

## Liposome Preparation

### Introduction:

Liposomes are just hollow spheres of lipids, i.e. some lipids form membranes that close on themselves forming liposomes. The main component of liposome membranes is dipalmitoyl phosphatidil choline (DPPC, PC or EPC- *egg phosph...* choline). In principle, one could make liposomes using PC only. However, some other compounds are added in order to improve stability or other structural properties. Two compounds are added: dipalmitoyl phosphatidil glycerol (DPPG or PG) and cholesterol. Apparently, cholesterol has the effect of making the membrane less permeable by filling up holes or disruptions. More on composition is explained in the first chapter of the pink book and in *Damage to liposomal lipids: protection by antioxidants and cholesterol-mediated dehydration*, by Samuni et al.

The membrane two different phases. One, at low temperatures (say, between 0C and the transition temperature) is kind of a rigid phase. Above the transition temperature, the membrane is in the *gel phase*. The difference in between these two phases seems to be the way in which liposomes are arranged in the membrane. Since the latter phase is somewhat more malleable, redispersion in buffer and extrusion should be both made above the transition temperature.

In the lab we have PC, PG and cholesterol. We have two types of PC and two types of PG. They differ in the number of carbons, which results, in turn, in a different transition temperature. The two PC are:

Name	Carbon #	Product #	Critical Temp (C)
Steroyl	18:0	850365	55
Palmitoyl	16:0	850355	41

The two PG are:

Distearoyl	18:0	840465	55
Dipalmitoyl	16:0	840455	41

Together with the carbon number is given the number of double bonds between carbons, in the format *carbons: double bonds* (i.e. the unsaturation of the molecule).

When choosing which PC goes with which PG one should look at two variables, the transition temperature and the miscibility. If both phospholipids have the same transition temperature, then the resulting membrane will also have the same transition temperature (however, the addition of cholesterol might change the transition temperature as explained in chapter 1 of the pink book). Miscibility is *perfect* for 16:0 with 16:0 and *high* for 18:0 with 16:0 (check table on page 186 of the Avanti Catalog). I always used 16:0 PC with 16:0 PG when preparing liposomes.

## **Composition:**

The pink book suggests the following composition (see below):

- 100 mg EPC
- 40mg cholesterol
- 10mg PG

The molar mass of each component is:

- EPC: 734g/mol
- PG: 745 g/mol
- Cholesterol: 387 g/mol

Hence, the molar ratio of this composition is roughly 10:8:1. This high ratio of cholesterol (almost 50% mol/mol) will have a huge effect on the phase transition (Pink book chapter 1).

In *Damage to liposomal...* they use the molar ratio 10:1 PC to cholesterol and no PG.

Another composition is suggested in *Neveux et al.*: 6:3:1 PC: cholesterol: PG.

I never used the composition without cholesterol, but I tried the other two and it didn't seem to be any difference regarding size or entrapment capability (i.e. size distributions are similar and both emit a flash of light when treated with triton X).

## **Buffer:**

A buffer solution is one which main feature is that its pH is fairly stable under addition of acid or basic compounds. Hepes or TRIS in water serve this purpose, both of which are available in the lab. However they are not interchangeable and for liposome preparations they always use Hepes.

The buffer is the solution in which phospholipids are resuspended (notice that phospholipids are *dissolved* in chloroform/methanol while *suspended* in water solutions. Biologists are very picky with this terminology, as it turns out) to form liposomes. In the buffer solution one also adds the compounds to be entrapped, in this case ATP. Nevaux et al. use 60mg/ml for their entrapment efficiency measurements. In *Damage to liposomal...* they also add the antioxidant Tempol (50mM for liposomes with cholesterol. 1M=1mol/liter).

## **Procedure:**

This is basically a transcription of the procedure outlined in the pink book (pp. 37-38), with a few comments of my own.

*i) Make up the lipid mixture containing 100 mg of egg PC, 40 mg of cholesterol and 10 mg of phosphatidil glycerol (PG), in 5ml of chloroform/methanol solvent mixture (2:1 vol/vol).*

The lipid mixture suggested here seems to have too much cholesterol. In fact, if one looks at the molar ratio it turns out that there's almost one molecule of cholesterol per molecule of egg PC. This high ratio might make the phase transition disappear, as is described in chapter 1 of the pink book. A seemingly more sensible ratio could be used, following Nevaux's paper, is 6:3:1 egg PC : cholesterol : PG. I add the three powders to the empty 100ml flask, add the chloroform (3.3ml) and then the methanol (1.7ml).

*ii) Introduce the lipid solution into a 250ml round bottom flask with a ground glass neck.*

*iii) Attach the flask to a rotary evaporator, evacuate, rotate about 60rpm and immerse the flask in a thermostatted water bath set at 30 C (or above the transition temperature of the lipids used)*

The powder doesn't seem to dissolve readily in the chloroform/methanol solution. Hence it turned out to be a good idea to let the flask rotate over the water bath (at a temperature above the transition temperature of the lipids which is ~ 41 C. I set it at 50 C) for about half an hour before starting the vacuum pump. A very low nitrogen flux could be set up to prevent too much oxygen to get dissolved. If this flux is too high the solvent will then evaporate. I set the nitrogen flux thorough the inlet tap, so it goes directly into the flask. Some of the solvent evaporates inevitably during this time, but the solution thermalizes and the lipids dissolve. Only after this time the vacuum pump should be turned on, together with the cooling coil. Allowing cold water to flow through the coil before turning on the vacuum results in water condensating inside the evaporator. Placing a clamp on the corresponding hose controls the amount of vacuum. The vacuum was set usually above 10inHg, around 15inHg. This can be further controlled by the nitrogen flux. Again, a high nitrogen flux will result in a too rapid evaporation of the solvent, an undesired situation given that one wants a uniform film to be deposited on the walls of the flask.

