Charlène De Conto
Chemistry Degree Project C
23rd of March- 5th of June 2015

UPPSALA UNIVERSITET

Effect of Ubiquinone 10 and Solanesol on liposome behaviour under osmotic stress.

Department of Chemistry- BMC (B7:2)
Supervisor : Victor Agmo Hernàndez and Emma Eriksson

Summary

In this project, the stability of the POPC liposomes modified with solanesol or ubiquinone is studied. POPC liposomes containing ubiquinone are known to be very resistant from previous work in this laboratory. Also, it has been shown that ubiquinone has a more stabilizing affect on liposomes than solanesol (a molecule similar to ubiquinone but without the quinone head group). However a recent published article has shown evidence that, both of them have the same effect on the response of liposomes, subjected to osmotic stress. In this project these apparently contradictory conclusions were tested in a system in which the size and composition of the liposomes was better controlled. In order to study the membrane stability, the liposomes were submitted to osmotic stress by addition of salt. First, the optimal induced osmotic stress to observe differences between the different composition of liposomes was established. It was found that with a concentration difference of 25mM NaCl, pure POPC liposomes were affected while the more stable POPC liposomes containing cholesterol was not. Secondly, the response to osmotic stress in this range was studied with liposomes containing ubiquinone and solanesol. This was done by employing a fluorometer and adding small volumes of a concentrated salt solution into a diluted solution of liposomes. The fluorometer allowed measuring both the decrease in the liposomes internal volume as well as changes in the membrane stability. The results shows that ubiquinone has a more stabilising effect on liposomes than solanesol in agreement with previous work with POPC liposomes. The new data in contradiction with other literature sources could be explained by the use of different lipid compositions. Also, the liposomes employed in the current project are better controlled in terms of size and composition. Finally, the experimental methods employed here are more reliable because they test the whole suspension instead of analysing single liposomes

Introduction

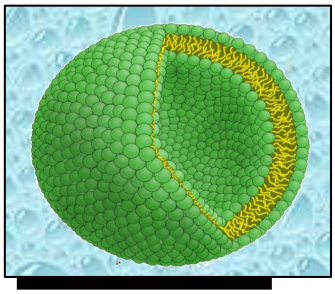

figure 1: Illustration of a liposome

A liposome is a nano sized vesicle with a spherical shape that is produced from phospholipids. The lipids in the liposome arrange in a bilayer structure in aqueous solution thanks to their amphiphilic nature, see figure1. Liposomes are used in several studies, much work is done within the development of new drug-delivery systems. [1]

In this project, liposomes formed by the lipid 1-palmitoyl-2oloeyl-sn-glycero-3phosphocholine (POPC, see figure 2a) and modified with a small amount of ubiquinone or solanesol are studied. Ubiquinone, also called Coenzyme Q10, is a very important biomolecule that has been studied extensively because of its participation in aerobic cell respiration and its function as an antioxidant. It has been recently shown that it is possible to incorporate Q10 into artificial liposomes in order to make them more resistant and stable [4]. However, one does not know the exact mechanism by which ubiquinone stabilizes the membrane, which is why the liposomes containing solanesol are also studied. Both of these two molecules have very similar structures with a long polyisoprenoid chain (10 units for ubiquinone and 9 units for solanesol) but they have different headgroups, see figure 2b and 2c. Comparing the effect of these two molecules could help discerning whether the increased stability is caused by the isoprenoid chain or by the quinone headgroup.

