JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

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Influence of the Preparation Route on the Supramolecular Organization of Lipids in a Vesicular System

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Supporting Information

ABSTRACT: A confocal fluorescence microscopy-based assay was used for studying the influence of the preparation route on the supramolecular organization of lipids in a vesicular system. In this work, vesicles composed of cholesterol and CTAB (1/1 mol %) or cholesterol and DOPC (2/8 mol %) and incorporating two membrane dyes were prepared by either a compressed fluid (CF)-based method (DELOS-susp) or a conventional film hydration procedure. They were subsequently immobilized and imaged individually using a confocal fluorescence microscope. Two integrated fluorescence intensities, I_{dye1} and I_{dye2}, were assigned to each tracked vesicle, and their ratio, I_{dye1}/I_{dye2}, was used for quantifying the degree of membrane inhomogeneity between individual vesicles within each sample. A distribution of I_{dye1}/I_{dye2} values was obtained for all the studied vesicular systems, indicating intrasample heterogeneity. The degree of inhomogeneity (*DI*) was similar for Chol/DOPC vesicles prepared by both procedures. In contrast, *DI* was more than double for the hydration method compared to the CF-based method in the case of Chol/CTAB vesicles, which can suffer from lipid demixing during film formation. These findings reveal a more homogeneous vesicle formation path by CFs, which warranted good homogeneity of the vesicular system, independently of the lipid mixture used.

Vesicles, especially liposomes, are one of the most studied self-assembled structures due to their diverse and important applications, which among others cover their use as cell membrane models,[1] reaction vessels,[2] and drug delivery systems (DDSs).[3] Despite their versatility, a high degree of structural homogeneity is crucial for optimal performance of vesicles as functional materials. Thus, the formation stage of these supramolecular entities must be tightly controlled to achieve a homogeneous self-assembling of the lipids constituting the vesicular membrane.[4] In this sense, compressed fluids (CFs) like compressed CO_2 have a great deal of promise as solvent media for material processing, since their unique characteristics, between those of liquid and gases, allow the achievement of materials presenting highly homogeneous structural characteristics at the macro-, micro, and supramolecular level.[5] For instance, depressurization of an expanded liquid organic solution (DELOS),[6] a CF-based process, has been demonstrated to provide crystalline solids with high polymorphic purity.[7] In contrast to solid crystalline materials and probably due to the lack of appropriate technical tools to do so, the capacity of CFs for providing superior supramolecular homogeneity remains practically unexplored for noncrystalline ordered materials like vesicles, which can be produced in one step by these easy scalable procedures.[8]

Recently, technical advances have allowed the monitoring of single vesicles within a population[2b,9] and have provided unique information on heterogeneous properties that were otherwise obviated due to ensemble averaging. Particularly, a recent confocal fluorescence microscopy study on the lipid composition of single vesicles has revealed intrasample compositional variations.[10] Using this methodology, in the present work we have studied the impact of the preparation route on the supramolecular organization of the lipids forming a vesicular system. We prepared vesicles with two lipid compositions by the CF-based one-step procedure or a multistep conventional film hydration technique. The first composition consisted of 1/1 mol % of cholesterol (Chol) and cationic surfactant CTAB, which can suffer phase separation, and the second one was a fully miscible mixture consisting of 2/8 mol % of Chol and DOPC. The degree of membrane inhomogeneity between individual vesicles within each sample and the supramolecular arrangement of the lipids was then characterized and compared. Our findings indicate a strong preparation route dependence on the compositional inhomogeneity and supramolecular organization for lipid mixtures that phase separate.

Vesicles composed by Cholesterol/CTAB/DSPE-PEG_{2000}-biotin/DiD-oil/NBD-6-cholesterol with 49.35/49.6/0.3/0.5/0.25 molar proportions were prepared by a DELOS-based method for vesicle production, named DELOS-susp,[8a] and a commonly used film hydration method[11](see Supporting Information (SI)). CryoTEM microscopy revealed that vesicles prepared by a multistep hydration procedure were a mixture between unilamellar, multilamellar, and multivesicular vesicles, whereas unilamellar structures were achieved by DELOS-susp. Size characterization by Nanoparticle Tracking Analysis (NTA)