*iv) Continue until all the liquid has evaporated from solution, and a dry lipid film has been deposited on the walls of the flask.*

*v) Continue for 15 min after the dry residue first appears.*

At some point, when most of the solvent has evaporated, bubbles start forming on the bottom of the flask. At low vacuum (< 10inHg) these bubbles are many and small. For high vacuum (~20inHg) a big portion of the film detaches from the bottom of the flask, and forms a big bubble which

eventually breaks. This weird behavior concerned me, so I emailed technical support from Avanti. This is my message and the response:

Hi, thanks for your help. I have further questions.

So, the foamy residue I get is still useful or I have to start over? I am using

nitrogen to purge the vacuum, 15 minutes before placing the solution in the

rotovap, and all over the evaporation process. Is there something else that

could be wrong? Is it possible that there's some contamination of the flasks?

I'm using Alconox to clean the flasks.

I am not sure why you are so concerned about your material. Everything you have described and observed is not unusual. Lipids can and often do foam during the rotovap process. When the solvents are removed you should get a white solid that is not very dense due to the presence of gas-filled cavities formed during the boiling process at low pressure. Think about it, lipids are very similar to soaps which definitely foam on the rotovap. Foaming is often called "bumping the product", which occurs for most materials on the rotovap when you drop the pressure too much and the entire sample boils violently. It is not necessary to flush your system with nitrogen prior to putting your material on, just purge the vacuum after you have removed your solvents. If you remove a solvent from a material that is a solid in the absence of solvent then wouldn't you expect to recover a solid after you remove the solvents?

I have no idea if you have contaminated your sample; if you didn't rinse your vessel thoroughly then yes it is possible.

If you are concerned that you have contaminated your sample then start over. Or, run a TLC on the mixture against pristine material to assess product purity. See the following links for helpful advice regarding TLC.

<http://www.avantilipids.com/TechnicalTlcAnalysisOfPhospholipids.asp?T=Thin%20Layer%20Chromatography%20Analysis%20of%20Phospholipids>

<http://www.avantilipids.com/TechnicalSolventSystemsLipidMigration.asp?T=Solvent%20Systems%20-%20Lipid%20Migration>

<http://www.avantilipids.com/TechnicalDetectionMethodsForThinLayerChromatography.html>

-The Avanti Technical Staff

So I ignored the bubbles and kept going.

- vi) *Stop rotation of the flask, and raise it from the water bath.*
- vii) *Isolate the evaporator from the vacuum source by closing the tap (not by switching vacuum off, in case suck-back of oil or water occurs)*
- viii) *Introduce nitrogen from a cylinder into the evaporator via the inlet tap -gradually raise nitrogen pressure at the cylinder head until there is no pressure differential between the inside and the outside of the flask. The pressure release valve between the cylinder and the evaporator should prevent build up of pressure inside the apparatus.*

- ix) *Remove the flask from the evaporator. Remove residual solvent by wrapping the flask in cling-film, fitting it on to the manifold of a lyophilizer, and subjecting it to high vacuum at room temperature for at least an hour. A gas-line filter (e.g. Millipore FG 50) should be interposed between the flask and lyophilizer, to avoid possible contamination.*

Since we don't have a lyophilizer in the lab, what I did was just keep the flask in the evaporator for at least another an hour after all the solvent has evaporated, in the thermal bath. To release the flask, tight up the clamp on the vacuum pump and increase gradually the nitrogen flux, until inner and outer pressures have equilibrated.

- x) *After releasing the vacuum and removing from the lyophilizer, flush the flask out with nitrogen, add 5ml of saline (containing solutes to be entrapped) and 0.5g of glass beads. (Ideally, the saline solution should be purged with nitrogen, or degassed under vacuum to remove traces of oxygen).*

I leave the buffer overnight (the night before!) inside the vacuum pot connected to the vacuum pump.

- xi) *Attach the flask to the evaporator again (flushed with nitrogen). Secure the flask in position with a clip or sticky tape, and rotate it at room temperature and pressure at the same speed as before (~60r.p.m.).*
- xii) *Leave the flask rotating for 30 min, or until all the lipid has been removed from the walls of the flask, and has given a homogeneous milky white suspension free of visible particles.*

Every time I prepared lipids, I got the white suspension but with some visible particles in it.