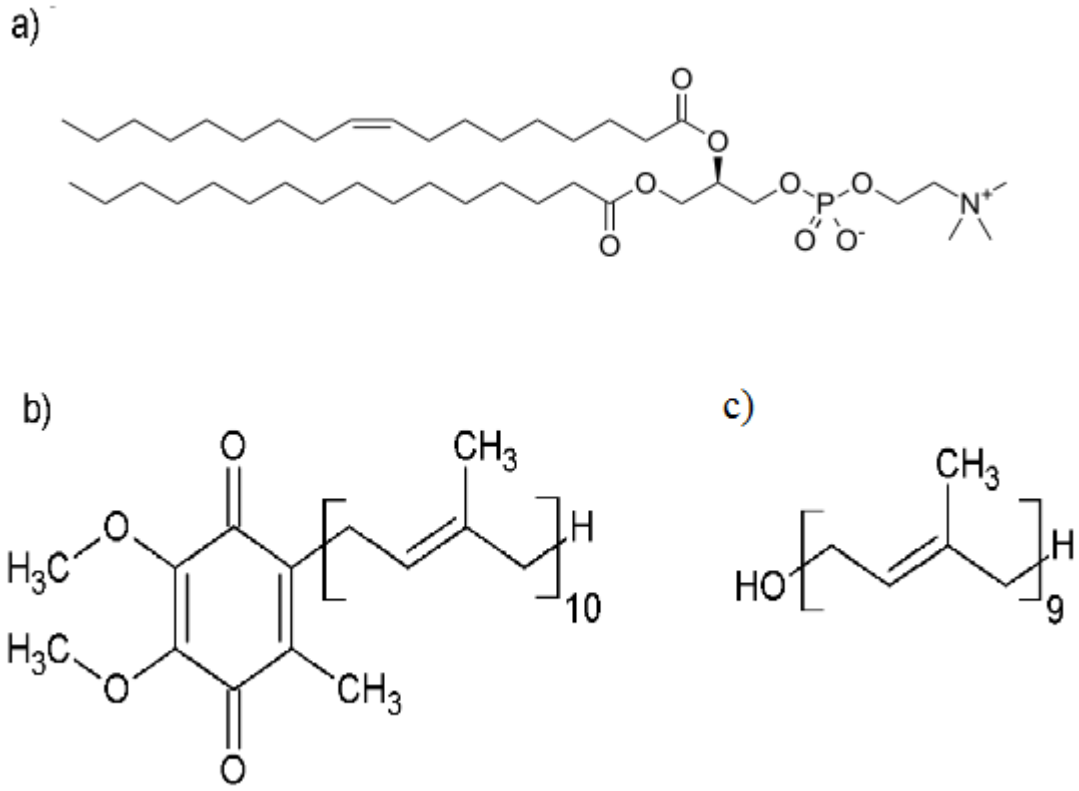

figure 2: a) is the structural formula of POPC ; b) is the structural formula of ubiquinone-10 ; c) is the structural formula of solanesol

A previous work about ubiquinone in liposomes has been done in this laboratory [4]. There, it was proved that solanesol did not have the same stabilising effect on POPC liposomes that ubiquinone had. The results show that the polyisoprenoid chain is probably not the origin to the stabilizing effect of the liposomes, but the quinone headgroup is. However, and in contrast with this results, a recent article[2] using a different method, has suggested that solanesol has the same stabilizing effect on liposome as ubiquinone. In this article, giant liposomes (around 2µm) were subjected to osmotic stress. However, theses model is not ideal. Among other problems, because of their big size, the liposomes are not very stable. Also, in such preparations, the amount of multi-lamellar vesicles is likely to be very large. This structures would respond differently than uni-lamellar vesicles. Finally, the optical microscopy method employed is not optimal to actually follow changes in size and shape of structures as flexible as liposomes. In order to have a more reliable system for these studies, the liposomes need to be smaller. In this project, by using a better controlled lipid membrane-based systems, the resistance to osmotic stress is studied for POPC liposomes with a diameter of 100nm. Liposomes were prepared from pure lipid as well as containing ubiquinone and solanesol. By adding a solution of salt or sugar in the solution of liposome, an osmotic difference is created, causing the water inside the liposome to go out, which make the liposome shrink, see figure 3.

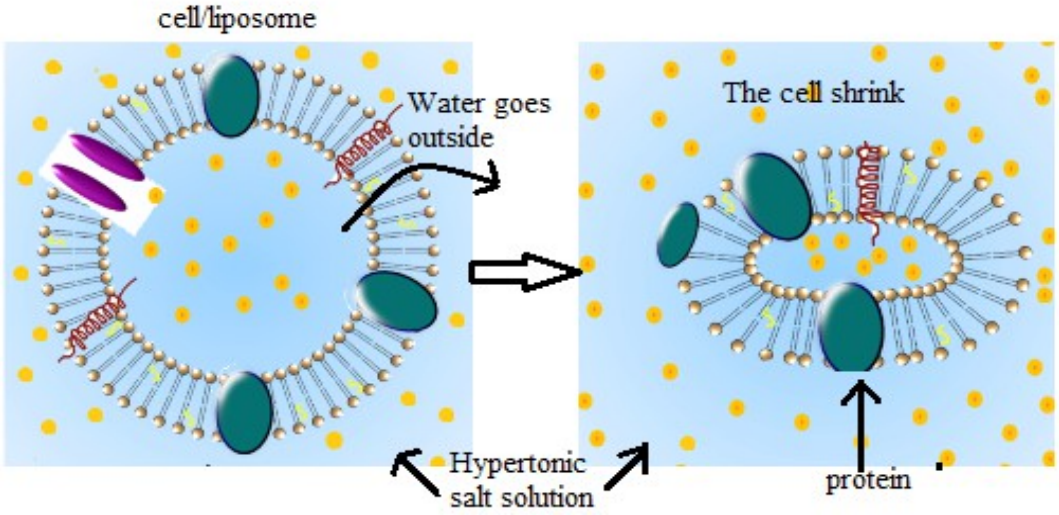

figure 3: qualitative explanation of what is happening to a liposome because of osmotic stress

A preliminary work was done prior to this work POPC liposomes were subjected to osmotic stress. By using the cryo-Transmission Electron Microscope (to help interpreting the results, see figure4), it was possible to see how the liposomes reacted to the addition of 1M NaCl. Here, it was proved that pure POPC liposomes and the POPC liposomes with ubiquinone or solanesol transformed into multi lamellar structures after the addition of salt, see figure5a. As a reference some liposomes were supplemented with cholesterol, these liposomes were affected in a different way, see figure 5b. The first aim of this project was to determine the concentration of salt affecting the pure POPC liposomes but not the liposomes containing cholesterol. Cholesterol is a biomolecule well known for increasing the stability of the membrane [5] and it has been proved that cholesterol has a very strong stabilizing effect on liposomes. Liposomes only composed of POPC are usually very unstable and will respond to osmotic stress. In order to obtain the right salt concentration, POPC liposomes composed on 40% of cholesterol and liposomes composed of pure POPC were studied by two different methods, the first one is using the Dynamic light scattering instrument (to measure the size) while the second method is fluorescence spectroscopy (see method section for the details). Once the appropriate working salt concentration was found, it was possible measure the quenching of the the POPC liposomes modified with around 4% ubiquinone or solanesol and compare the results.