Received: September 19, 2011
Published: December 28, 2011

showed that DELOS-susp procedure yields vesicles with an average diameter of 154 nm and with a narrower particle size distribution than those produced by hydration, which have an average diameter of 197 nm (see SI). Once prepared, the vesicles were located onto a glass surface which had been sequentially functionalized with PLL-PEG-biotin and avidin (Figure 1A).[12] The immobilization was accomplished through

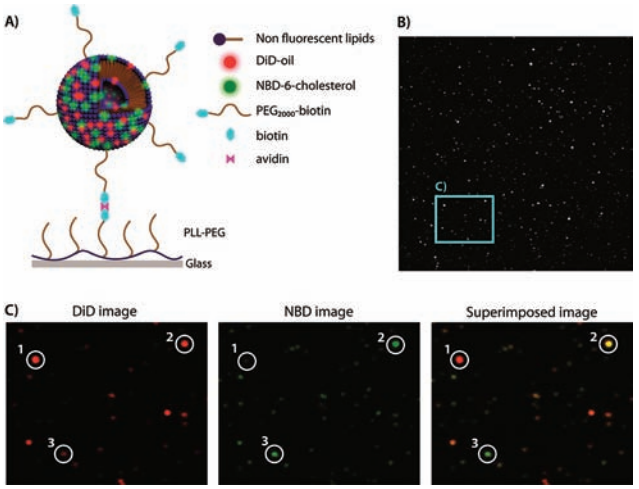

Figure 1. (A) Schematic illustration of one of the vesicles under study and its anchoring to a glass surface through a biotin−avidin union. (B) Typical fluorescence confocal microscopy image used for the membrane homogeneity studies (51.67 μm × 51.67 μm). (C) Zoom of image shown in (B) obtained for both membrane dyes at different channels and the corresponding superimposition. Vesicles 1, 2, and 3 have different I_{NBD}/I_{DiD} values indicating intrasample heterogeneity.

the avidin/biotin par thanks to the anchoring lipid (DSPE-PEG_{2000}-biotin) inserted in the membrane of the vesicles (see SI).

The amphiphilic DiD-oil (DiD) dye with high lipophilic character and the fluorescent cholesterol analogue, NBD-6-cholesterol (NBD), were used as molecular probes for studying variations in the membrane supramolecular structure of individual vesicles within a population. Sequential imaging of both dyes in different zones of the sample in combination with the use of particle tracking algorithms enabled the analysis of $\sim 10^3$ vesicles in a high-throughput manner (Figure 1B). Each vesicle was localized in both the DiD and the NBD channel and assigned corresponding integrated intensity values, I_{DiD} and I_{NBD}. The ratio of these two integrated intensities, I_{NBD}/I_{DiD}, was calculated for each vesicle and used to compare the membrane organization of individual vesicles within the ensemble. This ratio should be constant if all the individual vesicles had the same membrane composition and supramolecular organization. However, in agreement with a recently published study on a different vesicular system by Larsen et al.,[10] a distribution of I_{NBD}/I_{DiD} values was found for all the vesicular populations presented here (Figure 1C, Figure 2). This intrasample heterogeneity, expressed as the *Degree of Inhomogeneity (DI)*, was quantified dividing the standard deviation by the Mean of the Gaussian function fitted to the histogram of I_{NBD}/I_{DiD} values (see SI). As observed in Figure 2 and Table 1, the *DI* found for the Chol/CTAB system prepared by the multistep hydration method was more than double than that of the samples obtained by the one-step CF-based method (*DIs* of 1.08 ± 0.12 and 0.46 ± 0.04 for hydration method and

