We have developed a methodology for the analysis of liposomal membranes and their contents using near-IR Raman spectroscopy on liposomes held in an optical trap. We were able to detect a variety of membrane components including lipids, cholesterol, and small molecule solutes such as ethanol, DMSO and hexafluorisopropanol. The methodology is able to distinguish between solutes that equilibrate across the liposomal membrane from those that partition selectively into the lipid bilayer.

Optical entrapment, also known as laser tweezing, is an emerging technique that has the potential to be extremely useful for the analysis of liposome properties.1 This technique offers the possibility of manipulating liposomes in order to observe a number of phenomena that would otherwise be difficult to study, and has the advantage of being performed under physiologically relevant conditions. Optical tweezing of liposomes has already been described for applications involving micromanipulation, controlled fusion, and studies of single molecule reactions2 and enables measurements to be made on membrane processes with slow kinetics that require lengthy acquisition times. In this paper we describe a methodology for trapping single liposomes in order to obtain Raman spectra of the membrane components. Although methods for obtaining Raman spectra from lipids have been described, these rely on either measuring bulk properties, which may be difficult in cases where the number of liposomes per unit volume is low, or on detailed preparation procedures that enable supported membranes to be probed using conventional Raman microscopy.3 Raman tweezing has previously been applied in a few cases to the study of small particles,4 although little work has been done with liposomes. It is a technique particularly well suited to liposomes, as potentially any substance that interacts with, or partitions into, a membrane may be studied without the need for labelling and extended sample preparation. Detailed knowledge of the chemistry and biology of natural lipid membranes is essential for understanding a variety of phenomena, including membrane permeability, peptide–lipid interactions and blood–brain permeability. The Raman spectra that were collected are suitable for obtaining analytical information regarding membrane composition, and to determine the permeability of an added solute.

In our initial experiments we sought to obtain reference Raman spectra for typical examples of saturated and unsaturated lipids under conditions of physiological relevance, and selected dimyristoyl phosphatidylcholine (DMPC) and dioleoyl phosphatidylcholine (DOPC) respectively. Phosphate buffered saline (PBS) was used as the medium throughout our experiments due to the weak Raman signature of this buffer. Our optical setup comprised an Argon ion source of emission wavelength 514.5 nm that was directed to a ×63, water-immersion objective lens using a dichroic mirror housed in a Leica DM-IRB microscope. The tightly focused laser beam formed the optical trap, and backscattered Raman shifted light from the trapped liposome was collected using the same lens.

The probe laser beam wavelength was eliminated before the beam entered the spectrometer using a holographic notch filter. In a typical experiment, the power of the source was attenuated to 12 mW and Raman spectra obtained from a single trapped liposome by averaging the emission data from several scans over a period of 150 s. In all cases, background spectra were obtained by dislodging the liposome from the trap and recording the Raman emission from bulk solution. These background spectra subtracted from those obtained for the trapped liposome.

