POSITIVE LIPOSOME UPTAKE MECHANISMS

The effect of different uptake inhibitors on the uptake of positive liposomes

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Content

Abstract

The aim is to determine the effect of different uptake mechanism inhibitors on the uptake of zwitterionic liposomes by Hela cells to see how the liposomes are taken up. This was done by performing multiple experiments exposing the inhibitors to Hela cells at different time points. The results do not give a clear picture of the uptake mechanisms of the liposomes due to the observed increase in uptake for two inhibitors. It has been hypothesized the observation could be due to overcompensation by another pathway or an altered stability of the liposomes.

Introduction

Nanomedicine has multiple advantages over traditional medicine, allowing for better delivery of hydrophobic compounds, passing barriers and targeting tissues. This field is still young and therefore needs more development on all fronts [14]. The advantages mentioned allow for more specialized treatments with less side effects. This has led to a rise in research into the subject.

Liposomes are the most common type of nanoparticle used in clinical applications. They are composed of a lipid bilayer, usually made with anionic phospholipids. Some liposome applications have already been approved, with Doxil being the first approved by the FDA. This is a liposome delivering the drug doxorubicin. Doxil has less toxicity and better compliance with patients compared to doxorubicin on its own due to its pharmacokinetics [15]. After the approval of Doxil, multiple other nanomedicine formulations have been approved, sitting at 50 in 2017 and mainly consisting of liposomes, polymers and nanocrystals [16]. This low number is partially caused by the stability and reproducibility of the nanoparticles, according to E. Fröhlich [10]. The paper suggests to vary parameters of the nanoparticles, like the size and surface charge to improve on the characteristics like the pharmacokinetics.

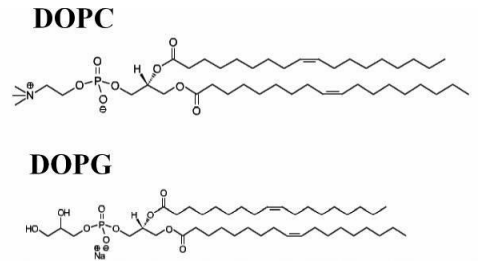


Figure 1: The structures of the phospholipids DOPC and DOPG [22]

More recently, the effect of zwitterionic components has been studied [2]. The negatively charged liposomes contain the anionic 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (DOPG), whereas the zwitterionic liposomes contain the zwitterionic 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC). These phospholipids are shown in figure 1.

By adding cholesterol to the liposomes, the leakage of its contents can be reduced. This is done by closing up the space in between the phospholipids [8]. This effect can also be achieved by having the liposomes in solid phase [7]. In the solid phase, the phospholipids in the bilayer move much less frequent compared to the liquid phase, making diffusion more difficult.

For the experiments described in this report, the positively charged liposomes are used. These are made with DOPC and DC-cholesterol at a ratio of 20:1. A positive charge on the liposomes enhances the drug delivery in exchange for more cytotoxicity [10], which makes sense considering a cell membrane is negatively charged [17]. The cytotoxicity is partially caused through the damaging of the plasma membrane. These liposomes are used to transfect genes into the cells. After the particle

is taken up, it can slow down the conversion of the endosome to a liposome until the gene has been released [10]. In a study by Montizaan D et al., the zwitterionic liposomes are also taken up more by Hela cells when compared to the uptake of negatively charged liposomes [2]. These observations suggest the potential of positively charged liposomes, opposed to the commonly used anionic liposomes. The enhanced uptake by cells and the aforementioned help in gene transfection could be worth the extra cytotoxicity. This toxicity can be reduced with small changes to the lipids, like covering the amine group [10].
The different net charge can have another effect on the pharmacokinetics. After the liposomes are administered to a patient, they come into contact with blood. Blood contains a lot of different biomolecules which can adhere to the liposomes, resulting in particles surrounded by biomolecules that form a corona. This corona appears to have an effect on the uptake mechanisms of the target cells [9] and can alter the net charge of the liposome [17]. Studies have consistently found a biomolecule corona on the surface of nanoparticles. The effect on the uptake mechanisms can be beneficial or detrimental, as certain recognition units can be covered by the biomolecules [21]. Considering the corona differs based on the surface charge, zwitterionic liposomes would likely have different blocked recognition units compared to the negatively charged liposomes.

The effect of the charge and biomolecule corona on its uptake mechanisms can be studied to gain a greater understanding of the process. The mechanisms by which liposomes, or other nanoparticles, are taken up are researched with different methods. It can be researched by inhibiting specific uptake pathways. This is done in the experiments of this report by using inhibitors. The inhibitor blocks a specific pathway, if the liposome uptake is affected the blocked pathway plays a role in the transport. It is also possible to block the pathways with, for example, RNA interference [1]. Using double stranded RNA, mRNA can be inhibited [20]. Through this method, important proteins associated with endocytosis can be inhibited. If the liposomes have been fluorescently marked, the uptake of the liposomes by the cells can then be measured by the use of flow cytometry. This will show the intensity of the particles present at each cell as the mean fluorescence intensity (mfi). Imaging is another way of studying the uptake of liposomes. The liposomes are fluorescently labelled and studied with a confocal microscope [18]. With this method, the location of the particles in the cells can be observed. This method can differentiate between the particles that have been taken up by the cells and the particles that are adhered to the plasma membrane, which cannot be done with flow cytometry. The different ways of studying the uptake mechanisms have their positive and negative sides. Therefore, it is important to utilize different ways of researching the uptake mechanisms, which results in a better understanding [17].

