Subscriber access provided by ISTANBUL TEKNIK UNIV

JACS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

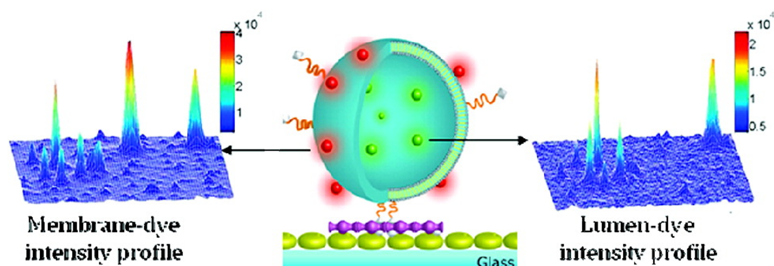
Communication

Encapsulation Efficiency Measured on Single Small Unilamellar Vesicles

Brian Lohse, Pierre-Yves Bolinger, and Dimitrios Stamou

J. Am. Chem. Soc., **2008**, 130 (44), 14372-14373 • DOI: 10.1021/ja805030w • Publication Date (Web): 09 October 2008

Downloaded from http://pubs.acs.org on February 9, 2009



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

ACS Publications High quality. High impact.

Journal of the American Chemical Society is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

J|A|C|S
COMMUNICATIONS

Published on Web 10/09/2008

Encapsulation Efficiency Measured on Single Small Unilamellar Vesicles

Brian Lohse, Pierre-Yves Bolinger, and Dimitrios Stamou*

Bio−Nanotechnology Laboratory, Department of Neuroscience and Pharmacology & Nano−Science Center, University of Copenhagen, Universitetsparken 5, 2100 Copenhagen, Denmark

Received July 1, 2008; E-mail: Stamou@nano.ku.dk

During the past two decades liposomes[1] have been studied intensively for their use as biocompatible drug delivery systems (DDS)[2] and transfection agents.[3] One of the critical parameters that have to be optimized in the development of a delivery system is the encapsulation efficiency (EE, ratio of encapsulant concentration inside the delivery vehicle over the loading solution).[4] So far EE has been regarded as a bulk property and was measured as the average of all DDS particles in a solution. Recent technical advances however[5] allowed the monitoring of single particles[4a,6] and are beginning to provide unique information on heterogeneous properties that were otherwise lost due to ensemble averaging. Here we focus on measuring the EE of *single* vesicles with sizes below the optical resolution. The measurements revealed an intrinsic intrasample EE variability that may be a property of other delivery formulations as well.

In order to be able to quantify the EE of lipid vesicles with single-vesicle resolution and to study changes in single vesicles over long periods (minutes to days) we immobilized vesicles on BSA passivated glass substrates (see Figure 1A). The vesicle composition was DOPC/DOPG/DSPE-PEG_{2000}-biotin/C_{18}-DiD (93.2:6:0.3:0.5 mol %). The vesicles were prepared using a standard rehydration procedure[7] and were loaded with CoroNa Green (M = 585.55 g/mol); see SI. They were then added onto surfaces functionalized sequentially with BSA-biotin and streptavidin. We used the well-established coupling method of biotin-streptavidin as it has been shown to maintain immobilized vesicles tight against concentration gradients of fluorophores and ions.[8]

Vesicles were labeled by a fluorophore in the membrane and a water soluble fluorophore in the lumen, Figure 1A. After immobilization, by washing the surface with a buffer of the same osmolarity we removed the nonencapsulated dye allowing for maximum contrast in fluorescent microscopy, Figure 1B (membrane dye) and 1C (lumen dye). Washing *in situ* circumvented the need for separation of the nonencapsulated material in an additional step, e.g., chromatography.[9]

Images similar to the ones shown in Figure 1B, C typically contained a few hundred vesicles and were analyzed using particle tracking algorithms. Each vesicle was localized in both channels and assigned two integrated intensity values. Since the vesicles are smaller than the optical resolution, their size cannot be measured directly. However the intensity of the membrane bound dye is proportional to the square of the diameter. We used the total fluorescent intensity of a vesicle to quantify its size, as recently described by Kunding et al.[6b] Knowing the size and therefore the volume of the vesicle allowed us to convert the total number of encapsulated dyes (calculated from the measured intensity in the lumen channel) to an absolute concentration for individual vesicles; see SI.