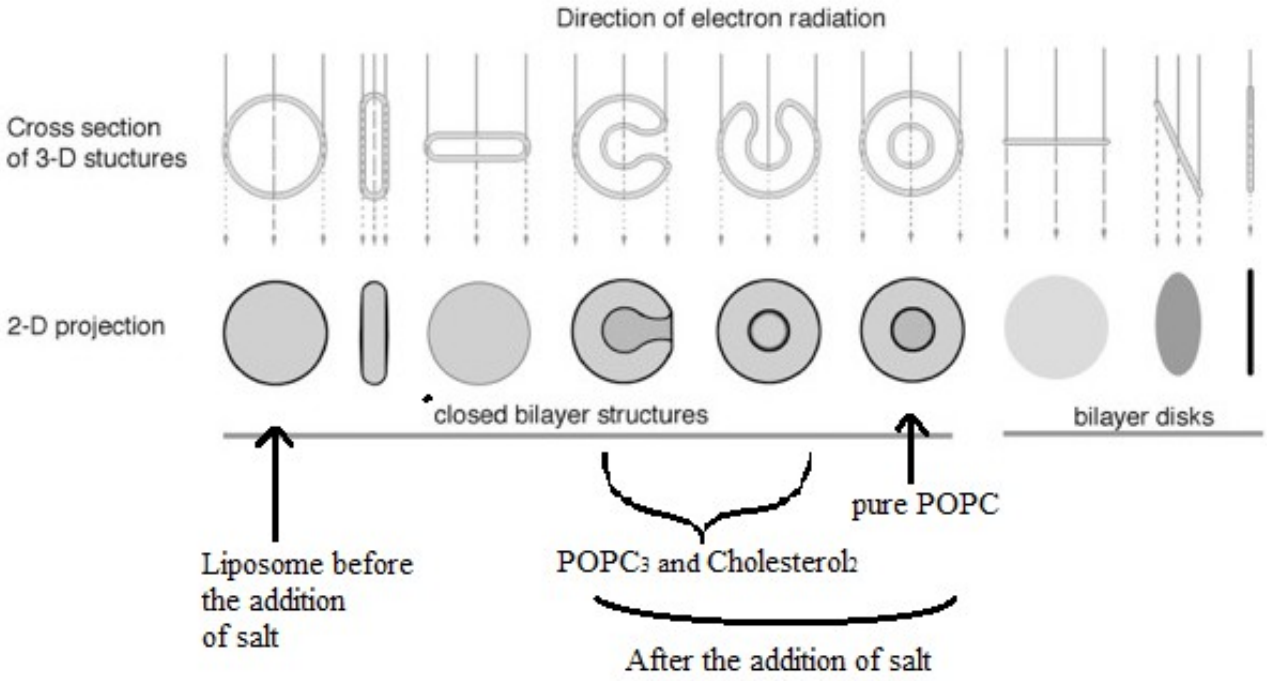

figure 4: Cryo-TEM projections. Using this picture, one can interpret the results obtained with the cryo-TEM for the liposomes composed on POPC and cholesterol and the pure POPC liposomes after the addition of salt.