To determine the importance of different active transport mechanisms, inhibitors interacting with different pathways are used in the experiments. The first inhibitor, sodium azide, is used to inhibit the ATP formation in the cells. This is done by inhibiting the oxidative phosphorylation, vital to ATP formation in cells [12]. ATP depletion results in the inhibition of active transport.
Chlorpromazine hydrochloride inhibits the clathrin-mediated endocytosis by binding to the protein AP2, which is important for the pathway to function [1]. This uptake mechanism seems to be largely responsible for the uptake of positive liposomes [17].
EIPA blocks micropinocytosis through the inhibition of Na^+/H^+ pumps on the plasma membrane. This results in acidification close to the membrane, interfering with both micropinocytosis directly and indirectly through the Rac1 and Cdc-42 pathways [1].
Cytochalasin D binds to F-actin, resulting in the inhibition of actin polymerization. This works similar to the final inhibitor, nocodazole. This molecule blocks tubulin, resulting in the inhibition of microtubule polymerization [1]. Microtubules play an important role in the transport of vesicles inside the cell, more so for clathrin-mediated endocytosis [19]. Both cytochalasin D and nocodazole

block multiple mechanisms of endocytosis and macropinocytosis. Therefore, these two compounds will show the involvement of actin and the microtubules in the liposome uptake but give a less precise indication of the exact uptake mechanisms used by the liposomes.

For the liposomes to successfully deliver its contents to the target, it needs to be taken up by the target cells. This is very likely done with active transport, endocytosis. Passive transport cannot take place, considering the liposomes are not small enough [5].
The types of endocytosis studied during the experiments are mainly clathrin-mediated endocytosis and macropinocytosis. These processes are both a type of pinocytosis. Clathrin-mediated endocytosis is started by the proteins AP-2 and AP180, which stimulate the assembly of clathrin. The resulting polymer then forms a vesicle in the plasma membrane, which is closed up by the enzyme dynamin. The resulting endosome is then transported further into the cell using the actin cytoskeleton. Unlike clathrin-mediated endocytosis, macropinocytosis does not rely on clathrin or dynamin. After the activation of signaling cascades through receptor activation, the cell can take up a particle. This process is performed with the actin cytoskeleton, which is used to envelop the particle with the membrane. The resulting vesicle is larger than the endosome formed after clathrin-mediated endocytosis [17].

A good understanding about the uptake mechanisms of liposomes allows for a better design and efficacy of the particle [1], which will consequently impact its pharmacokinetics in a positive way.

The aim of the experiments is to determine the effect of different uptake inhibitors on the uptake of zwitterionic liposomes. If an inhibitor of an uptake mechanism causes a reduction in liposome uptake, the blocked mechanism plays an important role in the liposome transport across the plasma membrane.
The sodium azide was first added to the liposomes to confirm whether the uptake of liposomes is indeed predominantly active transport. After this, the inhibitors of uptake mechanisms were added to exclude or confirm the role of the pathways associated with each inhibitor. The inhibitors focus mainly on the clathrin-mediated endocytosis and micropinocytosis, as those are the most dominant mechanisms.
This leads to the research question: what is the effect of different uptake mechanism inhibitors on the uptake of zwitterionic liposomes by Hela cells and what does this say about the mechanisms used by the liposomes? Based on the effect of the inhibitors, the uptake of zwitterionic liposomes by the Hela cells can be better understood. The better understanding will allow for the optimization of the nanoparticles, resulting in more optimal pharmacokinetics.

Materials and methods

Cell culture

During the experiments described, Hela cells were used. The cells were grown in an incubator at 37 °C at 5% CO_2, using a T75 flask. For this, complete medium (cMEM) was used, made with MEM (Gibco) and 10% v/v fetal bovine serum (FBS) (Gibco). The cells used originated from in between passage p11 + 4 and p11 + 18.

Liposome properties

The positively charged liposomes used have a size of 100 nm, and are composed of DOPC and DC-cholesterol (distributed 20:1 respectively). Dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern

Panalytical, UK) was performed with the second batch of liposomes. From this analysis a diameter of 113.7, 110.7 and 124.3 nm was found when dissolved in water, PBS and cMEM respectively. The zeta potential was also measured: 27.2, 4,7 and 0 mV when dissolved in water, PBS and cMEM respectively. These particles were fluorescently labelled with 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DII) which have an excitation wavelength of 550 nm and an emission wavelength of 564 nm. The stock concentrations of the liposomes were 10.6 mg/mL and 10.87 mg/mL across all experiments. The liposome concentration in the wells of a 24-well plate during incubation was 50 µg/mL.
The liposome uptake was measured at different time points between 1 and 5 hours for every experiment besides the first experiment resulting in a concentration-effect curve.

