J. Biophotonics **5**, No. 1, 40–46 (2012) / **DOI** 10.1002/jbio.201100058

Journal of BIOPHOTONICS

FULL ARTICLE

Membrane composition of jetted lipid vesicles: a Raman spectroscopy study

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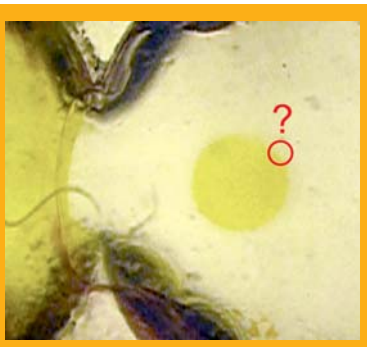
Received 21 June 2011, revised 2 August 2011, accepted 7 September 2011
Published online 5 October 2011

Key words: phospholipid vesicles, microfluidic jetting, biological membranes, membrane composition, Raman scattering

Microfluidic jetting is a promising method to produce giant unilamellar phospholipid vesicles for mimicking living cells in biomedical studies. We have investigated the chemical composition of membranes of vesicles prepared using this approach by means of Raman scattering spectroscopy. The membranes of all jetted vesicles are found to contain residuals of the organic solvent decane used in the preparation of the initial planar membrane. The decane inclusions are randomly distributed over the vesicle surface area and vary in thickness from a few to several tens of nanometers. Our findings point out that the membrane properties of jetted vesicles may differ considerably from those of vesicles prepared by other methods and from those of living cells.



A jetted phospholipid vesicle filled with fluorescein solution.

1. Introduction

Phospholipid vesicles are widely used to mimic the complex biological membrane of living cells in biological and medical studies [1]. To be used as model cells, these vesicles must fulfill certain requirements and therefore the preparation methods must be able to control (i) membrane unilamellarity and composition, (ii) vesicle size and (iii) encapsulation of arbitrary chemicals or biomolecules inside the vesicles. Furthermore, the possibility to incorporate functional biomolecules into the membrane (e.g. transmembrane proteins) is required. Most of the standard vesicle preparation protocols fulfill only some of these requirements and thus do not provide the desired flexibility for tailoring the vesicles' properties [2]. For example, hydration of phospholipids from a solid surface results in the growth of predominantly multilamellar vesicles with a broad size distribution [3]. The hydration under an externally applied electric field, so called electroformation [4], can be used to produce unilamellar vesicles, but it is difficult to control vesicle size with the electroformation method. The size uniformity can be improved significantly by extruding vesicles through a filter with pores a few hundred nanometers in size. However, this limits the vesicle diameter to the pore size and encapsulation of molecules is not possible [5].

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To fulfill all of the conditions required to mimic living cells with artificially prepared phospholipid vesicles, an elegant microfluidic technique for the preparation of giant vesicles has recently been proposed [6]. Similar to blowing soap bubbles from a soap film, the method is based on the microfluidic jetting of an arbitrary aqueous solution onto a planar lipid bilayer. The lipid bilayer, stabilized by an organic solvent (e.g. decane or chloroform), is initially formed between two water droplets in a double well chamber [7]. The bilayer represents a material source for the membrane of jetted vesicles. The microfluidic jetting approach has attracted increasing attention because of its versatility and high degree of control over the vesicle properties. The vesicle size can be controlled precisely by tuning the jet parameters [8]. The composition of the encapsulated solution and of the vesicle membrane can be adjusted too [9]. Although the jetting process is well understood and a number of important proof-of-concept experiments have been reported, the actual composition of the membrane of jetted vesicles has not yet been examined.

Here, we report on the systematic study of the membrane composition of giant phospholipid vesicles prepared by using the jetting method, using a combination of optical dark-field microscopy and Raman micro-spectroscopy. Most of the jetted vesicles appear perfectly unilamellar when imaged in an optical microscope, without a sign of membrane thickening, Raman scattering spectroscopy however reveals a considerable amount of residuals of the organic solvent *n*-decane, a vital component in the jetting process, providing membrane stability. It was found that the *n*-decane inclusions are distributed randomly over the surface area of the vesicles and vary considerably in thickness.