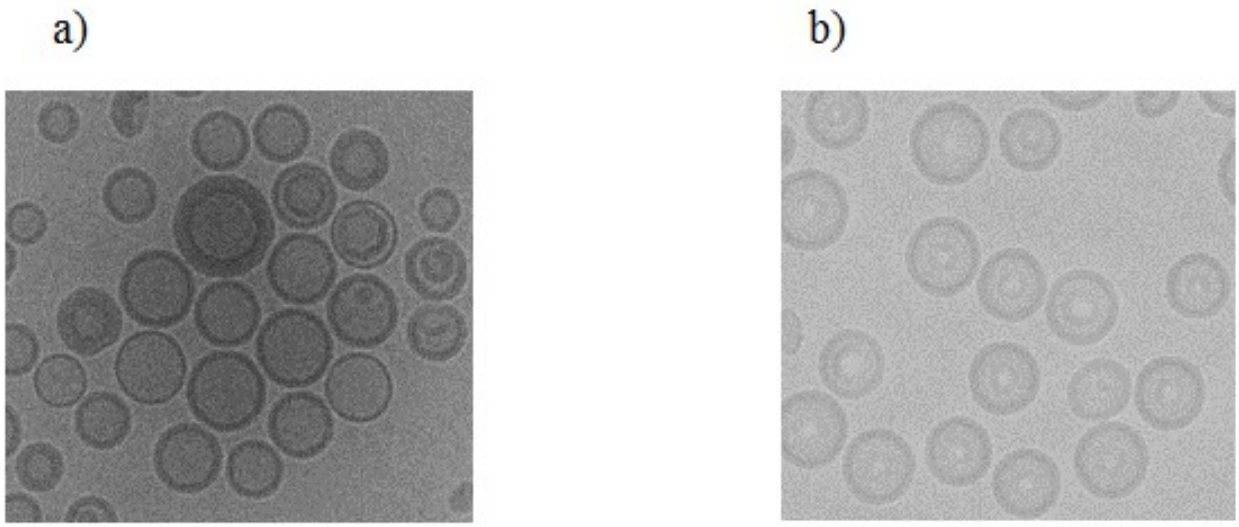

figure 5: a) POPC+ 1M NaCl. The uni-lamellar liposomes have transformed into multilamellar b)POPC+ cholesterol+ 1M NaCl. Because of a too strong membrane, the liposomes haven't transformed into multi-lamellar, but they are "half way".

Method

- Preparation of the liposomes

Four compositions of liposomes will be studied: POPC, (3:2) POPC and cholesterol, (25:1) POPC and solanesol, and (25:1) POPC and ubiquinone. First, one needs to create the lipid film by weighting the components corresponding to the aimed composition of the liposome, dissolving them in chloroform ($CHCl_3$) and removing the solvent using Nitrogen gas. The lipid film formed need to stay over night in a vacuum chamber to remove traces of solvent. Thereafter, the liposomes can be prepared. If the DLS instrument is used: 1mL of extra pure water (MQ-water) is added to the lipid film, however if the fluorometer is used: 1mL of a solution of 5-(6)-carboxyfluorescein (CF) is added to the lipid film. The solution is five times frozen in liquid Nitrogen, heated in a hot water bath and vortexed, before being prepared by extrusion with a double syringe and a filter of 100nm or 200nm. The solution of liposomes created need to stay a night on the bench. This is needed in order to guarantee that the liposomes suspension becomes homogeneous [6]. The third day, the solution of liposome can be used for the measurement. The liposomes extruded with a solution of CF need to be separated before use with a gel filtration column for size separation eluted by PBS. This column is used in order to obtain a solution with CF inside the liposome but without CF outside the liposome. This gel filtration column is separating the particles depending on their size, the bigger the particle is, the faster it will "pass through" the column. Since the liposomes formed should have a diameter around 100 nm, the first particles that we will obtain will be the liposomes that we need.

- Dynamic light scattering

This method allows us to determine the size distribution of the liposomes in an aqueous suspension. The liposomes motion in water follow the brownian motion, causing fluctuations in the intensity of the light scattering. From this fluctuations the size of the liposomes with and without osmotic stress is characterized.

- Fluorescence Spectroscopy

With the help of fluorescence Spectroscopy one can measure the fluorescence of a sample at one excitation wavelength and measure the emission at another wavelength. In this project the two wavelengths chosen are 495nm and 520nm, respectively. The liposomes are filled with a solution of carboxyfluorescein (CF), a fluorescent dye with self-quenching properties at high concentrations. Indeed, it is believed that at high concentrations CF dimerizes, and that the dimers are not fluorescent.[7] When the liposomes are subjected to osmotic stress two opposing processes affecting the fluorescence intensity are occurring: quenching and leaking. The quenching corresponds to the shrinkage of the liposomes, causing a decrease of their internal volume and, therefore an increase of CF concentration inside the liposomes, leading to fluorescence quenching. Whereas the leakage correspond to the formation of holes in the liposomes and the release of small amounts of CF which, once in the outer solution, are found at a concentration low enough to avoid quenching but high enough to generate a fluorescence signal. By adding salt in the solution the concentration inside the liposome is increasing which decreases the intensity in fluorescence: this is the quenching. The concentration in the liposome is also decreasing when the CF leaks from the liposome, which can be seen as an initial increase of the intensity of fluorescence due to less quenching. By varying the concentration of the solution of CF inside the liposome, it is possible to investigate on both effects separately. When the liposomes are filled with a concentrated solution of CF (100mM), only the leakage is measured. Both reactions are occurring, but the intensity of fluorescence cannot decrease because of the quenching, since it is already at the lowest intensity, see figure 6. Whereas, if the liposomes are filled with a dilute solution of CF (15mM), both reactions are measured. By using the leakage measurement from the liposomes filled with 100mM,

one is able to correct the measurement found for the liposomes with 15mM and get the right quenching results. One can suppose that the shrinkage may change the concentrations, but this change does not need to be taken in consideration because it is assume to be very little.