Inhibitor experiments

The cell concentration in the wells was 50000 cells per well, which were filled 24 hours before the experiment. A 24-well plate (Greiner Bio-One, AT) was used. During pre-incubation, the cells were exposed to the inhibitors for 20 minutes, except for the second experiment with sodium azide (NaAz) and the uptake kinetics experiment with 5-(N-ethyl-N-isopropyl)amiloride (EIPA), which were incubated for 90 minutes and 30 minutes respectively. The optimal concentration [1] of each inhibitor in the wells was 5 mg/mL for NaAz, 10 µg/mL for chlorpromazine hydrochloride (Sigma-Aldrich, USA), 100 µM for EIPA (Sigma-Aldrich, USA), 2.5 µg/mL for cytochalasin D (ThermoFisher Scientific, USA) and 5 µg/mL for nocodazole (Biovision, USA).
After pre-incubation, the inhibitor was added to the cells again together with the liposomes as described in the section liposome properties.
For the experiment with EIPA, dextran was used as an efficacy test of the inhibitor. Its well concentration was 250 µg/mL.
During the experiment about liposome stability, the pre-incubation was done the same way. However, after pre-incubation the inhibitors were no longer present, which could give a better idea about the effect of the inhibitors on the liposomes.

Flow cytometry

After the incubation at different time points, the cells were rinsed with cMEM. Consequently, the cells were rinsed two times with phosphate saline buffer (PBS). Afterwards, 0.05% trypsin/EDTA in PBS (TEP) was added to the wells. The well plate was shaken at 37 °C to let TEP loosen the cells. cMEM was then added the wells, after which the cells were collected and centrifuged. The supernatant was afterwards replaced with PBS, so the cells could be measured with a flow cytometer (Cytoflex, Beckman Coulter, USA) with channel 585/42 BP. The raw data obtained from the flow cytometer was then processed using FlowJo (FlowJo LLC, USA).
The mean fluorescence intensity obtained with FlowJo was plotted against the period of incubation with liposomes. The experiments were performed in duplicate. Therefore, the data points are plotted separately with a line through the average. For the experiments with an inhibitor, the uptake of liposomes in the presence of an inhibitor was normalized for the uptake with no inhibitor present. These values are shown in bar charts. With the data from the experiment testing the liposome stability only the bar chart could be made, as only one time point was used.

During the second NaAz (figure 2C/D) and cytochalasin D (figure 5C/D) experiments and the liposome stability test (figure 7), the cells were fixated and measured the next day. Fixating was done by adding formaldehyde instead of PBS to the cells after the centrifuge. This was then stored in the dark

at room temperature for 30 minutes. Afterwards, the suspension was combined with PBS and centrifuged. Finally, the supernatant was removed and the cells were resuspended in PBS. This suspension could be stored in a fridge covered with parafilm and measured the next day with the flow cytometer.

Results and discussion

Flow cytometry is used for measuring the fluorescence of the marked liposomes. The suspension containing the cells to be measured is transported to the laser beam using sheath fluid. The fluid is pressurized, causing the cells to pass the laser beam one at a time. This allows for a more accurate measurement. The light of the laser beam is scattered forward (FSC) and sideward (SSC). FSC indicates the size of the cells, whereas the SSC indicates the complexity of the cells. Besides these parameters, the flow cytometer can also measure the fluorescence of any fluorescent markers if excited at the excitation wavelength. This parameter shows the presence of fluorescent marked particles in the cells [13].

With the data obtained, a light scatter plot can be made in Flowjo by setting the FSC against the SSC as seen in figure 2A/B. In this figure, it is shown how the viable cells are gated. With the filtered data, another light scatter plot can be made with FSC-H and FSC-A, as seen in figure 10. Figure 10 also shows how the single cells were gated. The filtered data obtained is the mean fluorescence intensity used in the graphs seen in the results.

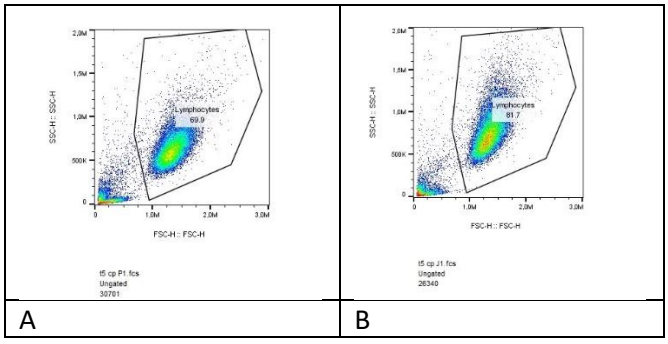


Figure 2: A and B depict the light scatter plot of the data points at an incubation time of 5 hours and belongs to the graph of figure 5A, with the SSC-H on the y-axis and the FSC-H on the x-axis (the H indicates height).