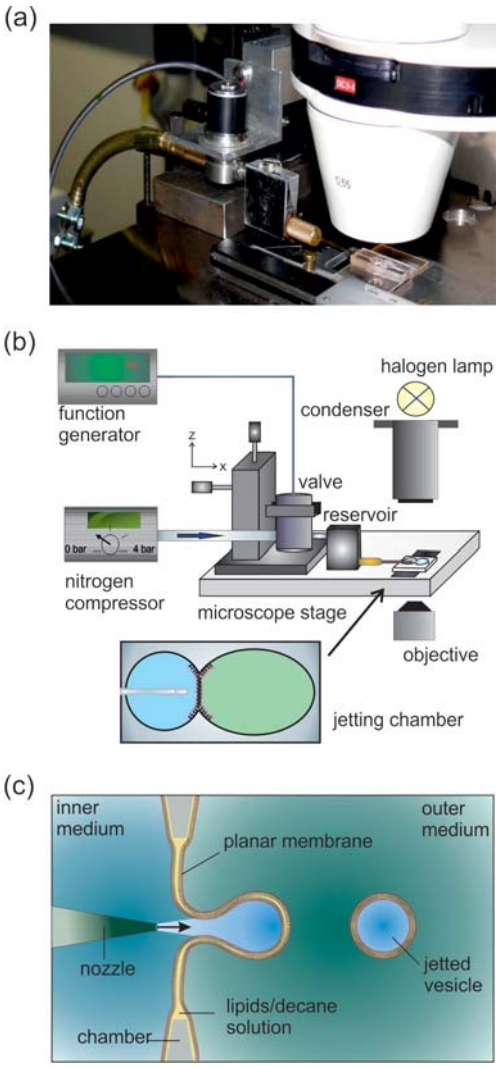


Figure 1 (online color at: www.biophotonics-journal.org) Microfluidic jetting. (**a**) Photograph and (**b**) schematic of the experimental setup used for microfluidic jetting. (**c**) Sketch of the jetting process.

2. Experimental

Figure 1 illustrates the experimental setup and the principle of the microfluidic jetting method adopted with slight modifications from [6]. In short, a planar lipid membrane is formed between two tips of an eight-shaped microfluidic chamber and separates two droplets of aqueous solutions. A pulsed fluidic jet flow is directed from a reservoir through a micropipette onto the planar membrane. The vesicle is formed by the jet and detaches from the membrane which then recovers its original planar form. Thus the jetting process can be repeated, enabling a high-throughput vesicle production by a sequence of jet pulses. The vesicle itself is loaded with a mixture of the jetted reservoir liquid and the solution from the left half of the chamber.

2.1 Planar membrane formation

To prepare the lipid bilayer-forming solution, a solution of 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC) in chloroform (20 mg/ml) from Avanti Polar Lipids Inc. (# 850375) was dried under a nitrogen flow, leaving a dry lipid film on the bottle walls. After drying, the lipids were re-dissolved in *n*-Decane (anhydrous (>99%), Sigma Aldrich) to a concentration of 25 mg/ml. The hydrophobic solvent *n*-Decane serves as a physical stabilizer for the energetically unfavorable planar membrane structure of the lipids between the two tips in the chamber and two aqueous media on both sides of the membrane. Alternatively, the membrane could be stained with a fluorescent dye, by adding 1,2-Dioleoyl-*sn*-Glycero-

3-Phophoethanolamine-*N*-(lissamine Rhodamine B sulfonyl) (ammonium salt) [Rhodamine Lipid] (Avanti Polar Lipids Inc.) to the DOPC stock solution in the ratio of [Rhodamine Lipid]/[DOPC]=1/199. To form the membrane between the two chamber tips, 15 µl of the phospholipid solution are dropped into the chamber. Then 30 µl of the inner medium solution is added to the chamber side with the needle port. Finally 90 µl of the solution for the outer medium is filled into the other chamber side. The phospholipids stained by a Rhodamine dye were purchased from Avanti Polar Lipids Inc.