- xiii) *Allow the suspension to stand for a further 2 h at room temperature (or at a temperature above the transition temperature of the lipids) in order to complete the swelling process.*

### **Extrusion:**

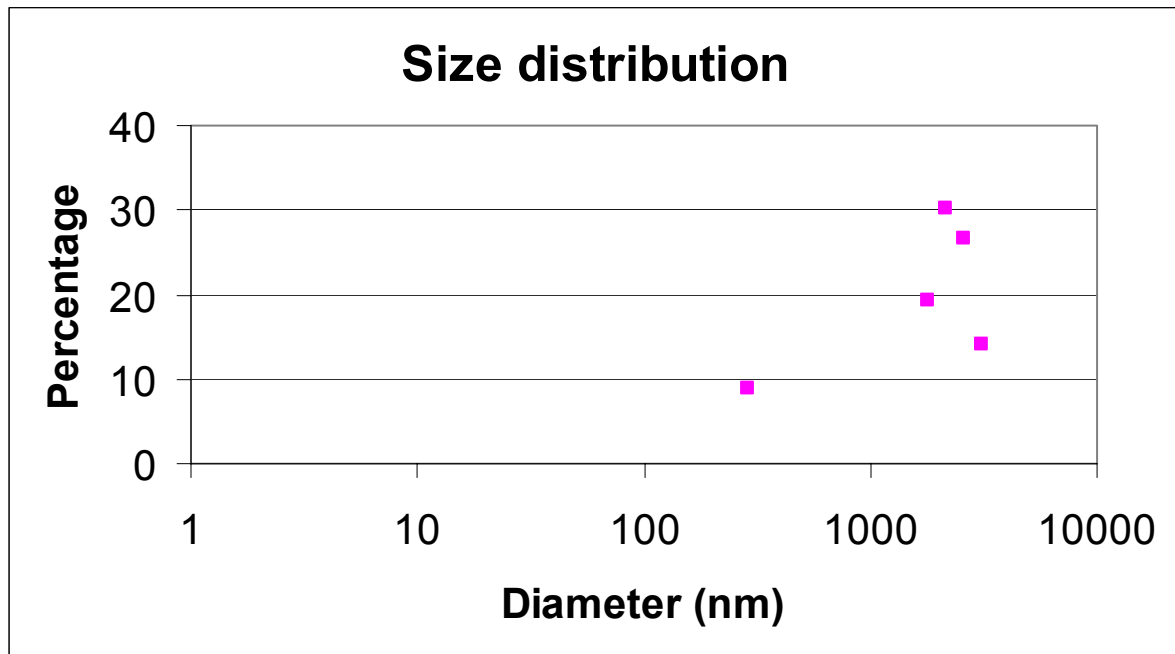
Mounting the extruder is pretty straightforward and is clearly explained in the corresponding manual. The extruding process should be carried on on the hot plate at a temperature above the transition temperature (I usually used 48 C, since anything higher

than that started to burn my fingers). Each syringe should not be loaded with more than 500ul: the more lipid suspension the more pressure needs to be applied. Eleven passes through the filter is enough to get a narrow distribution around 170nm of diameter (the odd number of passes is to finish on the opposite syringe, leaving everything that never made it through the filter in the first syringe). I used the same filter for three different 500ul samples, always starting on the same side of the filter and loading them in the same syringe. Liposomes are then put in the 1.5ml flasks, capped and stored in the fridge (0-5 C).

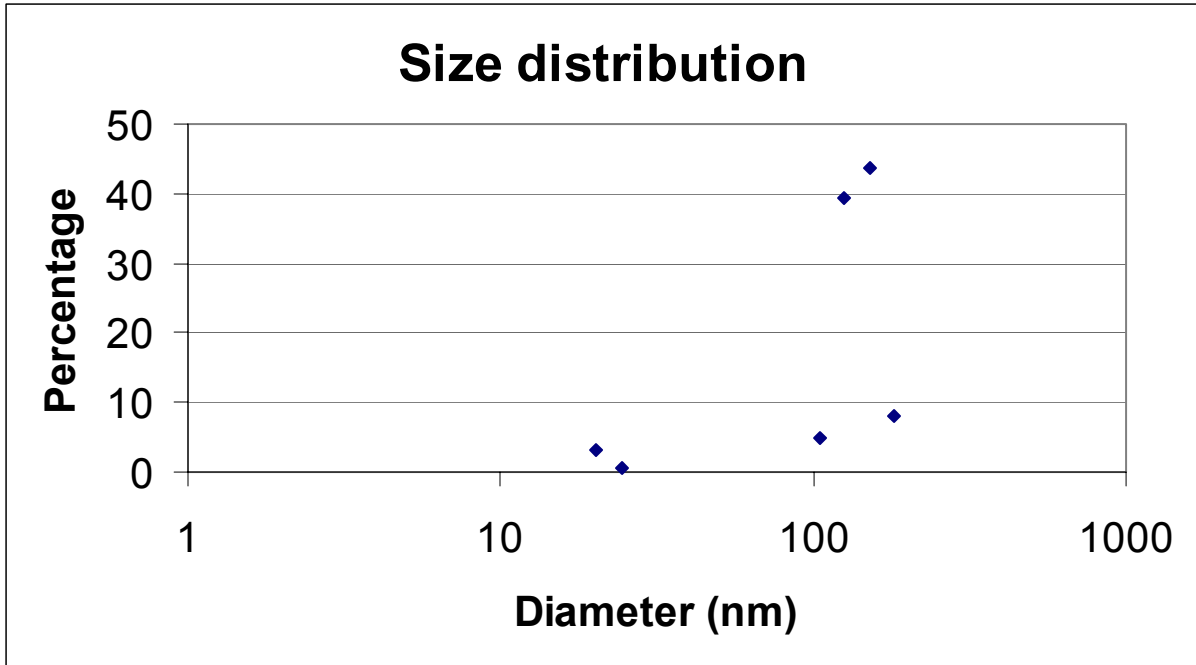
### Sizing:

Liposomes are sized using the Dynamic Light Scattering apparatus in the Biophysics Facility in the 4<sup>th</sup> floor of Cunnings. The lab guy teaches you how to use the machine, which is pretty easy. The samples cannot be sized as they are since the liposome concentration is huge. To get a reliable measurement the sample has to be dissolved 1,000-10,000 times. This facility counts with micropipettes and ependorf flasks, needed to perform the dilutions. It's a good idea to dilute in the same buffer as before to prevent liposomes to swell due to osmotic pressure.