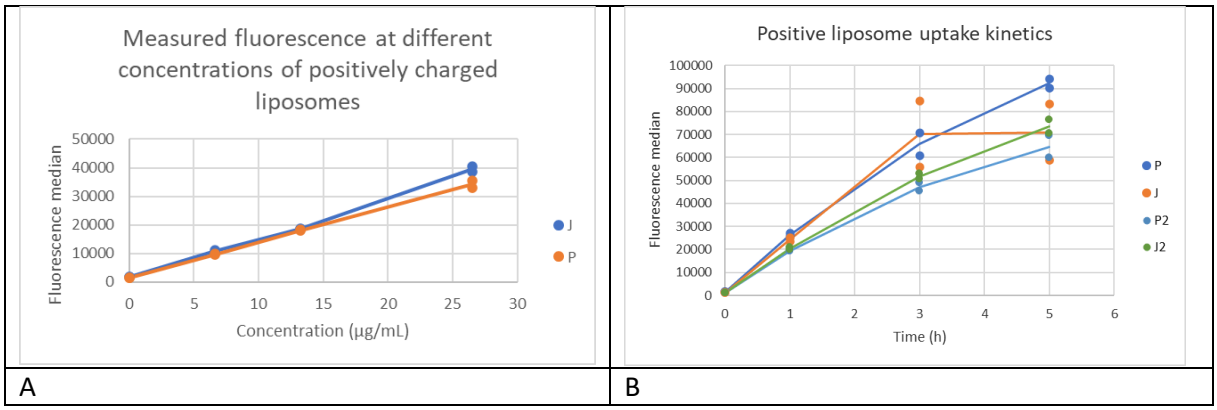


Figure 3: A) The mean fluorescence intensity is plotted against the positive liposome concentration. The Hela cells have been exposed for 4 hours to liposomes at different concentrations. The cell fluorescence was measured using the flow cytometer, as shown in the methods and materials. B) The mean fluorescence intensity is plotted against the positive liposome

concentration. The Hela cells have been exposed for 1, 3 and 5 hours to 50 µg/mL liposomes. The cell fluorescence was measured with a flow cytometer. The results are the median fluorescence of duplicate samples with a line going through the their average obtained in two replicate experiments.

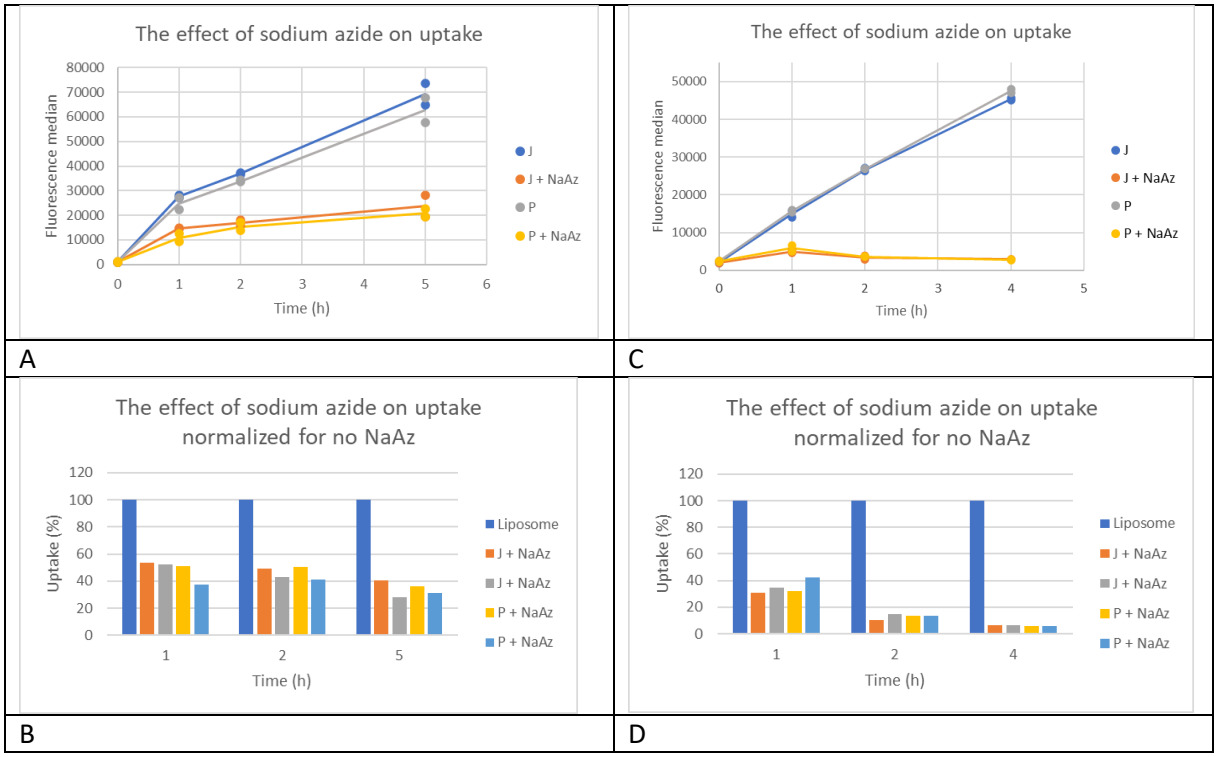
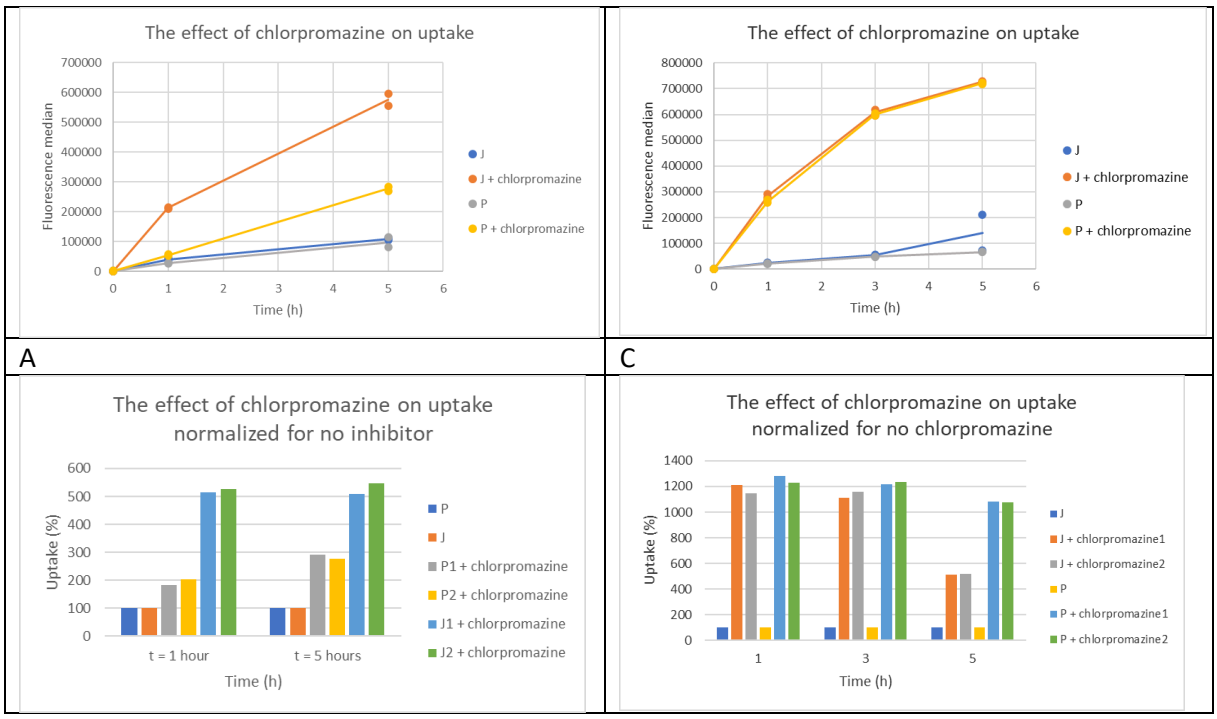