2.2 Microfluidic jetting

The jetting chamber is fabricated from a rectangular piece of acrylic glass (length = 2.5 cm; width = 1.5 cm; height = 3 mm) by drilling and milling two overlapping cylindrical grooves (depth = 3 mm). As a result two sharp tips separated by 1.25 mm are formed. A hole with a diameter of 1.2 mm is drilled into one side of the chamber in order to incorporate the micropipette. The acrylic glass chamber is glued onto a microscope cover slide. Borosilicate glass tubes (Science Products) with an outer diameter of 1.2 mm, a wall thickness of 0.4 mm and a length of 8 cm were processed with a micropipette puller (P-2000, Shutter Instrument Co.) to prepare micropipettes with an inner nozzle diameter ranging from 40 to 80 µm. An inverted optical microscope (Axiovert 135 TV, Carl Zeiss) was used to image the jetting process. The reservoir containing the jetting solution is connected to a magnetic valve (3/2 065, Asco). The opening time of the valve is controlled by a function generator (Hewlett-Packard 33120A). The valve is connected to a nitrogen compressor to control the jet pressure between 0.4 and 1.7 bars. To control the position of the needle inside the jetting chamber the valve is mounted on top of a *xyz* micro manipulator which itself is attached to the top of the microscope stage. To perform the Raman spectroscopy jetted vesicles were gently sucked by a pipette from the jetting chamber and transferred to another optical microscope.

2.3 Spectroscopy and imaging

Raman spectroscopy and imaging of the vesicles were performed using an upright optical microscope (Axioscope.A1, Carl Zeiss) equipped with an oil immersion dark-field condenser (NA 1.2–1.4) and two objective lenses (10× (NA 0.25) and 100× (NA 0.75)). A He–Ne laser (Electro-Optics Inc.) operating at 633 nm was used to perform Raman scattering measurements. In all experiments the laser power was kept at 5 mW. Optical signals collected by the microscope objectives were directed either to a spectrometer (Acton SP-2500, Princeton Instruments) equipped with a nitrogen-cooled CCD camera (Spec-10, Princeton Instruments) or to a (digital camera FZ-50, Panasonic). A 633 nm razor-edge long pass filter (Semrock) was used to filter out elastic scattering of the laser light. To prevent bursting of the vesicles on the glass substrate during the measurement, the substrates were covered with a layer polymer Polydiallyldimethylammonium chloride (PDADMAC) [10] purchased from the Sigma Aldrich. To induce vesicle sinking and immobilization on a substrate during the Raman scattering measurements solutions of 300 mM Sucrose and Glucose were used as inner and outer medium, respectively.

3. Results and discussion

Figure 2 illustrates three main advantages of the jetting method for vesicle preparation. First of all, a large number of vesicles with a very narrow size distribution can be created in one experiment by applying a sequence of jet pulses (Figure 2a). For example the vesicle size deviated by only 9% using the parameters 1.7 bars jet pressure, 40 µm nozzle diameter and 10 ms jetting duration. Second, the membrane composition of jetted vesicles can be changed easily. For example, phospholipid molecules functionalized with a fluorescent dye can be incorporated into the vesicles' membrane without modifying the jetting protocol (Figure 2b). Finally, the vesicles can be produced to encapsulate an arbitrary aqueous solution,

