Study of lipid vesicles using nanoparticle tracking analysis and quartz crystal microbalance

Nastaran Fallah Randjbar, Mathias Arvidsson, Carl Strandby

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Abstract

One way to model behaviour and properties of cell membranes is to study lipid vesicles. This report discusses two common methods to examine lipid vesicles, a common model for cell membranes. The first method is nanoparticle tracking analysis, NTA, that can be used to relate the Brownian motion of nanoparticles to their size. The second method is quartz crystal microbalance, QCM, which can be used to study a process of particle interactions by measuring a change of mass of particles bound to the crystal surface. In the first experiment, NTA was utilized to determine the size distribution of lipid vesicles in a mixture. In the second experiment, QCM was used to determine the concentration of lipid vesicles with attached biotin in a mixture consisting of vesicles with and without biotin. The average size of lipid vesicles was determined to be 87 ± 1.3 nm in scattering mode. In fluorescent mode the average size of vesicles with attached fluorophores was measured to be 56.4 ± 3.2 with NTA. The concentration of lipid vesicles with attached biotin was decided to be 1 ± 0.3 % using QCM.

1 Introduction

There are many applications where it is important to understand the behaviour of cell membranes and how they interact with other particles. These interactions can be modelled with lipid vesicles, a name for a membrane-like sphere of a lipid bilayer. With these lipid vesicles one can model the interactions of a virus and a cell membrane, used for example in the identification of antigens [1] in medical diagnostics. The following two subsections describe two methods, nanoparticle tracking analysis, NTA, and quartz crystal microbalance, QCM, that are used to study lipid vesicles. Two experiments were carried out, first of which used NTA to measure the size distribution of lipid vesicles in a mixture, and the second one determined the concentrations of different types of vesicles in a mixture using QCM.

1.1 Nanoparticle tracking analysis

The theory behind nanoparticle tracking analysis is that a particle subject to Brownian motion will move randomly with a speed that can be related to the particle size -smaller particles move faster, while larger ones move slower in a liquid-. By analyzing a series of frames and measuring the average distance a particle travels between two frames, the particle diameter is calculated using the equation

$$d = \frac{\mathrm{k_B}T}{3\pi\eta}\frac{4}{\overline{(x,y)}^2} = \frac{4\mathrm{k_B}T}{3\pi\eta\overline{(x,y)}^2}, \tag{1}$$

where k_B is the Boltzmann constans, T is the temperature and η is the fluid viscosity [2]. The motion of the particles can be measured in both scattering and fluorescent mode.

The difference between these two modes is that even though the excitation LASER still illuminates all particles, only the light from particles that have been fluorescently labelled reach the camera in the fluorescent mode(often through long-pass filters). As a result, F-NTA enables identification of a sought particular sub-population of all the present particles [3]. The advantage to measuring in fluorescent mode is that one is able

to detect smaller particles due to the fluorescence intensity being considerably higher than the intensity of light scattering.

1.2 Quartz crystal microbalance

Quartz is known as a piezoelectric crystal, which means it responds mechanically to applied voltages. More specifically, a shear motion is induced during an applied voltage. The resonance frequency of the crystal depends on its mass. When molecules bind to the quartz crystal, the mass changes effectively, and in conjunction the resonance frequency changes. This interaction makes it possible to measure mass changes and molecular bindings simply by measuring the resonance frequency and the way in which it changes. The process is governed by the Saubrey equation

$$\Delta m = \frac{A}{n} \cdot \Delta f, \tag{2}$$

where Δm is the planar density with units ng/cm^2, Δf is the change in frequency, and A is a constant with value $A = -17{,}7\,\mathrm{ng/cm^2}$ [4], n is the harmonic of the resonating crystal [5]. For our measurements n =3.

As more particles bind to the quartz crystal surface, increasing the mass, the resonance frequency adjusts accordingly. The key is to isolate a certain binding process and measure the frequency change. If the mass of the binding substance is known, the number of binding particles can be calculated.