Typical results are shown below:  
Before extrusion:



After extrusion:



The effects of the extrusion are clear from the graphs.

## Column Chromatography

After the preparation process, the concentration of ATP inside the liposomes should be the same as the concentration outside them. This concentration,  $50\text{mg/ml} = 9.1\text{E-}5\text{mol/ml}$  is a huge concentration (almost 6 orders of magnitude higher than the concentration required to saturate the detector). Hence, a method to get rid of this external ATP is needed. This method is that of the column chromatography.

The working principle of column chromatography is explained elsewhere (for instance in the printouts I'm handing in together with this report!). Here I'll detail the procedure.

Mount the column on the support, leaving some space under the tap to place the flasks. Close the tap. In a beaker, place the necessary amount of sephadex (10 dry grams per 10 hydrated ml). Add one column volume of buffer + 30%. Leave standing at room temperature for 3 hs. to allow hydration. Remove the supernatant with a pipette (i.e. the buffer standing on top of the, now hydrated, sephadex). Add buffer to make a 75% dispersion (dry mass of sephadex in grams)/(ml buffer) (i.e. for every 7.5 dry grams of sephadex one put initially, add 10 ml of buffer). Pour into the chromatography column being careful not to trap air bubbles. Add on top of the column at least 1ml of liposome suspension. Open the tap. Buffer will start dropping out. It's very important not to allow the sephadex to dry, since it will stop working. Hence, one should have enough buffer to pour into the column as buffer comes out through the tap. Collect in 1ml samples. At some point liposomes will start coming out. The resulting liposome dispersion will be diluted. Different concentrations of liposomes are then obtained. Usually it's easy to distinguish them, the more concentration, the more opaque. I always used the most opaque dispersion that came out of the column.

## ATP Bioluminescent Assays

The procedure to perform ATP concentration determination using luciferin-luciferase is thoroughly explained in the product data sheet, together with the way each individual component is recomposed and stored. Hence, I'll describe only the experiments I performed and the results I got.

### Calibration Curve

To measure a calibration curve one just needs to follow the usual procedure: for different concentrations of ATP, add the same amount of luciferin-luciferase (L+L) and measure the number of photons emitted for each concentration. It seems sensible to assume that the number of photons will be proportional to both, the concentration of ATP and the concentration of L+L, i.e.

$$I \propto [ATP] \cdot [L + L]$$

Hence, if the concentration of L+L is kept constant, the log of the intensity (number of photons) as a function of the log of ATP concentration should be a straight line with slope 1.

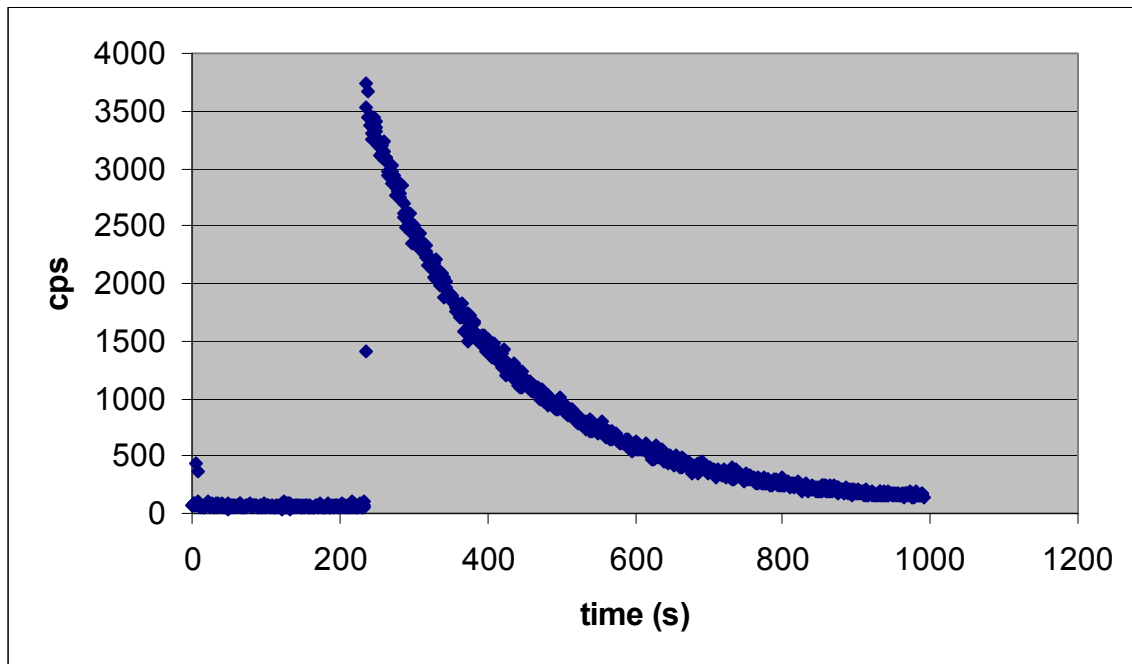
The bioluminescence kit includes a sample of .9mg of ATP (the weight is indicated in the flask). I recomposed this sample by adding 1ml of distilled, deionized water (DD water) directly to the original flask. I performed dilutions of this sample (.9mg ATP = 1.63E-6 moles, in 1ml is 1.64mM ATP, a huge concentration!) by taking 200ul of a given sample, by means of the 40-200ul micropipette and adding 900ul of DD water using the 1ml syringe and the small needle (blue). The 1.5ml flasks were used. Hence, the dilution factor is 200/1100 = 0.18 each time. It turns out that the needle has a small volume of about 15ul (hard to measure precisely though), leading to a systematic error in the dilution factor. After finishing all the dilutions, the flasks were capped using the sealing caps, and stored in the fridge (0-5 C)