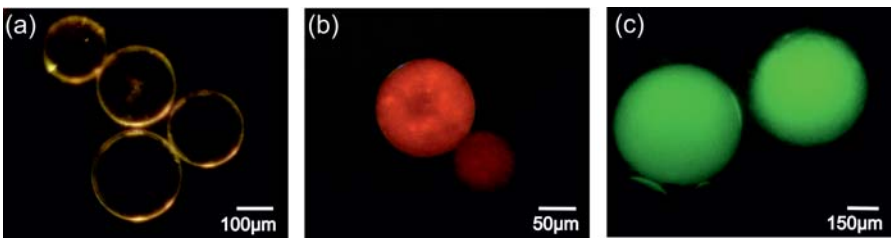


Figure 2 (online color at: www.biophotonics-journal.org) Giant phospholipid vesicles prepared by the jetting method. (**a**) A dark-field image of sucrose-filled vesicles. (**b**) Fluorescence image of the vesicles with the membrane stained by a Rhodamine dye. (**c**) Fluorescence image of the vesicles filled with a fluorescein solution.

different from the surrounding medium. To test this, we have jetted a 10 mM fluorescein solution onto a planar membrane, which resulted in fluorescein-filled vesicles (Figure 2c).

Vesicles produced by the jetting method and by means of the electroformation method [4], [11] appear to be identical, when examined under a microscope with both dark-field and bright-field illumination. Considering that the electroformation method is known to produce predominantly unilamellar vesicles, one might conclude that the jetting method also leads to primarily unilamellar vesicles with a membrane consisting only of phospholipid molecules. Considering that decane is used to dissolve the phospholipid molecules and to stabilize the membrane formed between two aqueous phases (Figure 1c), it is reasonable to question the purity of the membrane of jetted vesicles. Obviously, only vesicles containing no inclusions of organic solvent inside the lipid bilayer membrane can adequately mimic the lipidic part of the plasma membrane of living cells. We have performed Raman scattering measurements on the membranes of vesicles prepared by the jetting method, to gain an insight into the actual membrane composition.

Raman spectroscopy is a technique which enables distinguishing between different chemical compounds without labeling them, by comparing excitation of compound-specific vibration modes. To be able to determine whether decane is included in the jetted vesicles' membranes, we first recorded the Raman scattering spectra of pure DOPC and decane (Figure 3a, b). The spectra of both molecules are very similar due to the chemical similarity between the decane and the hydrophobic part of the DOPC molecules. For example, the Raman peak at 1440 cm^{-1}, corresponding to the scissor-type bending mode of the methylene group, is present in both DOPC [12] and decane [13] spectra. In contrast to this, the Raman line at 1650 cm^{-1}, corresponding to the C=C double-bond stretching, is only present in DOPC [14]. Thus it is possible to use the intensity ratio of the Raman peaks I_{1440}/I_{1650} to verify and quantify the presence of decane inside a DOPC bilayer. Due to its chemical structure, a sample of pure DOPC displays an intensity ratio $I_{1440}/I_{1650} = 2.4$, as determined in Raman scattering measurements. Adding decane to the DOPC shifts the ratio to larger values because decane contributes only to the Raman signal at 1440 cm^{-1}, but not at 1650 cm^{-1}.

Figure 3c (black curve) illustrates a typical Raman scattering spectrum of a jetted vesicle's membrane, as it is depicted in Figure 3d. In this particular case the intensity ratio $I_{1440}/I_{1650} = 5.3$, which clearly indicates that decane molecules are present in the vesicle membrane.

To quantify the amount of decane the following algorithm is used. The DOPC spectrum is normalized to 1 at 1650 cm^{-1}, which results in an intensity equal to 2.4 at 1440 cm^{-1}. The decane spectrum is normalized to the same value of 2.4 at the 1440 cm^{-1}. The measured membrane spectrum (Figure 3d, black curve) is normalized to 1 at 1650 cm^{-1} and fitted by a linear combination of the bare decane and the bare DOPC spectra $I^{norm}_{Membrane}(\nu) = I^{norm}_{DOPC}(\nu) + \eta \cdot I^{norm}_{Decane}(\nu)$ (Figure 3d, red curve). The prefactor η defines the relative contribution of the decane and DOPC molecules to the total Raman scattering signal of the vesicle membrane. The contribution of