The process of interest in this experiment is the binding of the protein streptavidin to biotin elated lipids in the lipid vesicles. Streptavidin has a strong binding affinity for the amino-acid biotin, which can be attached to lipids. Only a fraction of lipids contain biotin and the vesicles are produced using mostly normal lipids. First, however, a uniform layer of lipids must be formed on the quartz crystal. By adding lipid vesicles to the surface, the spheres will eventually burst and form a lipid bilayer, onto which the streptavidin can bind. The number of streptavidin molecules that binds to the surface is given by

$$\#\mathrm{Streptavidin}/cm^2 = \frac{\Delta m}{M_{str}} N_A. \tag{3}$$

Where Δm is the measured mass change, $M_{str} = 55 \cdot 10^3$g/mole is the molar mass for streptavidin [6], and $N_A = 6.022 \cdot 10^{23}$ is Avogadro's number. The number of lipids per cm^2 in the lipid bilayer is given solely by their size, which is approximately $0{,}5\,\mathrm{nm^2}$ [7]. Corresponding to $2 \cdot 10^{14}$lipids/cm^2.

2 Experimental procedure

2.1 Nanoparticle Tracking Analysis

The NTA measurements were made with the NTA-device NANOSIGHT LM10. The solution of lipid vesicles contained 98% POPC solution with lipid vesicles, 1% lipids with attached biotin and 1% lipids with an attached fluorophore. The solution was filtered and diluted 4000 times with a saline solution. The measurement device was cleaned with MilliQ water, ethanol and residue free tissues. When assembled, the pipes were rinsed with buffer and the device was set to measure an area of interest where moving particles were identified. Then the prepared solution with lipid vesicles was pumped through the device with an automatic pump. At first with a high flow rate, and when the flow rate was measured to be constant, it was reduced and the contrast and focus of the device's camera adjusted. At this stage five measurements were taken in scattering mode, and subsequently another five in fluorescent mode.

2.2 QCM-D measurements

In order to perform the full QCM-measusrements, several solutions to be applied to the crystal were prepared. First a solution of ionized water was prepared to use for the different solutions. The water was put in a vacuum chamber for twenty minutes for any air bubbles to be removed. A buffer of phosphate buffered saline

(PBS) was prepared using the ionized water. The PBS is a mixture that recreates the alkalinity of normal cells to prevent the vesicles from bursting spontaneously. Using the buffer, the first solution was prepared containing 0,1 mg/mL of biotin-elated vesicles. The second mixture also used the PBS-buffer as a base and then Streptavidin was added to a concentration of 15 molars. The third and final solution was a mixture of normal vesicles and biotin-elated vesicles with a ratio of 99:1 (98:1:1 Normal vesciles: PE-cap-biotin; PE-aF-488).

The next major step was cleaning the QCM-chip through 3 separate procedures. First the chip was vibrated clean in an ultrasound machine. The chip was submerged in a cup of distilled water and then the cup was set in a waterbath of the ultrasound machine which was run for 15 minutes. Second the chip was rinsed using distilled water, then etanol, then distilled water again. The final step in the cleaning procedure utilized a plasma cleaner. The rinsed chip was dried using a residue-free tissue, and put in a glass cup. The chip was then placed inside of the plasma cleaner, and a vacuum was initiated. Once the machine had stabilized, oxygen was supplied, which at the low pressure and current temperature turned into a plasma which surrounded the chip. After 15 minutes the machine was turned off, and the clean chip was extracted.

The measurement was performed using a "q.sense" machine which identifies the resonance frequency of the QCM-chip even as particles bind to it. The chip was placed in the machine with matching electrodes. A pump began the flow of buffer to the chip. The program was started, and given some time to stabilize and to locate the resonance frequency. A discard cup was used to gather the used solutions. A tube was inserted in the desired solution to pump it to the chip, and each time the solution was switched the tube was wiped clean to not contaminate any of the solutions. The measurement began with only supplying the chip with buffer, then swapping to pump biotin elated vesicles. Then the solution was swapped back to buffer to verify completion of the bilayer. Next the streptavidin solution was applied to the chip, and then finally vesicles with biotin were added again.