This way I got 12 samples, the first one being the original concentration: 1.64mM, the next one 0.18 less concentrated, etc. and the 12<sup>th</sup> of a concentration of 1.01E-8mM. Each flask, except for the first and the last one, ended up containing 900ul of solution.

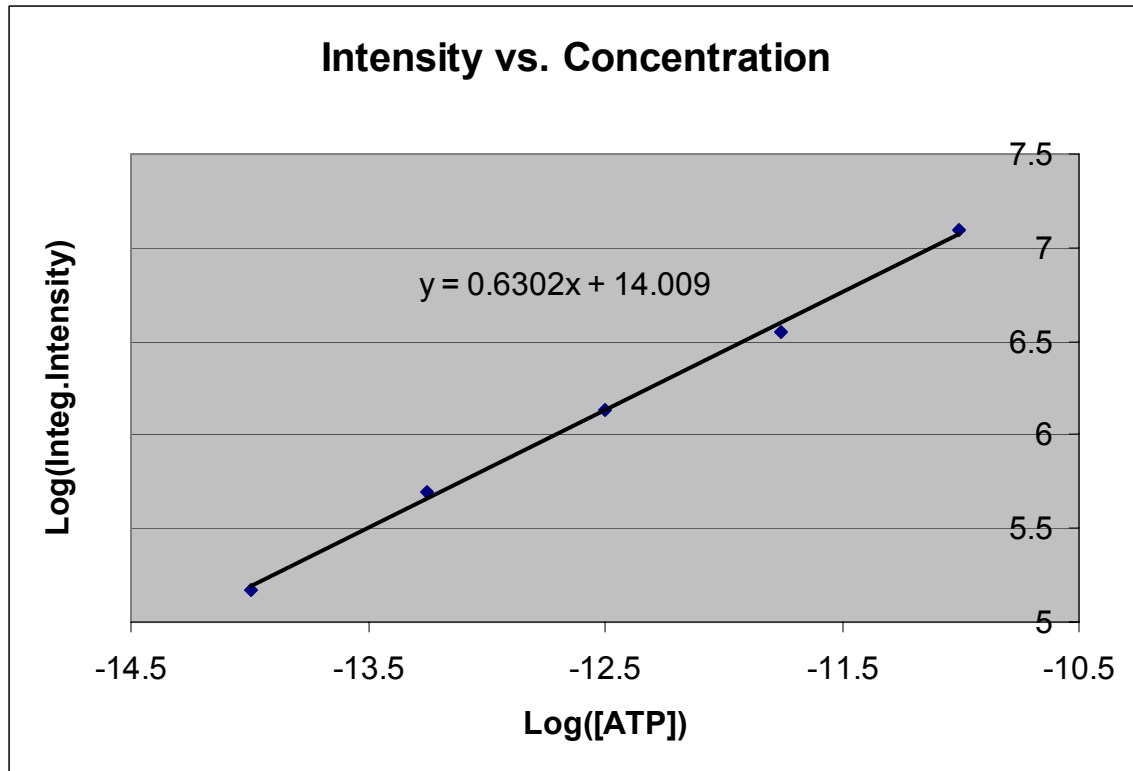
L+L was recomposed by adding 5ml DD water to the original flask, and then, let stand for an hour as recommended in the product data sheet. 200ul of L+L were transferred to a 1.5ml flask, capped with the white caps. Using a syringe needle two holes were performed on the cap. One of them will hold the needle when placed in the detector, and the other is to prevent build up of pressure when the ATP solution is added. The L+L flask was then placed into the detector. The detector was then started (using the lab view program c:\Nicolas\Hamafire2.vi). A background of about 200cps was commonly observed.

