Technical note

Rapid Quantification of Cardiolipin and DOPC Lipid and Vesicle Concentration

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A novel approach to quantification of cardiolipin and DOPC lipid and vesicle concentration that is rapid and inexpensive is described. Traditional approaches to quantifying vesicle concentration destroy sample and are often time consuming. Using common laboratory equipment and software, lipid vesicles were reliably quantified allowing for immediate use without significant sample loss. Once calibrated, only absorbance measurements with a UV-Vis spectrophotometer are necessary as input into a Matlab program, which calculates the corresponding vesicle and lipid concentration. Fast and accurate concentration determination for preparations of vesicles is essential for analytical titration experiments necessary for protein/vesicle binding curves.

Keywords: Cardiolipin, DOPC, POPC, POPG, Vesicle quantification, Lipid quantification

Introduction:

Vesicles are made and used in a variety of biologically relevant experiments to elucidate and characterize membrane associated interactions. Existing methods for vesicle quantification are time consuming and costly. Commercially available particle-counting equipment can enumerate vesicles as small as 10 nm, but these devices are expensive. Phosphate analysis assays and inductively coupled plasma atomic emission spectroscopy (ICP) can measure phosphate headgroup concentration but are time consuming and destroy large amounts of sample [1–3] and thus are impractical for routine use. Because of these limitations, measurement of protein/membrane binding affinities via analytical vesicle titrations is expensive. Due to lipid oxidation in solution and membrane stability [4,5], it is essential that lipid and vesicle concentration be measured expeditiously. Here, we report a rapid and reliable method for vesicle quantification.

This quantification technique can be performed immediately before vesicle titrations of protein samples and does not destroy any of the vesicle stock solution. After initial validation, this technique requires only 15 minutes to quantify vesicles and lipids and uses common laboratory equipment and software. In this report, we apply the method to measurement of the concentration of 100% 18:1 cardiolipin (1',3'-bis[1,2-dioleoyl-*sn*-glycero-3-phospho]-*sn*-glycerol, TOCL) vesicles of known radii, we then use existing physical parameters to extend this method to DOPC analysis demonstrating that the method is readily adapted for use with vesicles composed of any lipid with appropriate validation or calibration.

Theoretical Calculations

Quantifying lipids is difficult because they have no absorbance signature in a convenient region of the UV-Visible spectrum (Figure S1). While lipids, themselves, do not characteristically absorb visible light, supramolecular structures like lipid vesicles scatter light in a size, wavelength, concentration and media dependent manner known as Mie scattering [6]. A UV-Vis spectrophotometer can be used to evaluate Mie Scattering as a function of wavelength. Knowing particle size, the wavelength and angle of exposure, as well as the refractive index of the medium, the concentration of particles in solution can be calculated. Methods for using Mie scattering theory to measure nanoparticle concentration [6,7] have been modified for application to cardiolipin and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) vesicles so that vesicle and lipid concentration can be determined.

This method involves calculating the scattering cross section of vesicles of known size at an appropriate wavelength using Mie scattering theory, measuring the absorbance of the vesicle with a UV-Vis spectrophotometer and solving Beer's law for the particle concentration. Matlab code is provided in the supplementary information for the required calculations. To accurately calculate scattering due to vesicles, vesicle size, angle and wavelength of absorbance and the index of refraction of the medium are required. Furthermore, with appropriate validation or calibration, the number of lipids per vesicle can be determined, allowing total lipid concentration to be calculated.

To relate absorbance to the calculated scattering off of a non-absorbing vesicle, Beer's law must be expressed in terms of the scattering extinction coefficient, C_{ext} [6,7];

$$A(\lambda) = d \sum_i N_i C_{ext,i}(\lambda) \quad \text{Eqn. 1}$$

where d is sample path length, N is the number of scattering centers and A is the total absorbance measured at a specific wavelength, λ .

The scattering extinction coefficient is expressed as [6,7]

$$C_{ext} = \frac{\lambda^2}{2\pi} \sum_{n=1}^{\infty} (2n+1) \text{Re}\{a_n + b_n\} \quad \text{Eqn. 2}$$

where a_n and b_n are the differential expressions of Bessel functions

$$a_n = \frac{m\psi_n(mx)\psi'_n(x) - \psi_n(x)\psi'_n(mx)}{m\psi_n(mx)\xi'_n(x) - \xi_n(x)\psi'_n(mx)} \quad \text{Eqn. 3a}$$

$$b_n = \frac{\psi_n(mx)\psi'_n(x) - m\psi_n(x)\psi'_n(mx)}{\psi_n(mx)\xi'_n(x) - m\xi_n(x)\psi'_n(mx)} \quad \text{Eqn. 3b}$$

where ψ_n is the Ricatti-Bessel Function, $j(n)$, of the first kind, and ξ_n is the Hankel function, $h(n)$, of the first kind. The value, x is the size parameter relating vesicle radius, R , to wavelength;

$$x = \frac{2\pi \text{Re}(m)R}{\lambda} \quad \text{Eqn. 4}$$

where wavelength, λ , is reported in the same units as the vesicle radius. The value m is the relative refractive index represented by the real, n_{Re} , and complex, n_{Im} , parts of the refractive index of the lipid and water.