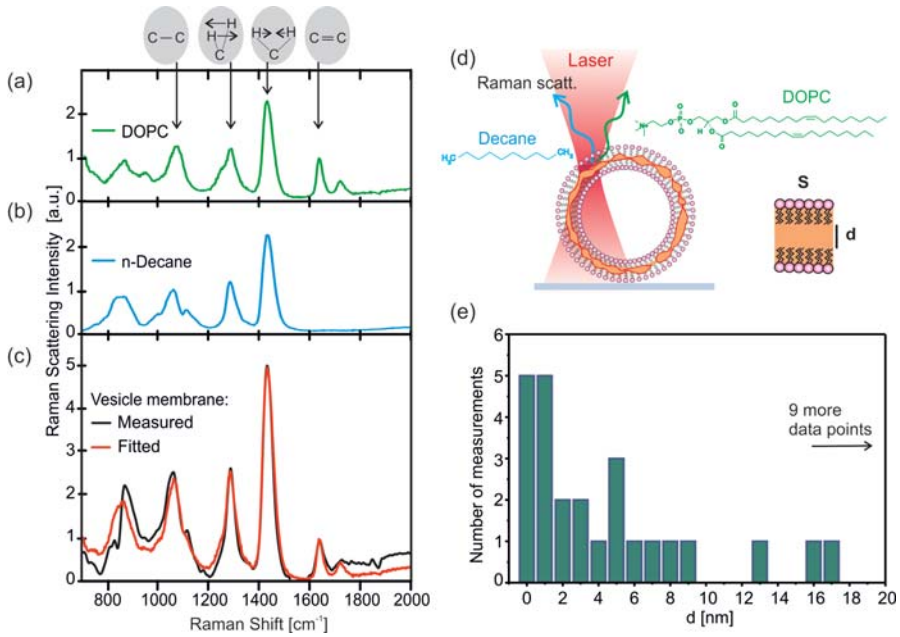


Figure 3 (online color at: www.biophotonics-journal.org) Raman spectroscopy reveals decane in jetted vesicles. Raman spectra of (**a**) DOPC, (**b**) decane and (**c**) the membrane of a jetted vesicle. The measured membrane spectrum (black curve) is normalized to 1 at 1650 cm^{-1} and fitted by a linear combination of the decane and the DOPC spectra ($I^{norm}_{Membrane}(\nu) = I^{norm}_{DOPC}(\nu) + 1.13 \cdot I^{norm}_{Decane}(\nu)$, red curve). (**d**) Sketch of the Raman scattering measurement of the membrane of a vesicle. (**e**) The number of measurements revealing decane inclusions with a given thickness summarizing 34 Raman scattering measurements.

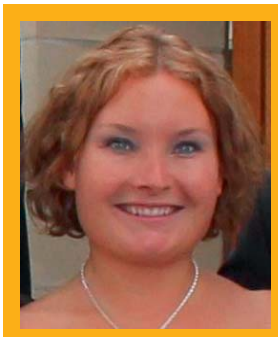
two monolayers of DOPC molecules to the membrane Raman scattering signal at 1440 cm^{-1} is given by I_{DOPC} (1440 cm^{-1}) $\propto 2 \cdot S \cdot \sigma \cdot n$, where S is the surface area illuminated by the laser beam, σ is the number surface density of DOPC molecules forming a fluidic layer and n is the number of vibrating scissor-like units in a single DOPC molecule. The contribution of decane molecules enclosed between these two lipid monolayers is I_{Decane} (1440 cm^{-1}) $\propto S \cdot d \cdot \varrho \cdot m$, where d is the thickness of the decane layer, ϱ is the number volume density of decane and m is the number of vibrating scissor-like units in a single decane molecule. Taking into account that $\frac{I_{Decane}(1440\,cm^{-1})}{I_{DOPC}(1440\,cm^{-1})} = \eta$, the thickness of the decane layer reads as $d = \eta \cdot \frac{2\sigma}{\varrho} \cdot \frac{n}{m}$. The value of η is extracted from the measured Raman spectra and all other parameters entering the relation are known material constants ($\sigma = 1.43 \times 10^{18}\,m^{-2}$ [15], [16], $\varrho = 3.09 \times 10^{27}\,m^{-3}$ [17]). The numbers of the scissor-type vibrations $n = 32$ and $m = 8$ are determined by counting the methelyne groups of a DOPC and of a decane molecule, respectively.