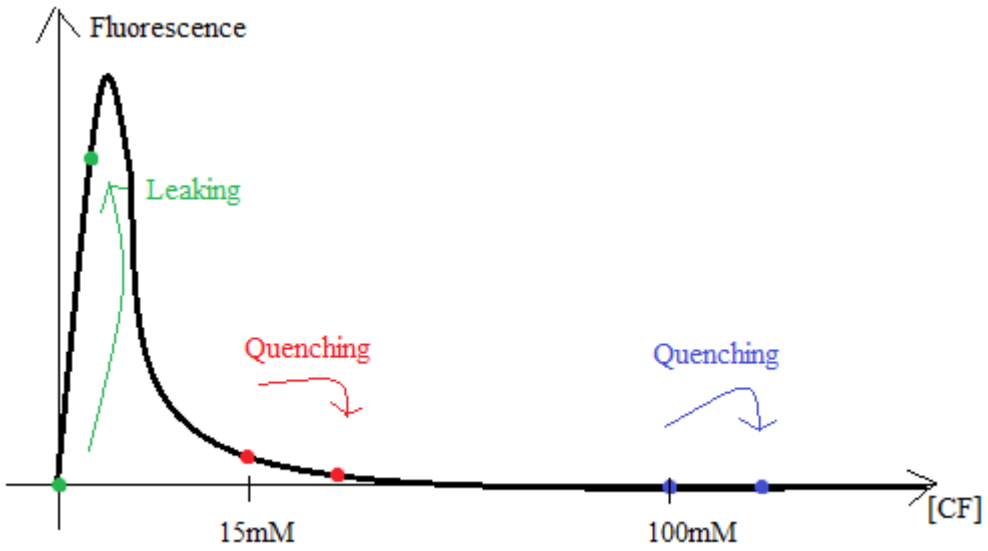

figure 6: Intensity of fluorescence against the concentration of CF in the solution

To determine the right concentration to be used in later investigations, an instant mixer equipment is used where two equal volumes of two different solutions are mixed. This means that the solution of liposomes diluted to 120µM and filled with a 15mM solution of CF is mixed with a solution of salt diluted to 20, 50, 100, 200 or 500mM in phosphate buffered saline (PBS) or a solution of PBS (composed of 150mM NaCl and 10mM Phosphate). This PBS solution is isotonic with the CF solutions employed.
In the second experiment, a small volume of a concentrated solution of salt is added in a dilute solution of liposome. This time, the leakage as well as the quenching of the liposomes are studied. In order to study the leakage of liposomes by addition of salt, a solution of 100mM CF is used and the solution of liposomes is diluted to 6µM. Whereas, in order to study the quenching of the liposome a solution of CF diluted to 15mM is used and the liposomes are diluted to 60µM. The solutions of liposomes are very diluted in order to avoid the filter effects.

- NaCl vs Sucrose

Experiments were repeated with sucrose instead of salt to characterize whether the small size of the Na and Cl ions could allow them to be transported through the membrane, thus decreasing the effective stress. Lipid membranes are well known as more impermeable for sucrose transport than for salt transport.

Results/discussion:

Determination of the right concentration of salt affecting the pure POPC liposomes but not the POPC liposomes containing cholesterol

In this part of the project, some manipulations have been performed with the liposomes composed on pure POPC and on (3:2)POPC and cholesterol. The liposomes only composed of POPC are not stable and they are supposed to react very easily to osmotic stress. On the contrary, the liposomes composed on (3:2)POPC and cholesterol should be the most stable liposomes studied in this project, it should be hard to make them shrink.

- Dynamic Light Scattering

The size of the liposomes was determined with the DLS instrument. The expected result was to get liposome radius around 100nm without any salt added since the filter used for this experiment were 200nm. For these experiments, 100 nm liposomes were preferred over 50 nm liposomes as changes in size would therefore be clearer. When salt is added to the solution, it was expected to get smaller radius and even smaller for the liposomes composed on pure POPC than with the one with (3:2) POPC and cholesterol. Unfortunately, the results were difficult to interpret, the radius found was either too small or too high. This could be due to the presence of a lot of multi lamellar liposomes, especially with the liposomes composed on (3:2)POPC and cholesterol. The second explanation is that we cannot know how the liposomes are reacting to osmotic stress, we are expecting that the liposomes are shrinking, so they will have a smaller radius, but it is possible that they are becoming flatter and in this case the radius will be higher, see visual explanation in figure 7.

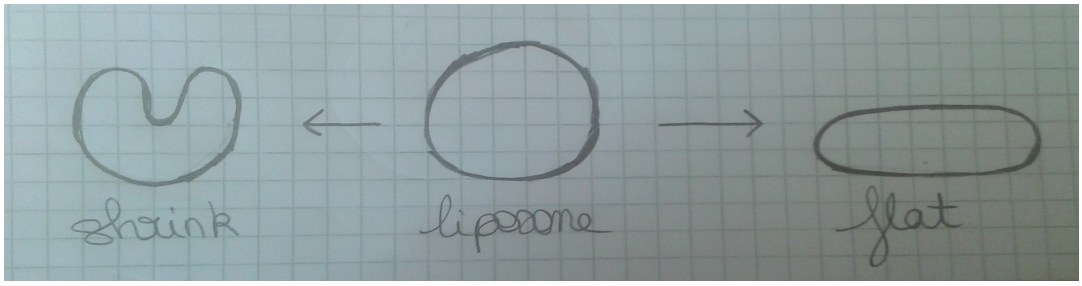

figure 7: qualitative explanation of the possible reactions of the liposome to osmotic stress : shrink or becoming flat.