All ATP samples were stored in ice during the measurements. A 1ml syringe (with a small, blue needle) was then filled with 200ul of a given ATP sample. The hose was then

pricked with the syringe needle, the extrusion pump turned on, and the 200ul injected. A quick rise was observed followed by decay. A typical measurement is shown bellow:

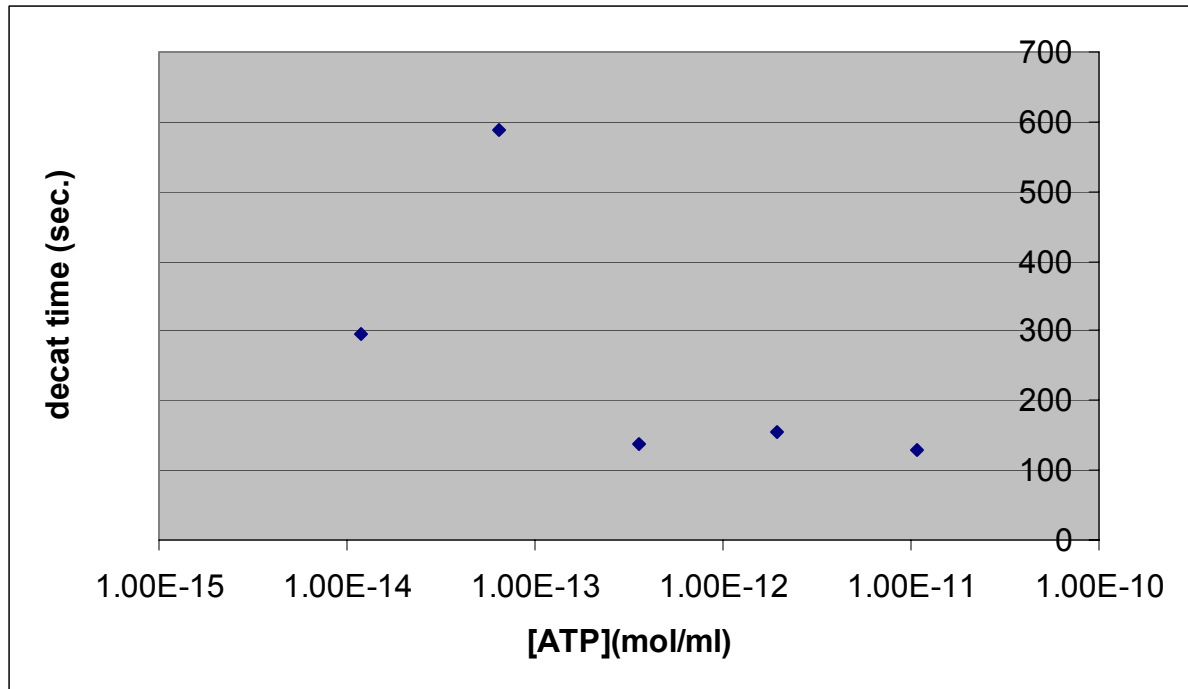


The first concentration I measured was the least concentrated, i.e. sample 12. Then I proceeded with samples 11th, 10th, 9th and 8th. Sample 6 ( $3.26 \times 10^{-10}$  mol/ml) already saturated the detector. After each sample was measured, a new flask with L+L was placed in the detector. It seems to be a good idea to measure lower concentrations first, since there's always some leftovers in the hose/needle. These leftovers will affect much more lower concentrations than higher concentrations. For each curve I integrated the number of photons for 6 seconds, 20 seconds after the addition of L+L (main peak). The following is the calibration curve obtained:



The slope of the fit is not 1 as expected, indicating the presence of some systematic error probably in the dilution factor (definitely not the one introduced by the dead volume of the needle). I went over the procedure many times but couldn't find the problem. After the experiment finished, 100ml DD water were run through the hose/needle at the pump's lowest speed by placing the free end in a 125ml water container. After the container was empty, the pump was kept running at a higher speed to make sure no water remained in the hose.

The decay times vary from sample to sample, but don't seem to be correlated to the concentrations, probably because it is more sensitive to the amount of L+L. The following is a graph of decay time vs. concentration:



As can be seen in the graph, the decay time is of the order of a few minutes.