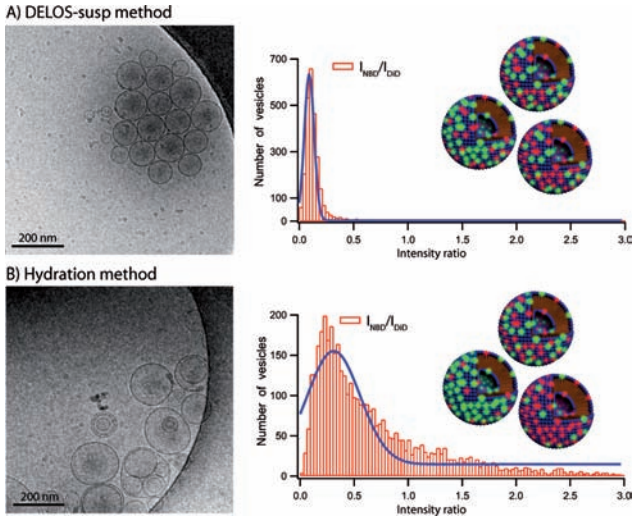

Figure 2. Cryo-TEM images and I_{NBD}/I_{DiD} histograms of Chol/CTAB (1/1 molar %) vesicular samples prepared by DELOS-susp (A) and the hydration method (B). A schematic representation of vesicles with different heterogeneities is also depicted.

Table 1. Degree of Inhomogeneity (DI), Mean, and % FRET Values Obtained by Confocal Fluorescence Microscopy for Chol/CTAB (1/1 molar %) Vesicular Systems Prepared by the DELOS-susp and the Hydration Methods

	Method	
	DELOS-susp[a]	Hydration[b]
DI	0.46 ± 0.04	1.08 ± 0.12
Mean	0.10 ± 0.01	0.33 ± 0.04
% FRET	43 ± 3	20.5 ± 0.7

[a]These results are the average between three samples prepared in different DELOS-susp experiments. [b]These results are the average between two samples prepared in different hydration experiments.

DELOS-susp, respectively). According to these values, DELOS-susp provides a more homogeneous path for the assembling of the lipids, leading to less disperse vesicular systems. This highlights the impact that the preparation route exerts not only on the particle size distribution and morphological uniformity of a vesicular formulation (cryoTEM images of Figure 2) but also in the vesicle to vesicle homogeneity regarding the supramolecular organization of the lipids in their membrane.

The greater heterogeneity encountered for vesicles prepared by hydration could be explained by lipid demixing during film formation. As reported by Buboltz et al.,[13] those preparation methods involving a solvent-free state, such as a lipid film, may favor demixing of membrane components and therefore an heterogeneous formation of the individual vesicles in an ensemble. In the case of Chol/CTAB (1/1 molar %) mixtures lipid demixing and formation of cholesterol-rich domains are probably promoted during film formation, explaining the large degree of compositional inhomogenity (*DI* = 1.08) achieved by the conventional hydration preparation route. In contrast, vesicles of the same composition prepared by DELOS-susp, which avoids any intermediary solvent-free state, showed higher compositional homogeneity.

To confirm this hypothesis, a fully miscible lipid mixture, Chol/DOPC (2/8 molar %),[14] was chosen to prepare vesicles by hydration and DELOS-susp procedures. Using the same dyes as molecular probes, the compositional homogeneity of

dx.doi.org/10.1021/ja2086678 | J. Am. Chem.Soc. 2012, 134, 1918−1921

samples, prepared by both methods, was analyzed following the previously explained procedure (see SI). After examination of two different samples for each preparation method, identical *DI* values were encountered for both preparation routes (*DI*s of 0.30 ± 0.04 and 0.30 ± 0.01 for DELOS-susp and hydration respectively). Whereas by DELOS-susp similar *DI* values were recorded for both lipid mixtures, a dramatic decrease (1.08 in Chol/CTAB vs 0.30 in Chol/DOPC) was found for the hydration method. Though more experiments may be required to fully elucidate the mechanistic explanation of these results, our findings generally support that the compositional heterogeneity for lipid compositions that can suffer phase separation may be amplified by preparation routes involving an intermediate solvent-free state such as in the case of film hydration methods. Since many lipid mixtures may suffer from demixing, methods like DELOS-susp that maintains lipids in solution during vesicle preparation would be a safer choice for achieving systems with superior homogeneity.