$$m = \frac{n_{Re,lipid} + n_{Im,lipid}}{n_{Re,water} + n_{Im,water}} \quad \text{Eqn. 5}$$

For cardiolipin. $n_{Re,lipid}$ was set to 1.46 [8], while $n_{Im,lipid}$ was approximated as $n_{Im,water}$ at 200 nm (1×10^{-7} , [9]). The wavelength dependence of $n_{Re,water}$ was calculated with Eqn. 6 [10],

$$n_{Re,water}(\lambda) = 1.31848 + \frac{6.662}{\lambda - 129.2} \quad \text{Eqn. 6}$$

while $n_{Im,water}$ was approximated using the value at 200 nm reported above. Equations 2-6 were programmed into Matlab (available from MathWorks, Natick, MA), to solve Eqn. 1 for N , allowing calculation of vesicle concentration. Absorbance measurements taken with a Beckman Coulter DU 800 UV-Visible spectrophotometer were used in the calculation. Wavelengths were selected such that the value of x in Equation 4 was between 1 and 10. Wavelengths of 300-400 nm are ideal for measuring vesicle scattering off of 100-200 nm diameter vesicles.

Method Validation:

Initial validation or calibration measurements are required before the Matlab vesicle concentration calculator (MVCC) can be used simply with absorbance data as input. Strong absorbing dyes were used to estimate vesicle and lipid concentration and relate these measurements to scattering measurements. All experiments were performed on a Beckman

Coulter DU 800 UV-Visible spectrophotometer where the spectral range from 200-800 nm was monitored. A Malvern Zetasizer Dynamic Light Scattering (DLS) instrument was used to monitor vesicle size throughout the validation process. If a DLS instrument is not available, particle size can be measured with a UV-Vis spectrophotometer [7].

To validate the MVCC, vesicles were first quantified by labelling their interior with a strongly absorbing dye and measuring dye content after external dye was removed by extensive washing. Knowing vesicle size (and thus internal volume) and initial dye concentration, vesicle concentration could be calculated from the decrease in dye absorbance after the wash step.

Vesicles were formed in the presence of fluorescein (a strongly absorbing dye) to determine vesicle concentration. External fluorescein was removed from extruded vesicles by buffer exchange (see Materials and methods in Supplementary Information) and the residual fluorescein concentration was calculated. This fluorescein concentration was related to inner volume of a vesicle to determine the total number of vesicles required to produce the fluorescein absorbance (Table 1).

Next, the vesicle concentration was found using Mie scattering theory. To extract scattering data from spectra taken of fluorescein-filled vesicles, the fluorescein absorbance was subtracted from spectra collected after buffer exchange (Figure S2). Scattering absorbance data between 300-400 nm were input into the MVCC because this spectral region has minimal fluorescein absorbance (Figure S2). The vesicle concentration obtained is reported in Table 1. The vesicle concentration found using fluorescein is within an error of less than 8% of the vesicle concentration found using Mie Scattering theory in three independent trials. Taking into account the error in approximating inner vesicle volume and extracting scattering absorbance from the raw absorbance data, the agreement is good, validating the use of Mie scattering theory to calculate vesicle concentration.

Having established that the number of vesicles per sample could be calculated, we carried out a second, independent set of experiments so that the MVCC could be calibrated to incorporate lipid density per vesicle to permit lipid concentration determination (see Materials

Table 1

Comparison of vesicle concentration calculated using fluorescein filled vesicles with that obtained from Mie scattering for the same vesicles.

Trial	Vesicle Concentration from Fluorescein Absorbance	Vesicle Concentration from Mie scattering	% Error
1	$4.8 \pm 0.1 \times 10^{17}$	$5.15 \pm 0.01 \times 10^{17}$	6.8
2	$5.0 \pm 0.1 \times 10^{17}$	$5.16 \pm 0.01 \times 10^{17}$	3.1
3	$4.8 \pm 0.1 \times 10^{17}$	$5.16 \pm 0.01 \times 10^{17}$	7.0

Concentrations reported in vesicles per L.

and methods in Supplementary Information). In these experiments, lipid concentration per vesicle was measured using fluorescently labelled lipid to correlate vesicle concentration to lipid concentration. NBD-labelled DOPC lipid was used to dope Cardiolipin vesicles and determine lipid concentration. Associated scattering data from absorbance measurements was used to calculate vesicle concentration and relate that to lipid concentration using Cardiolipin headgroup size as reported by Pan *et al.* [11]. The lipid concentrations obtained from NBD absorbance and from Mie scattering theory from three independent trials are within 5% (Table 2), confirming the validity of the surface area used for cardiolipin.