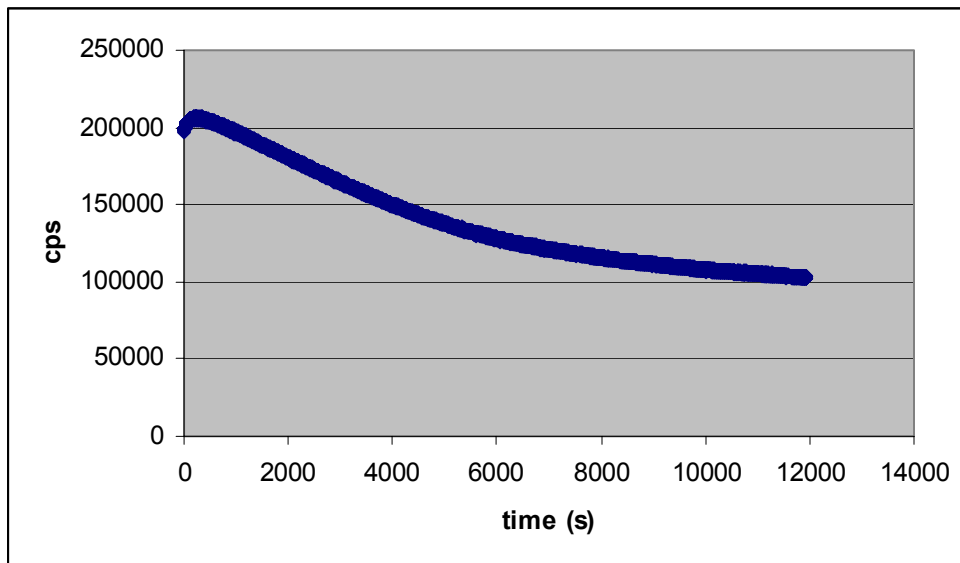
**Important Notice:**

Not all the 1.5ml flasks fit into the mouth of the detector. In fact, out of the two boxes of flasks, only 24 of them were found to fit. Hence, it's important to carry a cleansing scheme on them. If the flask contained liposomes, it was first washed withalconox and rinsed thoroughly with DD water (at least 10 times). As recommended in the Bioluminescence Kit data sheet, the flasks were left overnight soaking in an HCl 1N (3.7g/l) solution. They are rinsed thoroughly with DD water (at least 10 volumes) and left overnight submerged in DD water. Finally, they are allowed to dry. It should also be noted that only the white caps keep the flasks in position into the detector. The blue sealing caps allow the flasks to fall through. All the flasks that do not fit in the detector mouth were treated as disposable.

## Bioluminescence Assays and Liposomes

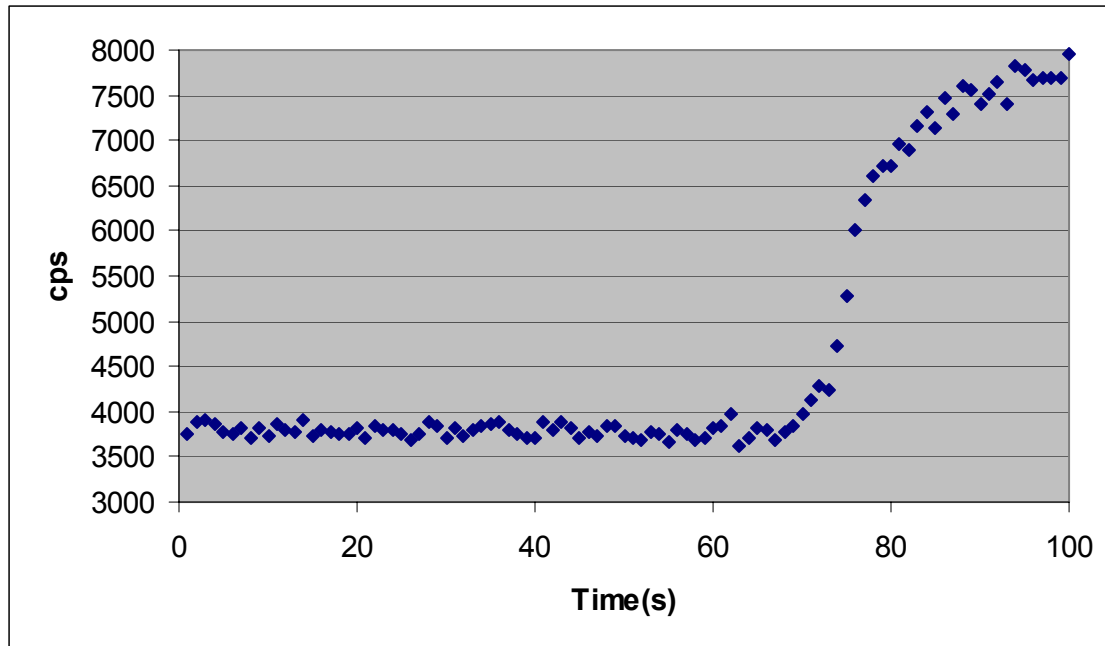
A bunch of bioluminescence assays were carried on using the samples with liposomes. The first question one may ask is whether the liposomes entrapped ATP at all. The easiest way to address this question is by adding L+L to a sample with liposomes, and verify that the count rate increases after treatment with Triton X (a detergent that breaks the liposomes open).

After passing through the chromatography column, 200ul of liposome dispersion were placed in a 1.5ml flask, and 200ul of L+L were added. The light emitted could be seen with the lights off (no wonder it would saturate the detector). Only after about 24hs the sample was ready to be measured in the detector. During this time samples were stored at room temperature wrapped in aluminum foil. A typical curve is shown below:



At first one might think that the initial raise might be due to a thermalization effect. But this option is discarded since samples are stored at room temperature. I think the raise is produced by the agitation to which the flask is subject to when placed into the detector. All the samples measured exhibited this initial raise.