- Fluorometer and instant mixing

This experiment is used to get an order of the concentration of salt needed to create osmotic stress in a range where differences between different liposome compositions can be observed. The osmotic stress subjected to the liposomes was studied by using the instant mixer. The intensity of fluorescence should decrease when the salt is added, and then increase again when the liposomes are leaking. The results found were not as expecting. The intensity was always decreasing even if no salt was added in the solution of liposomes. That might be due to a contamination of the cuvette or to the fact that even if the instant mixer is rinsed with the currently used solution a couple of time before the measurement, the cuvette cannot be perfectly cleaned. Nevertheless, it could be concluded that the right concentration of salt should be between 10 and 25mM in the cuvette. Also, something is happening with the liposomes (when increasing the salt concentration) composed on pure POPC but not so much with the one composed of (3:2)POPC and cholesterol, see table 1.

Table 1: Results obtained using the fluorescence spectrometer and the instant mixing. One can see that the relative intensity of fluorescence decrease more for the liposomes composed only on POPC than for the liposomes composed on (3:2) POPC and cholesterol

Composition of the liposome	Relative intensity of fluorescence with 10mM NaCl in the solution	Relative intensity of fluorescence with 25mM NaCl in the solution
POPC	0.98	0.84
(3:2)POPC+ cholesterol	0.95	0.92

- Fluorometer

In order to confirm the results found with the instant mixer, this method is used where small volumes of salt is added to a diluted solution of liposome. It could be expected that the intensity of fluorescence will decrease when the salt is added and that the intensity should stay constant or rather increase a little. In order to get the right concentration, the liposomes composed on (3:2)POPC and cholesterol shouldn't react to the addition of salt as fast as the one composed on pure POPC, which mean that the intensity of fluorescence should decrease faster with the pure POPC liposomes. The results obtained, seem contradictory, but it is needed to consider that leakage and quenching are happening simultaneously and giving opposing trends in the fluorescence intensity. From these experiments, however, it was possible to establish that the concentration of salt in the cuvette should be around 25mM in order to affect the POPC liposomes while the POPC:cholesterol remain virtually unaffected.
To be able to normalize the intensities from the above experiments, the intensity of the completely quenched suspension was determined. For this, the concentration of salt corresponding to the minimal fluorescence intensity needed to be known. It is observed that, after 250 mM in pure POPC liposomes, the decrease in fluorescence intensity corresponds only to the dilution of the sample and not to further shrinking of the liposomes. Therefore, 250 mM NaCl was chosen as the concentration at which full quenching could be obtained. Furthermore, the fluorescence intensity corresponding to maximum leakage was also determined for additional normalization purposes. To this end. the liposomes were destroyed by the addition of the detergent Triton X-100, thus causing the release of all the encapsulated CF.

Studies of the liposomes containing solanesol and ubiquinone

In this part, the same methods as before are used, the only difference is that the concentration of salt studied is only between 5 to 25mM in the cuvette expect 250 mM which is used to determine the fluorescence intensity at maximum quenching. Here the two aspects of the liposomes reaction are taken in consideration,which meaning that the leakage and quenching are studied separately. It is possible to correct the quenching results using the rate of the leakage.

- Leakage

In order to correct the results found with the liposomes extruded with 15mM CF, one have to make the assumption that the leakage rate is the same with 100mM CF than with 15mM CF. The second assumption is that the leakage is in a pseudo-zero order because the concentration is assumed to be constant inside the liposome and also because the leakage is calculated after the shrinkage of the liposomes. This also assume that the rate of leakage is constant in the short time window studied and it is confirmed by the observation of a linear leakage. Given that the leakage is very slow, this assumption is valid. By adding the salt in the cuvette, the intensity of fluorescence increases. The degree of leakage as a function of time for each samples and each concentration of salt is calculated, using the equation bellow.

$$X_{CF} = \frac{I_t - I_0}{I_{max} - I_0} \qquad Eq\ 1$$

I_0 correspond to the intensity obtained before the salt is added in the cuvette, it changes for every concentration of salt used. I_{max} correspond to the intensity reach when a detergent is added in the cuvette (here the tritton-X) (maximum possible leakage).

Using the calculated data, one can plot the normalized fluorescence (X_{CF}) against the time for each concentration of salt, the slope of each curve is then obtained. The slope correspond to the rate of leakage and it is plotted against the concentration of salt in the cuvette. Three replicates were made, the average of each slope were calculated, giving the figure 8 below.