Figure 4: The uptake kinetics of zwitterionic liposomes at the presence of sodium azide. A and C have the mean fluorescence intensity plotted against the incubation time. They show the effect of sodium azide on the uptake of the liposomes. The duplicate measurements have been plotted separately, with a line going through the average. B and D show the results of A and C after normalization for the results of Hela cells exposed to liposomes without sodium azide. Before the exposure to liposomes, the Hela cells were pre-exposed to sodium azide for 20 minutes and 90 minutes, for A/B and C/D respectively.



B	D

Figure 5: The uptake kinetics of zwitterionic liposomes at the presence of chlorpromazine hydrochloride. A and C have the mean fluorescence intensity plotted against the incubation time. They show the effect of chlorpromazine hydrochloride on the uptake of the liposomes. The duplicate measurements have been plotted separately, with a line going through the average. B and D show the results of A and C after normalization for the results of Hela cells exposed to liposomes without chlorpromazine hydrochloride. Before the exposure to liposomes, the Hela cells were pre-exposed to chlorpromazine hydrochloride for 20 minutes.

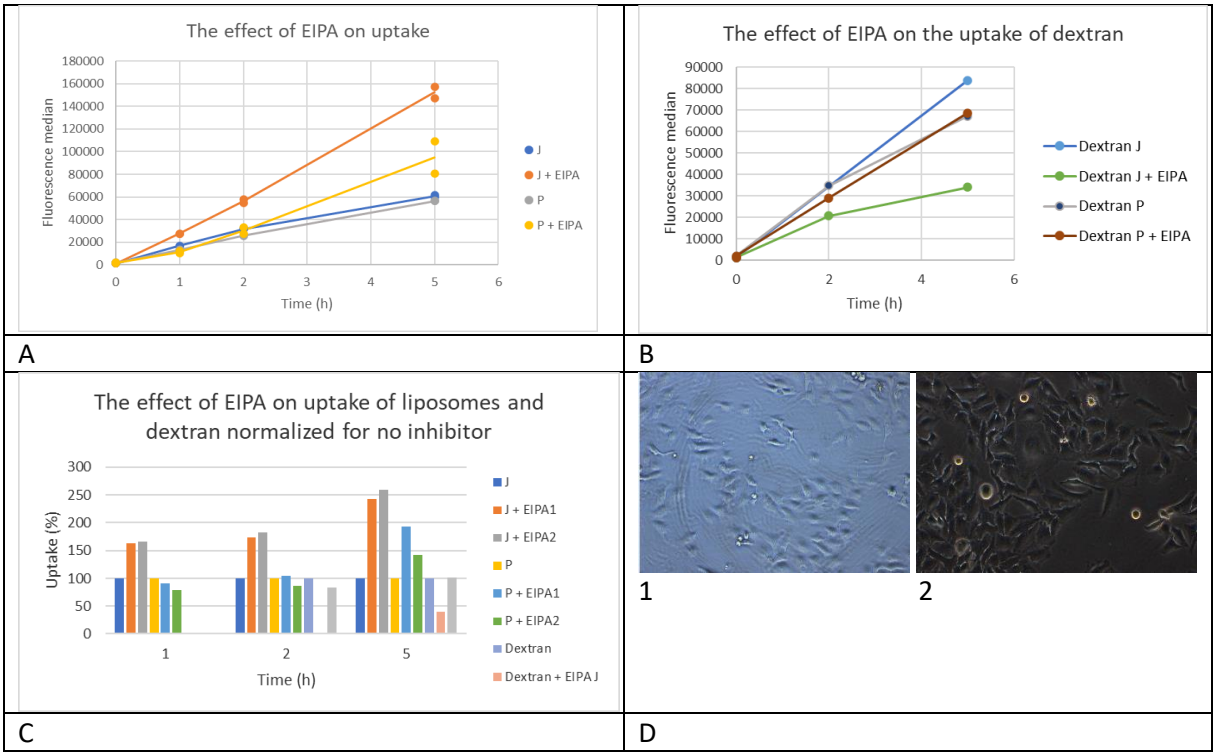


Figure 6: The uptake kinetics of zwitterionic liposomes at the presence of EIPA. A and B have the mean fluorescence intensity plotted against the incubation time. They show the effect of EIPA on the uptake of the liposomes and dextran respectively. The duplicate measurements have been plotted separately, with a line going through the average. C shows the results of A and B after normalization of the results for Hela cells exposed to liposomes or dextran without EIPA. Before the exposure to liposomes, the Hela cells were pre-exposed to EIPA for 30 minutes. D1 and D2 show the microscope images of Hela cells with EIPA and without EIPA respectively.