Raman scattering spectra were measured by focusing the laser at the vesicle surface as depicted in Figure 3d. For each vesicle measurement were taken at up to five different heights on the vesicle membrane (top, bottom, equator and two intermediate points at +/−45° above and below the vesicle equator). In total 34 Raman spectra have been recorded. The thickness of the decane residual layer enclosed within the lipid bilayer has been calculated from the Raman spectra according to the above-described algorithm. The results of this analysis are summarized in Figure 3e. We have not observed a single jetted vesicle that was completely free of decane residuals. However, some spots on the vesicle membrane were found to be comprised of a single lipid bilayer without any inclusions of decane (5 out of 34 measurements). The residual decane is distributed randomly over the vesicle surface. Our observation of the *n*-decane residuals in jetted vesicle are consistent with the known decane "microlenses" observed in classic black lipid membranes [18], [19] and liposomes [20]. Although one would expect a thicker layer of the less dense organic solvent at the top of the vesicle, we have not found a correlation between the thickness of the decane inclusions and the position on the vesicle surface. It is worth noticing that our results do not contradict the previously reported successful incorporation of functional transmembrane proteins into jetted vesicle membranes [8]. Indeed, although the membrane is heavily contaminated, a few decane-free spots have been detected. These locations may, in principle, provide a space for correctly functioning membrane proteins. Incorporation of the decane into the membrane is affected by the jetting parameters (e.g. jet pressure, jet duration). The piezoelectric approach [9] offers wider flexibility in controlling these characteristics and, therefore, is preferable to reduce the amount of the residual decane in lipid vesicles.

4. Conclusion

Using Raman spectroscopy we have investigated the chemical composition of the membrane of vesicles prepared using the microfluidic jetting method. Although the jetted vesicles appeared indistinguishable from unilamellar vesicles under the microscope, the membrane of all jetted vesicles is found to contain residuals of the organic solvent decane. The decane inclusions are randomly distributed over the vesicle surface area and vary in thickness from a few to several tens of nanometers. Since the thickness of the decane layer, less than 10 nm in most cases, is relatively thin the appearance of the vesicles under a microscope does not change significantly, but the bilayer properties may be altered considerably. Our findings have exceptional importance for biomedical studies aiming the investigation of cellular transmembrane processes by means of mimicking living cells by lipid vesicles. Since the presence of an organic solvent in a phospholipid bilayer will affect the functional properties of the membrane and of transmembrane proteins, it has to be carefully taken into account and monitored by e.g. Raman scattering spectroscopy.

Acknowledgements Financial support by the DFG through the Nanosystems Initiative Munich (NIM) and by the ERC through the Advanced Investigator Grant HYMEM is gratefully acknowledged. We are thankful to Stefan Niedermaier for technical assistance.



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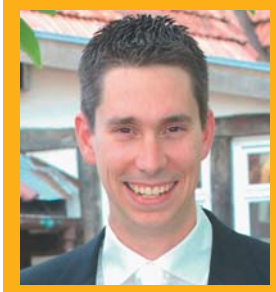

Alexander Urban studied physics at the University of Karlsruhe in Germany and at Heriot-Watt University in Edinburgh, UK. He obtained his Ph.D. in physics at the Photonics and Optoelectronics Group at the Ludwig-Maximilians University in Munich, Germany, where he is currently working as a postdoc. His main interests lie in the application of plasmonics of metallic nanoparticles for diverse applications, e.g biomedical studies and nanostructuring of substrates.

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