The confocal microscopy assay also provided interesting information concerning the supramolecular arrangement of the lipids in the bilayer. Despite the intrasample heterogeneities, the *Mean* I_{NBD}/I_{DiD} value is expected to be the same for two vesicular systems with the same bulk composition. However, whereas in the case of Chol/DOPC vesicles these values are equal within uncertainty by both methods (0.58 ± 0.04 and 0.60 ± 0.07 for DELOS-susp and hydration, respectively), Chol/CTAB vesicles prepared by DELOS-susp had a lower *Mean* I_{NBD}/I_{DiD} value than those prepared by the hydration method (0.10 ± 0.01 and 0.33 ± 0.04 for DELOS-susp and hydration methods, respectively). Since bulk fluorescence measurements of Chol/CTAB broken vesicles confirmed that vesicular systems had incorporated the same quantity of dyes independently of the method used to prepare them (see SI), the differences observed in the fluorescence of the single vesicles could be attributed to a distinct arrangement of the dyes in the membrane. Fluorescence resonance energy transfer (FRET) between NBD and DiD was used to study this hypothesis. This phenomenon, by which the donor (NBD) transfers part of the absorbed energy to the acceptor (DiD) for its emission as fluorescence, depends on the distance between the dyes and thus provides insights to the arrangement of the lipids in the vesicles prepared by the different methods. To perform this study FRET images (emission of DiD when exciting NBD) were taken together with images of the two dyes for each analyzed zone of the samples. Thus, apart from I_{NBD} and I_{DiD} values, I_{FRET} values were also assigned to each individual vesicle. The *% FRET* value yielded by each vesicle was calculated through eq 1,

$$FRET(\%) = \frac{I_{FRET}}{I_{NBD} + I_{FRET}} \times 100 \quad (1)$$

For comparison purposes, the mean value of the Gaussian curve fitted to the histogram of the *% FRET* values obtained for each vesicle was used (Table 1). The *% FRET* observed for Chol/CTAB samples prepared using compressed fluids (43 ± 3) was twice that found for the samples prepared by hydration (20.5 ± 0.7), which indicates a closer arrangement of the dyes that promotes a more efficient energy transfer.

The recorded decreased *% FRET* in the hydration samples is in agreement with our hypothesis of increased formation of cholesterol-rich domains by this method. Clustering of cholesterol in these domains would result in reduced interactions between NBD and DiD and thus in reduced *% FRET*. The reduced energy transfer in turn would increase the I_{NBD} values explaining the increased *Mean* I_{NBD}/I_{DiD} values encountered for the vesicles prepared by film hydration (see Table 1). For the fully miscible system of Chol/DOPC (2/8 mol %) we recorded identical *% FRET* values for both preparation routes, further supporting our hypothesis (see SI).

Monitoring single vesicles showed that the preparation method greatly influences the degree of membrane homogeneity between these lipid self-assembled entities. This information would normally be inaccessible in bulk experiments due to the ensemble averaging effect. Knowledge and precise control of the supramolecular organization of vesicles are instrumental in their use as functional materials. The findings of this work reinforced the idea of membrane heterogeneity between individual vesicles within the same ensemble, demonstrated the influence of the preparation route on the assembly of lipids as vesicles, and showed the potential of CF-based methods for providing more homogeneous noncrystalline ordered materials.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials, vesicle preparation and characterization methods, experimental procedures for fluorescence microscopy images collection and data treatment. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENTS

We would like to thank Andreas Kunding for his valuable help in data treatment. This work was supported by the "Plan Nacional de Investigación Científica y Desarrollo e Innovación Tecnológica", Iniciativa Ingenio 2010, Programa *Consolider*, under project EMOCIONA (CTQ2006-06333/BQU). We acknowledge financial support from Instituto de Salud Carlos III, through "Acciones CIBER". The Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN) is an initiative funded by the VI National R&D&I Plan 2008-2011, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions and financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund. The authors appreciate the financial support through the project "Development of nanomedicines for enzymatic replacement therapy in Fabry disease" granted by the Fundació Marató TV3. The authors wish to thank Pablo Castro for technical support in taking the cryo-TEM images. E.E. thanks CSIC for her PhD bursary. N.V. thanks Banco de Santander for the Chair of Knowledge and Technology Transfer Parc de Recerca UAB-Santander. D.S. acknowledges financial support from the Danish Councils for Independent and Strategic Research, the Lundbeck Foundation, the center of excellence CPNN, and the University of Copenhagen programs of excellence "Single molecule Nanoscience" and "UNIK - Synthetic Biology".

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