For the third lab, the measurement was repeated with a diluted streptavidin solution of concentration 1,5 µmol.

3 Results and discussion

3.1 Results of NTA measurement

Size distribution measurements made in scattering mode is shown in Figure 1 and Table 1. The mean size of the particles was measured to be 87.1 ± 1.3 nm and the average concentration of particles was determined to be 0.19 billion particles per milliliter.

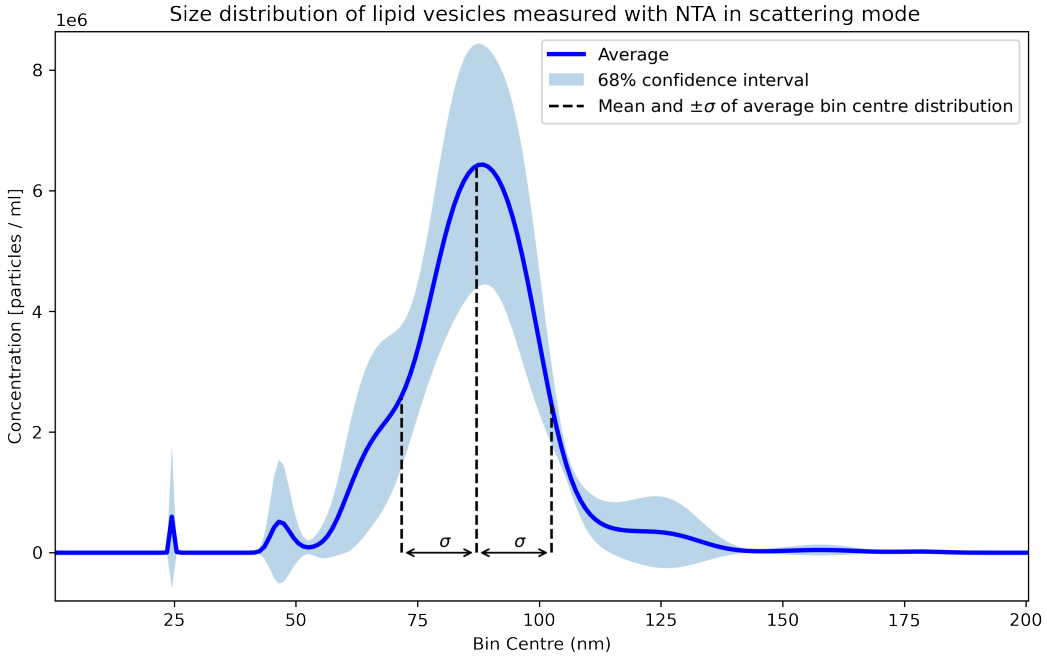

Figure 1: Average nanoparticle size measurement with light scattering (blue line) with a mean of 87.1 ± 1.3 nm, and average standard deviation 15.4 ± 0.9. Confidence interval is 68%

Table 1: Scattering measurement data produced by NTA software.

	Meas. 1	Meas. 2	Meas. 3	Meas. 4	Meas. 5	Average	Standard Error
Mean (nm)	85.3	84.5	91.7	86.6	87.2	87.1	1.3
Mode (nm)	87	94.8	81.6	87.7	89.8	88.2	2.1
Standard deviation (nm)	14.4	17.1	17.6	12.6	15.4	15.4	0.9
Concentration (Particles /ml)	1.78E+08	1.85E+08	1.84E+08	2.20E+08	2.28E+08	1.99E+08	1.04E+07

Corresponding figure and table for measurements in fluorescent mode can be seen in Figure 2 and Table 2. In fluorescent mode the mean particle size was measured to be 56.4 ± 3.2 nm and the average concentration $51,4$ million particles per milliliter. The measurement was labeled by the Nanosight device to contain heavy noise which is indicated by the measured concentrations varying from 18.1 to 76.3 million particles per mililiter and the 68% confidence interval in Figure 2 being noticeably wider than in Figure 1 from the scattering mode measurement.