Raman spectra were obtained from large unilamellar vesicles of diameter ~1 μm composed of pure DMPC and DOPC that exhibited characteristic emission bands. The salient features of the DMPC spectrum (Fig. 1A) were bands at 1060, 1085 and 1125 cm\(^{-1}\) (C–C skeletal stretching vibrations), 1296 cm\(^{-1}\) (CH\(_2\) twist) and 1445 cm\(^{-1}\) (CH\(_2\) bend). DOPC (Fig. 1B) exhibited additional Raman bands at 1264 cm\(^{-1}\) and 1655 cm\(^{-1}\) (cis C=C stretching vibration). Although these spectra have a lower signal-to-noise ratio than those reported,6 we routinely used a low laser power in order to minimise thermal effects on the liposomal membrane, at the expense of a slight increase in background noise. Liposomal membranes composed of DMPC/DOPC with an approximate DOPC content of 18 mol% were prepared and also subjected to Raman analysis (Fig. 2A). By performing a ratiometric subtraction of the peaks arising from DOPC using the spectrum of the authentic sample, we were able to determine the proportion of this lipid in these membranes to be 52 ± 2 mol%, significantly different from the intended composition. The precise reasons for this discrepancy are uncertain, and future work will be performed to try to determine whether it results from an inherent property of liposomes, or arises from...
as an artefact of the method used to prepare them. A similar analysis was performed on soybean phosphatidyethanolamine (SPE, Fig. 2B). By comparison of the intensities of the peaks at 1655 and 1450 cm⁻¹, we were able to estimate the olefin content of SPC to be 1.57 ± 0.09 times that of pure DOPC, based on the assumption that the spectrum for DOPC was characteristic of a cis- double bond-containing lipid. These values are consistent with published data for the composition of SPC, which contains a high content (43%) of the cis-9,12 unsaturated lipid linoleic acid.7 We were also keen to demonstrate the ability to detect the presence of cholesterol in membranes, particularly as cholesterol is a key modifier of membrane properties but has a less distinctive Raman spectrum than phospholipids. Consequently we prepared liposomes composed of 10% cholesterol/DMPK and obtained spectra for these mixtures from a series of trapped liposomes. We were able to subtract the DMPK signals and unambiguously identify cholesterol as a membrane component through the observation of peaks at 1438 and 1669 cm⁻¹ (Fig. 3B), in accordance with the literature data for this compound. The presence of several peaks of low relative intensity below 1400 cm⁻¹ is also typical of cholesterol.8 Additionally, as liposome samples are never monodisperse, we were able to trap a number of DMPK vesicles of varying size in order to examine the effects of particle size on Raman scattering intensity. Interestingly, scattering reached a maximum for particles of size 1 μm and then decreased slightly at larger sizes. The optimum size will depend primarily on the numerical aperture of the objective lens in the microscope and the size of the diffraction limited focal spot in comparison to the trapped object. In our experiments the focal spot was ~ 600 nm in length in the z-plane, smaller than the size of the liposomes for which maximum scattering was observed. Further work will be required to determine why these two values are different, although it should be noted that as the lipids are only present in a small layer of approximate diameter 10 nm at the surface of the liposome, the scattering intensity that we observe is determined not only by the quantity of lipid in the liposome, but also the distance of the liposomal membrane from the point of highest incident light intensity at the centre of the liposome. Thus, as liposomes become increasingly large, the potential scattering intensity is greater as more lipid becomes available to generate the Raman signal, however the actual scattering intensity decreases because the liposomal membrane is further from the focal point of the incident beam.

The ability to determine the partitioning of solutes into the lipid bilayer was of intrinsic interest to us. In order to examine the distribution of a solute added to the solution surrounding the vesicles, a sample of liposomes was diluted in PBS containing the solute (typically at a concentration of 2.5% v/v for liquids). After an overnight incubation period to ensure that equilibrium was obtained, Raman spectra of trapped liposomes were recorded, and a background spectrum obtained as normal. We performed analyses on three solutes, ethanol, dimethyl sulfoxide (DMSO) and hexafluoroisopropanol (HFIP). It was anticipated that the signals resulting from solutes that distributed freely throughout the sample by permeation across the lipid membrane would cancel out during the background subtraction. This proved to be the case for DMSO and ethanol. For HFIP however, a clear tendency to partition into the membrane was observed, as the Raman spectrum following background subtraction continued to display clear signals for this solute (Fig. 4).

From our data we have shown that Raman analysis of the lipid contents of optically entrapped liposomal membranes is a feasible methodology that can provide analytical data under physiologically relevant conditions that do not denature the membrane structure. Furthermore, these conditions are suitable for measurement of dynamic processes such as solute partitioning that would not be possible by any methodology that would require liposome processing (lyophilisation, centrifugation, dialysis or gel filtration) prior to analysis.

**Notes and references**


**Fig. 3** Raman spectrum obtained from liposomes composed of 10 mol% cholesterol/DMPK (A) and the same spectrum following subtraction of peaks arising from scattering by DMPK (B).

**Fig. 4** Raman spectra of 2.5% v/v HFIP/PBS (A) and DMPK liposomes following incubation in 2.5% v/v HFIP/PBS (B). Peaks arising from HFIP at 740 cm⁻¹ and 846 cm⁻¹ are indicated by arrows.