The presence of different vesicle sizes on the surface allowed us to record EE as a function of size for single vesicles. The results are plotted in Figure 2A and reveal a marked tendency for larger

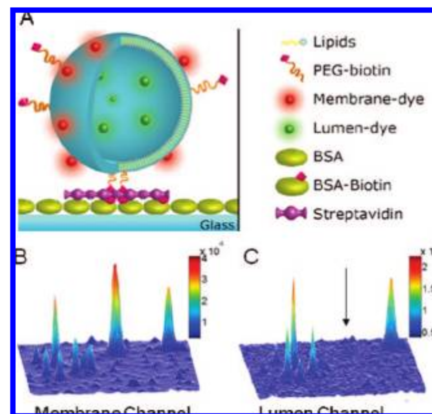


Figure 1. EE measurements on single vesicles. (A) Schematic illustration of EE measurements on single vesicles. Vesicles were randomly immobilized at a dilute density on glass substrates through streptavidin−biotin interactions as shown. They contained a lipid-dye (red) in the bilayer and a water soluble dye in the lumen (green). Double labeling allowed us to measure quantitatively the concentration of encapsulant in vesicles of different sizes. (B, C) Fluorescence microscopy images of single immobilized DOPC vesicles; see SI. B and C are the membrane (C_{18}-DiD) and lumen (CoroNa Green) channel, respectively. The arrow in C indicates the location of an empty vesicle (see text). The largest peak in B corresponds to a vesicle with a diameter of 380 nm as calculated from the measured fluorescence intensity (see SI).

vesicles to encapsulate lower concentrations of water soluble dye. EE decreases from ∼100% to a plateau value of ∼15% as a function of vesicle diameter (d). The size dependency of EE is inversely proportional ($1/d$) to the SUVs diameter, suggesting that the mechanism behind this observation is related to the surface-to-volume ratio of vesicles. Similar results were obtained for a few other encapsulants (carboxyfluorescein, Alexa 488 hydrazide) and lipids (POPC and DPPC); data not shown.

Next we carried out a number of control experiments to check for multilamellarity in our samples. Multilamellar vesicles (MLVs) could contribute to a change in the apparent encapsulant concentration in a vesicle. An MLV in our assay would appear as a single vesicle of larger diameter (higher lipid mass) and lower EE. Furthermore multilamellarity could potentially exhibit a size dependency. In good agreement with previous results,[6b] CryoTEM revealed that only a very small fraction of all vesicles (≤6%) contained 1−2 lamellas; see Figure S1. Furthermore we incubated the biotinylated vesicles with streptavidin. The streptavidin signal allowed us to quantify separately the amount of external lipid mass and relate it to the signal from the membrane dye in the vesicles. The ratio of the two signals was constant for all vesicle sizes demonstrating the absence of inner lamellas to any significant extent; see Figure S2.

To exclude the possibility that the observation is an artifact of the single particle image analysis we looked at a control sample,