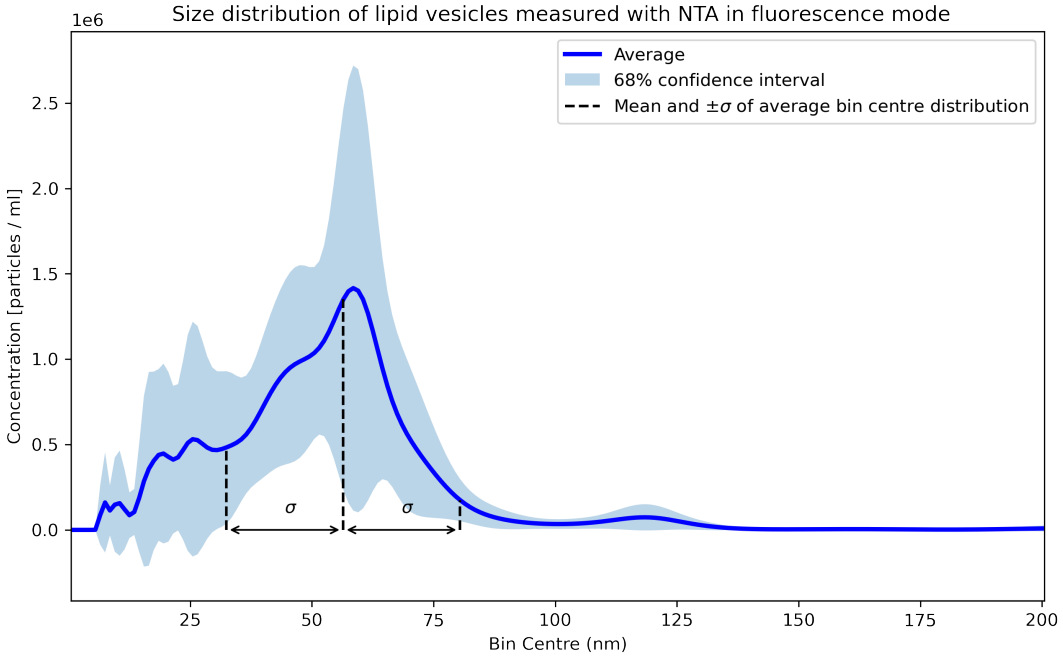

Figure 2: Average nanoparticle size measurement with light scattered from fluorescently labelled particles (blue line) with a mean of 56.4 ± 3.2 nm, and average standard deviation 24 ± 1.3. The noise level of the measurement was labeled by the nanosight device as high, which is also indicated by the 68% confidence interval being noticeably wider than in Figure 1 of the measurement made in scattering mode.

Table 2: Fluorescent measurement data produced by NTA software.

	Meas. 1	Meas. 2	Meas. 3	Meas. 4	Meas. 5	Average	Standard Error
Mean (nm)	61.7	66.1	50	53.6	50.5	56.4	3.2
Mode (nm)	47.7	46.6	58.8	63.4	47.8	52.9	3.5
Standard deviation (nm)	23	25	20.4	28.1	23.3	24	1.3
Concentration (Particles /ml)	1.81E+07	1.89E+07	7.63E+07	6.67E+07	7.60E+07	5.12E+07	1.35E+07

3.2 Results of QCM-D measurements

The measurement of frequency made with the q.sense device can be seen in full in Figure 3. The proportion of lipid vesicles with attached biotin was measured to be 0.64 ± 0.03 %, given the frequency drop of 9.92 Hz, measured when the streptavidin solution was introduced to the quartz crystal. To the left in Figure 3 the formation of the lipid bilayer is seen, as indicated by the drop in frequency as lipid vesicles bind to the quartz crystal, followed by a frequency increase as the vesicles burst and the lipid bilayer is formed. Then the frequency drops as streptavidin is introduced and binds to the biotin attached vesicles in the lipid bilayer. Lastly a third drop in frequency is seen as another solution of lipid vesicles with attached biotin is introduced and binds to the streptavidin.