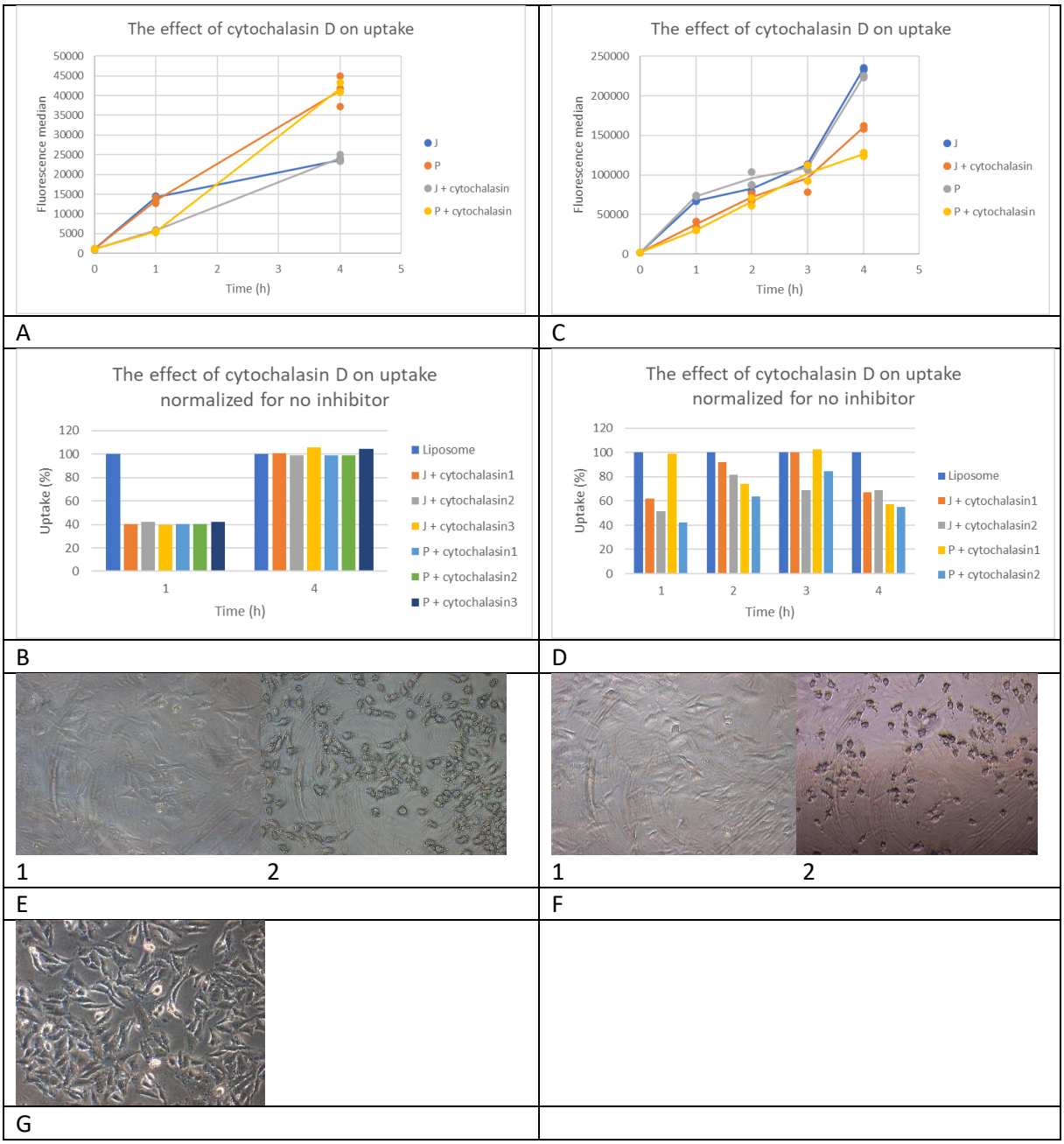


Figure 7: The uptake kinetics of zwitterionic liposomes at the presence of cytochalasin D. A and C have the mean fluorescence intensity plotted against the incubation time. They show the effect of cytochalasin D on the uptake of the liposomes. The duplicate measurements have been plotted separately, with a line going through the average. B and D show the results of A and C after normalization for the results of Hela cells exposed to liposomes without cytochalasin D. Before the exposure to liposomes, the Hela cells were pre-exposed to cytochalasin D for 20 minutes. E1 and E2 show the microscope images of Hela cells without the presence of cytochalasin and with its presence respectively at an incubation of 1 hour. F1 and F2 show Hela cells without and with cytochalasin respectively at an incubation of 4 hours. Both E and F belong to the data of A and D. G shows a microscope image of Hela cells without cytochalasin. In this image, a bacterial contamination is clearly visible.

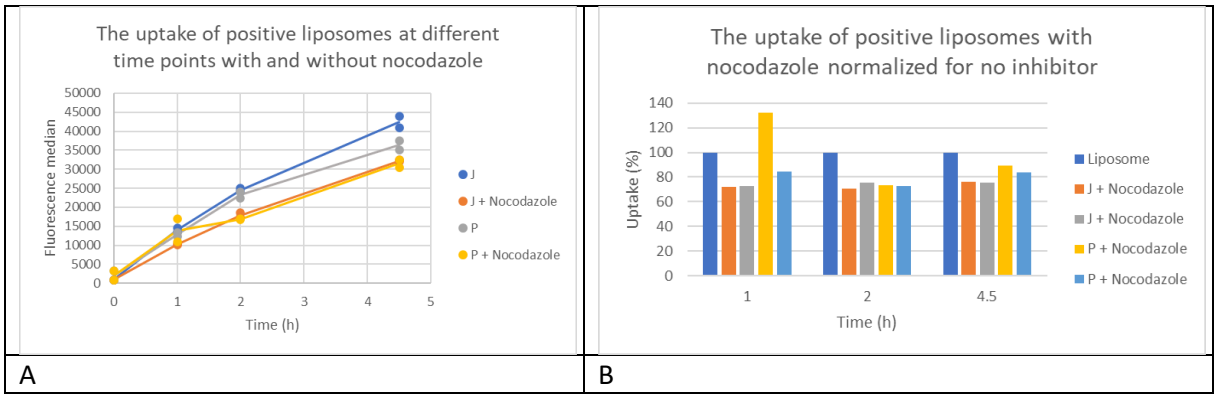


Figure 8: The uptake kinetics of zwitterionic liposomes at the presence of nocodazole. A has the mean fluorescence intensity plotted against the incubation time. It shows the effect of nocodazole on the