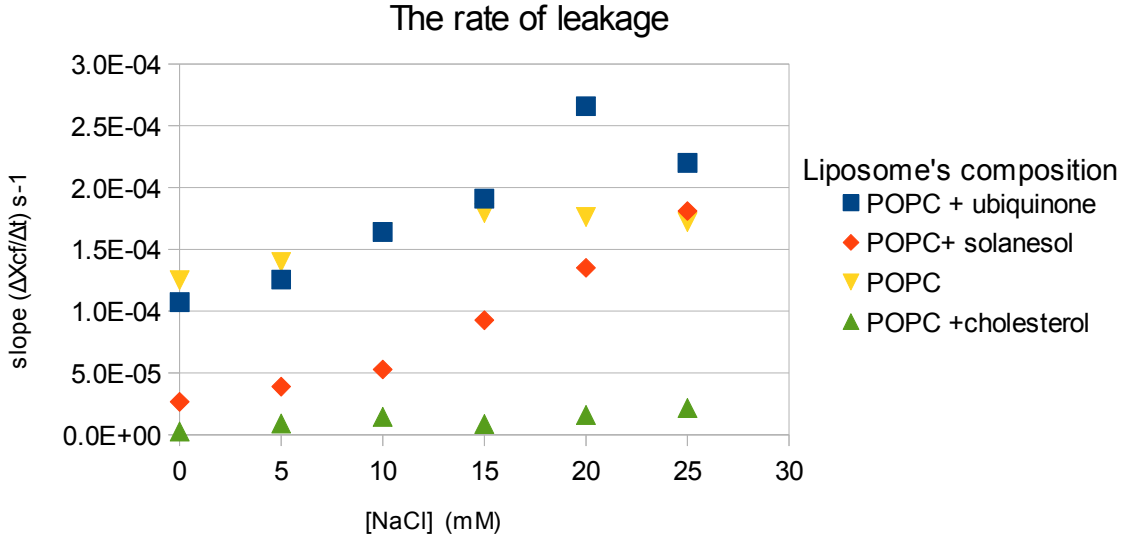

figure 8: the slope of the proportion of CF against the time is plotted against the concentration of salt in the cuvette.

The leakage for each composition appear to be very low, however one can notice that there is almost no leakage in the liposomes containing cholesterol. Surprisingly, the POPC liposomes containing ubiquinone together with the pure POPC liposomes are the one which are leaking the most.

– Quenching

By adding the salt to the sample, the intensity of fluorescence decreases directly and then stay constant or increase a little bit. Figure 9 shows how all the liposomes reacts after an addition of 25mM NaCl. Since the intensity of fluorescence at the beginning is not the same for each composition, the relative intensity is used.

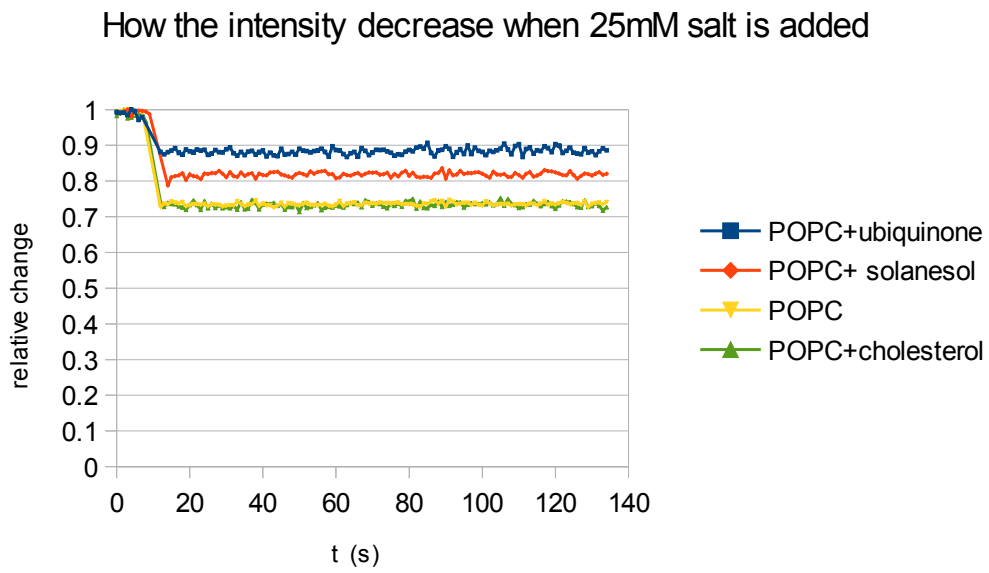

figure 9: 25mM NaCl is added in different solution of liposomes, the fluorescence is decreasing with a different rate for each composition.

In figure 13, the leakage is not been taken in consideration, but one can still notice that the liposomes containing ubiquinone are the ones who have less quenched. The liposomes composed of POPC and cholesterol are quenching more than expected, although they should be the ones which are quenching less and not the contrary. This difference is due to the fact that the leakage is not corrected in this figure. And as proved previously in figure 8, the POPC liposomes modified with cholesterol are the one which are quenching the less.

In order to retrieve the actual relative change in CF quenching by taking the leakage in consideration, some calculations have to be done. Firstly, the increase in intensity due to leakage for each concentration of salt is calculated with equation 2. Secondly, the average of the intensity of the 20 last measurement for each concentration of salt in the cuvette is calculated and finally the percentage of the CF which haven't quenched is calculated, see equation 3.

$$I_{leakage} = k * t * (I_{max} - I_0) \qquad \text{Eq 2}$$

k is the calculated rate of leakage, changing for each concentration of salt; t is the time taken for each measurement, around 160s; I_{max} is the intensity of fluorescence obtained by adding the detergent, triton X-100; I_0 is the intensity of fluorescence obtained before the addition of salt, it change for each concentration of salt in the cuvette.

$$\%CF_{haven't quenched} = \frac{(I_{20} - I_{leakage}) - I_{250}}{I_{max} - I_{250}} * 100 \qquad \text{Eq 3}$$

I_{20} is the intensity of the last measurements for each concentration, I_{250} correspond to the intensity minimal reached with 250mM NaCl.