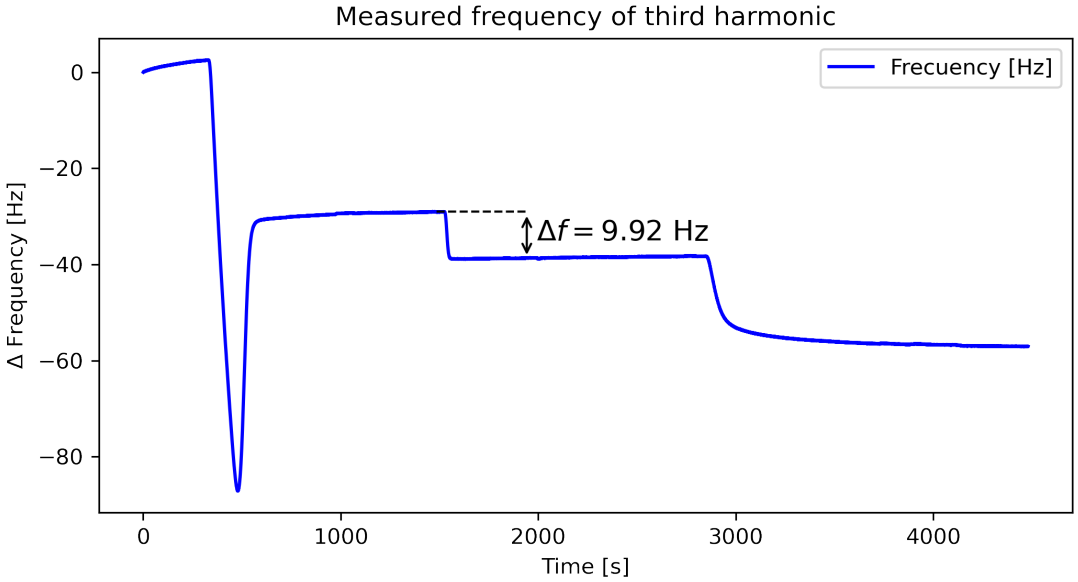

Figure 3: Measurement of variations in frequency as a result of attachment of lipid bilayer to the quartz crystal (first sudden drop) and attachment of streptavidin to the biotin lipids (second drop). The frequency drop of 9.92 Hz has provided the necessary information with the help of which the concentration of lipid vesicles was determined to be equal to 0.64 ± 0.03 %.

3.3 Discussion of NTA results

The measurements made in the devices scattering mode could be said to be satisfactory. Some uncertainties with the measurement remains as before the measurements were made, an air bubble was identified in the measurement device. This and residues from previous measurements could have caused inaccuracies and bright spots. These bright spots caused a less than optimal contrast between particles and background and thus a saturation of the measurements. This effect was most noticeable during the fluorescent measurements, which was also labeled by the measurement device as measurements with high noise. A possible cause of which could have been a too high concentration of fluorescent vesicles.

An important point to ponder is the significant difference in the size of the particles measured in scattering and fluorescent mode. Particles in the second experiment had a noticeably smaller diameter. This could be due to the fact that using the factor of fluorescence in measurements enables detection and tracking of smaller sized particles, decreasing the total average diameter. However, due to the noise of the measurement this effect is not as clear as the experiment attempted to show.

3.4 Discussion of QCM results

The proportion of lipids with attached biotin was found to be 0.64 ± 0.03 % which is not unreasonable. It was expected that the normal lipids would make up a large majority. One main uncertainty of the result comes from the fact that each streptavidin molecule possibly can bind two biotin molecules, which was mentioned in the preparation lecture before the lab. It is however unknown what proportion molecules actually bind to two biotin, and it would seem unlikely that two biotin-lipids would be spaced the same length apart as the distance between the binding spots on streptavidin. However the lipid bilayer could be seen as a two-dimenstional fluid, allowing the biotin-lipids to move around, meaning that given enough time all streptavidin molecules would bind to two biotin. However this process of binding already attached streptavidin is competing with new streptavidin reaching the surface, meaning the proportion of streptavidin that actually binds to two

biotin molecules is uncertain. One could however make a case for doubling the proportion of lipids with attached biotin.