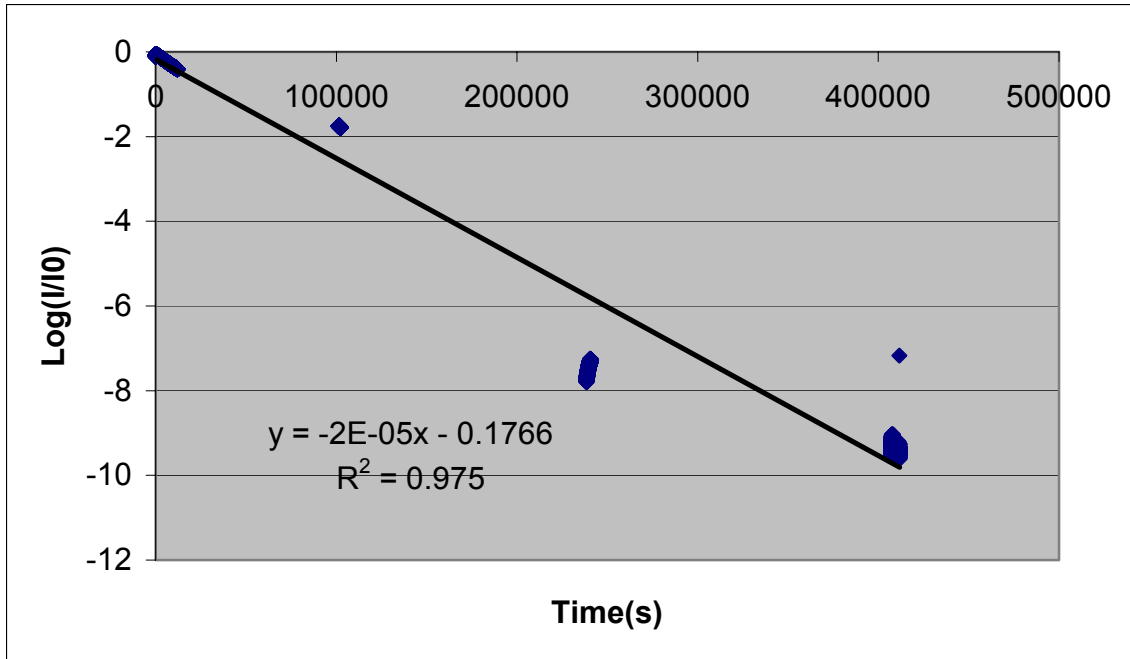
After treatment with Triton X the count rate clearly rises, shown by the following graph:



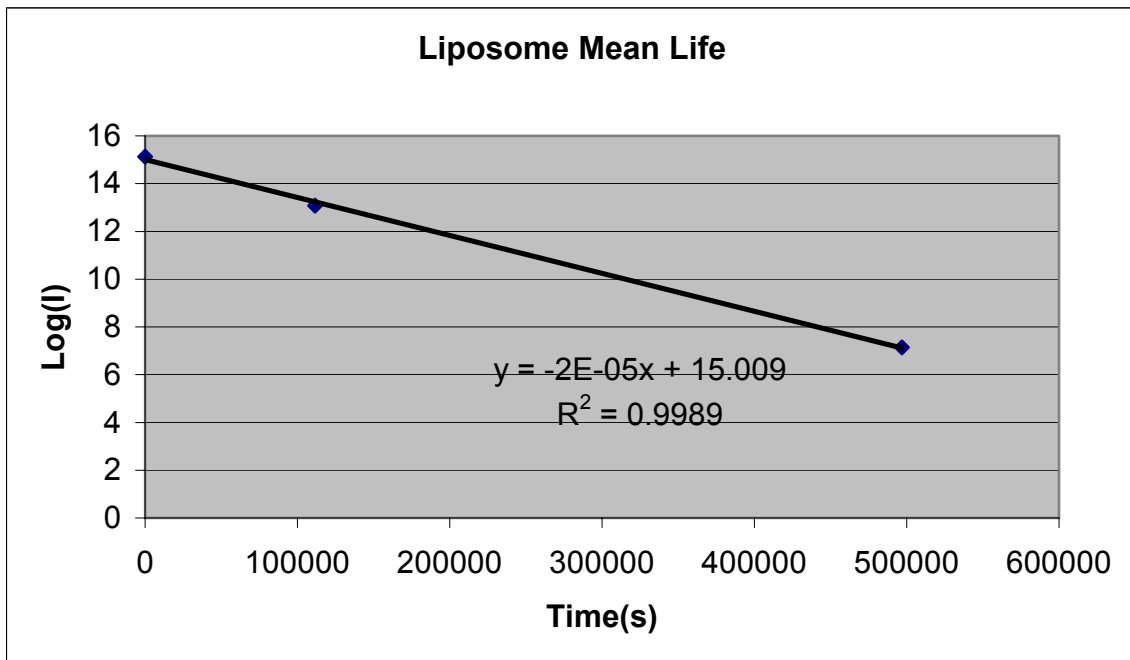
This graph shows that ATP was effectively entrapped into the liposomes.

Another puzzling feature is the extremely long decay time as compared with the characteristic times of the previous bioluminescence assays with ATP alone, the former being of the order of days, while the latter being a few minutes. A plausible explanation is that the light emitted in the samples with liposomes does not come from ATP outside the liposomes, but from ATP released from the liposomes, either by just leaking out of them or because of spontaneous breakdown. To address this question, one could measure the decay time of liposomes. This can be done by measuring in successive days the amount of light emitted by different samples, prepared the same day, after treatment with detergent. This experiment was carried out as explained bellow.

4 samples with 1.5ul of liposome suspension where placed in 4 different flasks, and 1.5ul of L+L were added to each of them. The light these samples emitted could be seen with the lights off. After a day, the intensity of the light emitted by each flask was recorded. These intensities were then used to “normalize” the fore coming measurements. Each day, the intensity from a given flask was measured for about 3hrs, after which the sample was treated with 200ul of Triton X. The following graph shows the relative intensity before treatment with detergent as a function of time:



The following graph shows the peak intensity, after treatment with Triton X, as a function of time:



Both graphs exhibit the same decay constant, suggesting that the background observed in liposomes with L+L samples is due to the decay of liposomes. This results also rules out the possibility of ATP leaking out of the liposomes, which shouldn't have the same decay constant.