Since the intensity of fluorescence without any salt is different for each composition of liposomes, an other calculation is done in order to give the relative change (see equation4), with this calculation one is able to compare the obtained results. All the data are given in table 2.

$$relative\ change = \frac{\%_{CFconc.salt}}{\%_{CFconc.salt=0\text{mM}}} \qquad Eq\ 4$$

Table 2: The ratio from 0 to 1 of remaining fluorescence (not quenched) in the trapped CF solution each composition of liposome and each concentration of salt in the cuvette

[NaCl] (mM)	POPC	POPC+cholesterol	POPC+solanesol	POPC+ ubiquinone
0	1.00	1.00	1.00	1.00
5	0.94	0.87	0.96	0.96
10	0.88	0.83	0.94	0.90
15	0.79	0.73	0.85	0.85
20	0.74	0.66	0.77	0.78
25	0.64	0.63	0.72	0.78

Looking at the results with the relative changes, one can notice that there is something unusual with the liposomes composed on (3:2)POPC and cholesterol. Indeed it was expecting that these liposomes would quench less, but this is due to the assumption that the minimal intensity is reached for a concentration of 250mM salt in the cuvette. Whereas as shown in an other work, see figures 6 and 7, the liposomes composed of POPC and cholesterol are very resistant, and even with 1M NaCl in the solution the liposomes do not create multi-lamellar structures. However, if we only look at the results for the three other liposomes, one can conclude that the liposomes composed on POPC and ubiquinone are more resistant to osmotic stress. Solanesol, on the other hand, seems to decrease the degree of quenching, although its effect is not as pronounced as that of ubiquinone. A t-test is done and prove that there is a significant difference between the liposomes containing solanesol and the one containing ubiquinone, this prove that ubiquinone and solanesol don't have the same stabilizing effect on POPC liposomes.

- Control test

In order to know if the intensity is really decreasing because of the liposome shrinkage or the dilution of the sample, a CF dilution test is done. 2mL of a diluted solution of CF is added to a cuvette, the concentration of this solution correspond to the concentration of CF which should be inside the liposome (around 1.6µM). To this cuvette small volumes of salt are added and the intensity of fluorescence is measured. By adding the salt to the cuvette, the intensity of fluorescence stayed constant which means that nothing is happening with the CF solution. A non-significant decrease in intensity also appears when the concentration of 250mM NaCl is reached, this diminution of the intensity is due to the big volume of salt added in the cuvette. Since the intensity only decreased 5%, one can conclude that the results obtained do not need to be corrected for this.

NaCl VS Sucrose

The aim of this part is to know if the results will be different when replacing the small chloride and sodium ion by a big sugar molecule. The solution of sucrose is very viscous and have is own osmotic factor that we need to take in consideration if some calculations are done. Using the same approach as with salt, one can notice that the solution in the cuvette is not perfectly mixed which is why a second method is used. In this second case, liposomes are added to sugar solutions instead of sugar being added to the liposomes. With this method, the result cannot be used because instead of decreasing more and more by adding the sucrose, the intensity was decreasing a lot and followed by an increase.
This part of the project was suspended, since a reliable parameter for normalization of the data was not available. But it would be possible to do it with the utilisation of a “clean” instant mixer, where two equals volumes of a dilute solution of sugar and a dilute solution of liposome are mixed or if the cuvettes used were perfectly washed between each measurement.

Conclusion

The concentration of salt in the solution, affecting the POPC liposomes but not the liposomes composed of (3:2) POPC and cholesterol is determined to 25mM. It has also been shown that the DLS instrument is not the most suitable for this experiment, on the contrary the fluorometer appear to be a better method. In the recent article[2], besides using a DLS instrument and giant liposomes, they claimed that ubiquinone and solanesol have the same stabilizing effect on liposomes, which have been proved to be false. By using the fluorescence spectroscopy instrument and the right concentration of salt, the POPC liposomes containing ubiquinone and solanesol have been studied in a better way. Using liposomes filled with a 100mM solution of CF, the rate of leakage have been found, which helped to correct the results obtained with the liposomes filled with a diluted solution of CF. The calculated percentage of liposomes who haven't quenched shows that ubiquinone have a stronger stabilizing effect on liposomes than solanesol. One can then conclude that the ubiquinone stabilizing effect is due to its head group. Furthermore, a conclusion can be done about the work on the leakage, indeed it is very important and essential to take in consideration the leaking part. In this project, it was unavoidable to take this into account since the POPC liposomes are known to be very soft. Unfortunately, this aspect of liposomes reactions are rarely taken in consideration.[2] This work shows the importance of carefully characterising the leakage under osmotic stress, if the leakage is ignored (as it is usually done), misguiding results can be obtained.

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