10.1021/ja805030w CCC: $40.75
© 2008 American Chemical Society

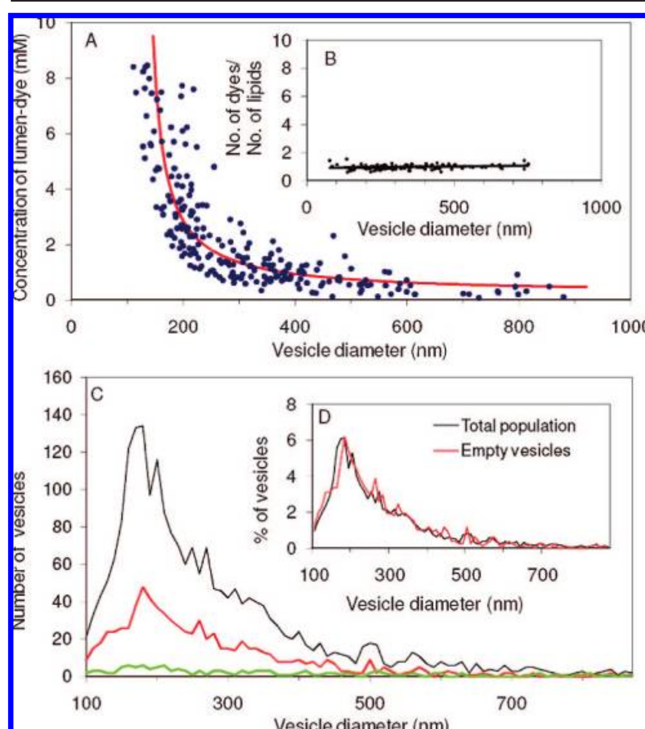


Figure 2. EE measurements as a function of vesicle size. (A) Concentration of lumen-dye as a function of diameter; each point represents a single vesicle. The red line is a $1/d$ fit to the data. Vesicles were freeze−thawed 15 times. (B) Control showing the ratio of two lipid dyes (DHPE and C_{18}−CF) versus diameter. (C) Number of loaded and empty vesicles as a function of vesicle diameter (black line) and number of empty vesicles after 1 and 15 freeze−thaw and extrusion cycles (red line and green line, respectively). (D) Normalized histograms of vesicle sizes for the total (loaded and empty) and empty vesicle population, respectively.

vesicles carrying two fluorophores (DHPE-Texas Red and C_{18}-CF) in their membrane, and performed the same data treatment. A plot of the number of dyes per number of lipids as a function of size in Figure 2B shows a constant relationship with a slope close to zero, ruling out any artifacts due to image processing. The significant standard deviation from the fit (red line) in Figure 2A is higher than that in Figure 2B by a factor of ∼8. This indicates that superimposed to the $1/d$ tendency and the noise there is an additional intrinsic vesicle-to-vesicle variability in EE for vesicles having similar diameters.

During data treatment we surprisingly found a large number of empty vesicles (signal below the detection limit); see arrow in Figure 1C. The distribution of empty vesicles within the total population of vesicles is shown as a function of size in Figure 2C. Normalization of the two populations (Figure 2D) reveals that there is no preference for empty vesicles to exist at a certain size.

To exclude that the empty vesicles resulted from fusion of SUVs on the glass surface we used procedures (extrusion and freeze−thawing) that are documented to change EE[10] and tried to establish a systematic relation between sample treatment in bulk and our observations on immobilized SUVs. Indeed we observed that the number of empty vesicles can be reduced from ∼50% for nontreated samples to ∼35% and ∼5% for samples that were freeze−thawed and extruded 1 and 15 times, respectively; see Figure 2C. These results demonstrate that empty vesicles exist in substantial numbers in rehydrated samples and are not the result of fusion upon immobilization. They also highlight that freeze−thawing is partly increasing the average EE as measured in bulk assays by simply changing the ratio of "empty" to "loaded" vesicles. We also observed that freeze−thawing increased the actual EE of loaded vesicles but mostly for diameters greater than 400 nm (see Figure S3). The size dependent EE persisted even after 15 freeze−thawing cycles, Figure 2A.

