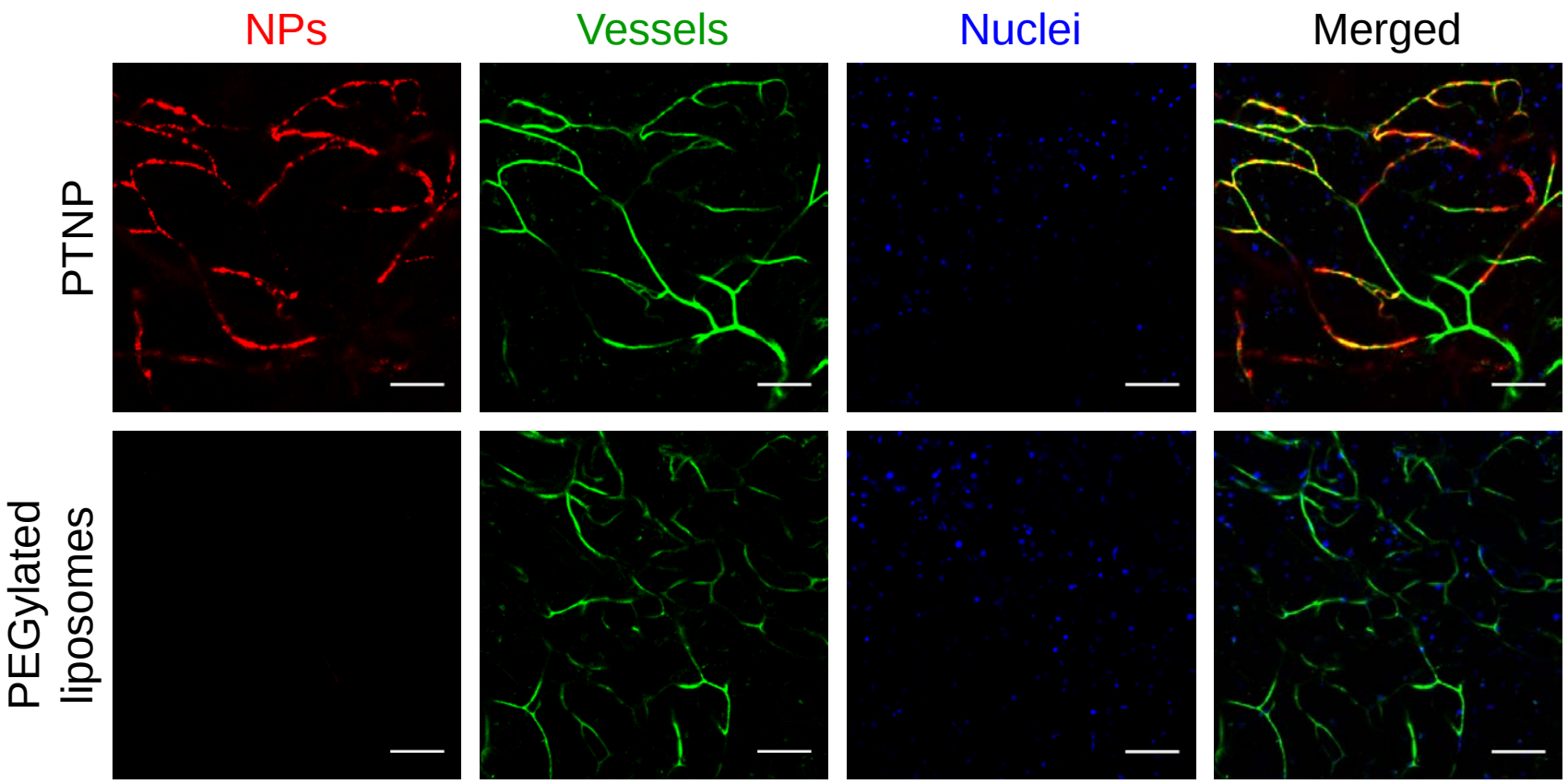

Fig.3