The size of the streptavidin molecules also create an upper limit on the number of molecules that fit in the measured area of $1\,\mathrm{cm}^2$, essentially creating an upper limit on the proportion of biotin-lipids to be measured. The surface area of the streptavidin molecules is about $25\,\mathrm{nm}^2$[6]. Meaning we effectively would not be able to tell if the proportion exceeded 4 %, luckily the proportion we found was far from the limit, meaning the size of the molecules shouldn't have affected the results strongly.

4 Conclusions

Two techniques for studying lipid vesicles and bilayers was examined. The first, nanoparticle tracking analysis was performed to measure brownian motion of particles and using this to calculate the particle size. As such, the diameter of the lipid vesicles was measured to be 87.1 ± 1.3 nm in scattering mode. However the fluorescent mode measurement was subject to noise saturation and the resulting diameter of the vesicles with attached fluorophores of 56.4 ± 3.2 nm is relatively uncertain. The second experiment utilized quartz crystal microbalance to examine the formation of a lipid bilayer and the the concentration of lipid vesicles with attached biotin to vesicles without. This concentration was found to be $0.64 \pm 0.03\%$. The results for these two experiments indicates the techniques utilities in studying lipid vesicles and their behaviour.

References

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[7]

5 Appendix A

5.1 An Invention Inspired By QCM

5.1.1 Piezoimmunosensor

A piezoimmunosensor places antibodies on a quartz crystal microbalance (QCM) to detect how minute changes occur in mass, which in turn is detected as a change in resonance frequency of the crystal [1]. This invention is an apparatus with one or more piezoelectric mass sensors, here QCM, to be used for diagnostic and analytic purposes. Most importantly, to detect immunochemical analytes that are relevant to that diagnostic purpose. Thus allowing the detection of particles in real time, with relatively few disadvantages of previous biosensor related inventions. Each of the piezoimmunosensors mass sensors includes a piezoelectric crystal with a bed of recombinant antibodies made up of polypeptides that help make up proteins by binding numerous amino acids that are specific for a particular antigen together. The binding of antigens to the antibodies changes the mass of the receptor surface and this change in mass is shown as a change in resonant frequency of the crystal.

5.1.2 Patent Key claims, and discussion

- Claim 1: The piezoelectric sensor surfaces utilize preformed anti-body receptors.
- Claim 2: The invention uses multiple sensor surfaces, each surface formed for one specific target substance to be detected. These target substances are generally anti-bodies.
- Claim 8 + 9: One of the apparatus layers is aimed towards binding streptavidin to a biotin layer, in claim 9 the surface first allows the binding of biotin to form a layer, in order to bind streptavidin.
- Claim 10: Specifies the receptor of the apparatus as an electrode for the piezoelectric mass sensor.
- Claim 11: Specifies the piezoelectric mass sensor as a Quartz crystal microbalance.
- Claim 12: Specifies the procedure for performing measurements with the apparatus. Many of the following claims such as claims: 13,14,15,16,18,19,20,21,22 expand on the method described in claim 12.
- Claim 23: Describes the components of a kit to perform measurements. Included in the kit are for example the apparatus with the piezoelectric mass sensor. This claim and the following claims 24-33 seem to specifiy how the invention / product is to be distributed. For example claim 33 is very similar to claim 11 and reads: "The kit of claim 23 wherein the receptor layer comprises an electrode for the piezoelectric mass sensor".

Claim 10 combined with claim 11 were specifically interesting as they claim apparatuses using all types of piezoelectric mass sensor, while only specifying that they will use a QCM-chip as mass sensor. This seems to be only to hinder others from creating very similar machines, even though the inventors have no intention to make similar appartuses using other piezoelectic mass sensors. Furthermore claim 12 and 23 are similar in that they specifiy how the invention is to be used and distributed, and the claims following expand on the information provided in 12 and 23 respectively.