In this communication we show how the encapsulation of a fluorescent dye can be correlated with the fluorescence of a lipid membrane dye to quantitatively determine the EE of single vesicles with diameters down to 100 nm. This new method revealed two important aspects of EE that are averaged out in bulk measurements: (i) the existence of empty vesicles and (ii) an inverse relation between EE and vesicle diameter. The molecular mechanism behind these two observations is not clear at this stage though we speculate it is related to the process of vesicle formation during dry-lipid-film rehydration (the most commonly used vesicle preparation technique).[1,2,4b,7,10,11] Further studies are needed to establish if these results can be generalized for other preparation techniques and delivery systems. Understanding EE on the nanoscale opens up new routes for the attempt to optimize it, a topic which is of broad relevance for drug delivery, and related disciplines like medicine and biotechnology. These results are also particularly relevant for the growing number of studies that manipulate and observe single vesicles.[4a,6,11]

Acknowledgment. The authors wish to thank Dr. N. Hatzakis, Dr. P. M. Bendix, and A. Kunding for valuable discussions and help. This work was supported by the Danish Councils for Scientific and Strategic Research and partly by the European Union FP6-2004-IST-4 STREP program NEMOSLAB.

Supporting Information Available: Materials and methods, control experiments, and cryoTEM images. This information is available free of charge via the Internet at http://pubs.acs.org/.

References

(1) (a) Bangham, A. D.; Standish, M. M.; Miller, N. *Nature* **1965**, *208*, 1295–1297. (b) Bangham, A. D. *Annu. Rev. Biochem.* **1972**, *41*, 753–776.
(2) (a) Lasic, D. D.; Needham, D. *Chem. Rev.* **1995**, *95*, 2601–2628. (b) Gregoriadis, G.; Florence, A. T. *Drugs* **1993**, *45*, 15–28. (c) Torchilin, V. P. *Nat. Rev. Drug. Discov.* **2005**, *4*, 145–160.
(3) (a) Kogure, K.; Akita, H.; Yamada, Y.; Harashima, H. *Adv. Drug Delivery Rev.* **2008**, *60*, 559–571. (b) Torchilin, V. P.; Levchenko, T. S.; Rammohan, R.; Volodina, N.; Papahadjopoulos-Sternberg, B.; D'Souza, G. G. M. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (4), 1972–1977.
(4) (a) Sun, B.; Chiu, D. T. *Anal. Chem.* **2005**, *77*, 2770–2776. (b) Edwards, K. A.; Baeumner, A. J. *Talanta* **2006**, *68*, 1432–1441.
(5) (a) Christensen, S. M.; Stamou, D. *Soft Matter* **2007**, *3* (7), 828–836. (b) Bolinger, P.-Y.; Stamou, D.; Vogel, H. *J. Am. Chem. Soc.* **2004**, *126*, 8594–8595. (c) Rhoades, E.; Gussakovsky, E.; Haran, G. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3197–3202.
(6) (a) Kuyper, C. L.; Kuo, J. S.; Mutch, S. A.; Chiu, D. T. *J. Am. Chem. Soc.* **2006**, *128*, 3233–3240. (b) Kunding, A. H.; Mortensen, M. W.; Christensen, S. M.; Stamou, D. *Biophys. J.* **2008**, *95*, 1176–1188.
(7) Olson, F.; Hunt, C. A.; Szoka, F. C.; Vail, W. J.; Papahadjopoulos, D. *Biochim. Biophys. Acta* **1979**, *557*, 9–23.
(8) Stamou, D.; Duschl, C.; Delamarche, E.; Vogel, H. *Angew. Chem., Int. Ed.* **2003**, *42*, 5580–5583.
(9) Lesieur, S.; Grabielle-Madelmont, C.; Paternostre, M.; Ollivon, M. *Chem. Phys. Lipids* **1993**, *64*, 57–82.
(10) Colletier, J.-P.; Chaize, B.; Winterhalter, M.; Fournier, D. *BMC Biotechnol.* **2002**, *2* (9), 1–8.
(11) (a) Cisse, I; Okumus, B.; Joo, Chirlmin; Ha, T. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (31), 12646–12650. (b) Chan, Y. H. M.; Lenz, P.; Boxer, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (48), 18913–18918. (c) Liu, T.; Tucker, W. C.; Bhalla, A.; Chapman, E. R.; Weisshaar, J. C. *Biophys. J.* **2005**, *89*, 2458–2472.

JA805030W