uptake of the liposomes. The duplicate measurements have been plotted separately, with a line going through the average. B shows the results of A after normalization of the results for Hela cells exposed to liposomes without nocodazole. Before the exposure to liposomes, the Hela cells were pre-exposed to nocodazole for 20 minutes.

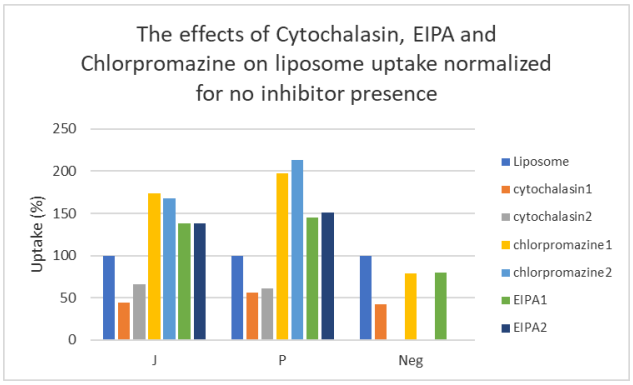


Figure 9: The uptake kinetics of zwitterionic liposomes after pre-exposure with cytochalasin D, EIPA and chlorpromazine hydrochloride. The figure shows the results of Hela cells pre-exposed to cytochalasin D, EIPA and chlorpromazine hydrochloride after normalization for the results of Hela cells exposed to liposomes without cytochalasin D, EIPA and chlorpromazine hydrochloride. Before the exposure to liposomes, the Hela cells were pre-exposed to cytochalasin D, EIPA and chlorpromazine hydrochloride for 20 minutes. Cytochalasin D, EIPA and chlorpromazine hydrochloride were not present during the exposure of Hela cells to the liposomes. The bars of Neg show the same, with anionic liposomes instead of zwitterionic liposomes. Experiments done by two other students showed anionic liposome uptake is inhibited by all three inhibitors. Therefore, the negative liposomes function as an efficacy control.