Table 2

Comparison of lipid concentration calculated using NBD doped vesicles with that obtained from Mie scattering for the same vesicles.

Trial	Lipid Concentration from NBD Absorbance, M	Lipid Concentration from Mie scattering, M	% Error
1	$8.5 \pm 0.3 \times 10^{-4}$	$8.41 \pm 0.04 \times 10^{-4}$	1.1
2	$8.4 \pm 0.2 \times 10^{-4}$	$8.20 \pm 0.03 \times 10^{-4}$	2.4
3	$8.5 \pm 0.2 \times 10^{-4}$	$8.16 \pm 0.02 \times 10^{-4}$	4.4

Error evaluated as standard deviation of multiple spectra collected for each trial.

As an independent external method for lipid concentration calibration, ICP was used to measure phosphorous content of samples of pure cardiolipin vesicles. Samples were prepared for ICP as outlined in Supplementary Information. The correspondence between cardiolipin concentration obtained by phosphorous analysis and cardiolipin concentration calculated with the MVCC using the experimental value for cardiolipin surface area [11] to evaluate lipid density was consistently within <5% (Table S1).

To demonstrate the versatility of the MVCC, samples of DOPC were also submitted for ICP analysis and compared to MVCC calculated concentrations (Table S1). DOPC bilayer thickness, headgroup surface area [11] and index of refraction [12] were used to calculate DOPC lipid concentration from scattering. Correspondence between the MVCC and ICP values was within <3% demonstrating the calculator's ability to accurately measure lipid concentration given the corresponding lipid physical parameters.

Lipid concentration as determined using the reported manufacturer concentration was compared to MVCC findings to analyze the necessity of a vesicle calculator in quantitative experiments. Significant deviations in concentration based on manufacturer specified lipid mass or concentration and ICP analysis are reported for all DOPC and TOCL samples in Table S2 (Supplementary Information). Error in concentration based on starting material and final ICP measurements were as high as 28%, whereas the correspondence between the ICP and MVCC values was always <5% (Table S1, Supplementary Information). Because of the vesicle formation process used, lipid had the potential of being lost in several steps demonstrating that

concentration based on manufacturer reported lipid mass does not necessarily reflect final experimental concentration. This finding supports the use of the MVCC in reliably determining lipid concentration for quantitative experiments.

Conclusion

The Mie scattering-based method for vesicle quantification was designed to provide a quick, reliable, and cost effective analytical method, which after initial validation by absorbance and/or phosphorous analysis, can be implemented immediately before any experiment involving use of extruded vesicles. The method allows vesicle concentration to be accurately measured simply using UV-Vis measurements. Knowing the surface area of the lipid (validated or calibrated by absorbance or by phosphorous analysis), lipid concentration can also be obtained with the MVCC. Due to the simplicity of the experimental setup, vesicle and lipid concentrations may be calculated quickly and without sample destruction, providing a reliable alternative for more time-consuming methods.

The wavelength measurement range is only limited by the size of the vesicle (thus size parameter, x) and constraints of the detection device. When using extruded vesicles or vesicles with a well-defined radius, as was done here, DLS measurements are not necessary once vesicle size is established, although occasional spot-checking of the consistency of the extrusion process is recommended. While the region of 300-400 nm was used to measure vesicle scattering and calculate concentration, any convenient wavelength in the UV-Visible spectrum may be used provided the constraints on size parameter, x , are met from Eqn. 4. This technique uses the mean vesicle radius to determine C_{ext} for a scattering center. Table S3 provides wavelength ranges for testing dependent on vesicle size for various vesicle compositions. It should be noted that for samples with large standard deviations in vesicle radius, theoretical vesicle calculations may have higher error.

The technique may be adopted for any pure lipid system. The above outlined technique should also work for mixed lipid systems, provided the refractive index of the mixed lipid vesicle is available. Finally, any buffer that does not appreciably change the index of refraction may be used provided it does not absorb significantly at the wavelength of detection. Modifications may be made to incorporate the adjusted index of refraction of the buffering media if needed.

A simplified version of the MVCC written in .m file format can be found in the supplementary information with associated instructions on the installation and running of the file. This version uses reported index of refraction, headgroup size and bilayer thickness to calculate lipid concentration for TOCL, DOPC, POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol)) [8, 11-15].

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