The experiments were done by myself and another student, Philip. Therefore, everything has been replicated at least once. In the figures, J refers to my results, and P refers to the results of Philip.

First, the liposome mean fluorescence intensity (mfi) was determined at different liposome concentrations. The results can be seen in figure 3A. The liposome uptake increases at a higher concentration, which was as expected. From these results, it was decided to use a liposome concentration of 50 µg/mL in the wells, as it gave a good amount of fluorescence.
Consequently, the liposome uptake at different incubation periods was determined. In figure 3B, the results can be seen of two experiments, as it has been replicated. The line of J, part of the first experiment, has a large difference between the data points at 3 and 5 hours. This was likely caused by a human error, as it was not replicated in the other three lines. Besides those errors, the results have a good replicability. After these results showed a clear increase in uptake over time, it was

decided to combine the liposomes with an inhibitor, sodium chloride. This experiment would give a good indication whether the uptake of the liposomes is mainly passive or active transport. The results depicted in figure 4A/B show a reduction in liposome uptake. However, the reduction of around 60%, which could mean sodium azide inhibits the liposome uptake only partially. This lead to the decision to repeat the experiment, the results of which are depicted in figure 4C/D. For the repetition, the pre-incubation with sodium azide was lengthened to 90 minutes. The reduction of around 90% after 2 and 4 hours of incubation shows sodium azide causes full inhibition of liposome uptake, and does indicate the liposomes are taken up through active transport. The reduction in uptake could not have been caused by dead cells, as there were not many visible when looking through the microscope. After it was confirmed the cells take up liposomes through active transport, inhibitors of endocytosis were added to the liposomes. The first inhibitor to be used was chlorpromazine, which inhibits clathrin-mediated endocytosis. The results of this experiment (figure 5A/B) were very unexpected, as the inhibitor caused a large increase in liposome uptake. The liposome uptake at the presence of chlorpromazine was also very different between the replicates. Because of these reasons, it was decided to redo the experiment. The second experiment resulted in the same increase in liposome uptake, as depicted in figure 5C/D. For this experiment, and every experiment done after, a new liposome batch was used. The difference between the replicates was not present this time, meaning it was likely caused by a human error. Based on this observed increase in uptake, present with the new liposome batch as well, it was theorized the blocked pathway could cause an upregulation of another pathway which proved to be much more efficient. With this hypothesis, it was decided to try and block another uptake mechanism, micropinocytosis. This can be done with the inhibitor EIPA. Therefore EIPA was added to the liposomes. The results are visible in figure 6. Besides the liposomes, dextran was also measured on its own and in combination with EIPA as an efficacy control. The efficacy control does not give a clear image of the efficacy of EIPA, as both replicates give a different result. J indicates EIPA works, whereas P indicates it does not work. The reliability of J is not as good as that of P however, as it has one time point less due to a mistake during the experiment. EIPA in combination with the positive liposomes suggests an increase in uptake as well, smaller than exposure with chlorpromazine (150-250% instead of the approximately 1100% in the repeated chlorpromazine experiment). The results of EIPA were however not very reproduceable between the results of J and P. Therefore, the results cannot be used to get a definite conclusion. Considering an increase was observed when blocking clathrin-mediated endocytosis with chlorpromazine hydrochloride, cytochalasin D was added to the liposomes in the next experiment. This inhibitor blocks multiple different pathways. The results of this experiment indicated cytochalasin D could decrease the uptake of positive liposomes. The decrease was only visible after 1 hour however, as visible in figure 5A/B. This observation is not expected, as the uptake should be inhibited after 4 hours as well. In the image taken after 4 hours (figure 7F2), the effect of cytochalasin is visible but it is less obvious when compared to the image after 1 hour (figure 7E2). Because of these results, the experiment was redone with more time points. This experiment did show a decrease in liposome uptake at each time point, as seen in figure 7C/D. The reduction does fluctuate between 25 and 50%. The wells were contaminated with bacteria, however, which is shown in the microscope image of figure 7G. The light scatter plots of this experiment did not show a problem however, as the percentage of viable cells was in the same range of the other experiments. After the inhibition of actin polymerization did show a decrease in liposome uptake, it was decided to try using an inhibitor of microtubule polymerization, nocodazole. Microtubules are part of the cytoskeleton, just like actin, the inhibition of which could therefore also lead to reduced liposome uptake. The results of this experiment can be seen in figure 8. Nocodazole causes a decrease in uptake of about 25%. This is slightly lower than the decrease observed with the experiment with cytochalasin D. Considering the inhibitors that block multiple pathways do not show

an increase in liposome uptake by the Hela cells, it seems more likely the cause of the increased uptake observed with chlorpromazine hydrochloride is related to the previously mentioned hypothesis: the liposomes are taken up through another, more efficient pathway if one pathway is blocked.

Another possibility is an altered stability of the liposomes at the presence of chlorpromazine hydrochloride. Figure 9 shows the results of an experiment done to test whether the stability of the liposomes is influenced by the inhibitors. The Hela cells were first pre-exposed to the inhibitors cytochalasin D, chlorpromazine hydrochloride and EIPA for 20 minutes. Afterwards, the Hela cells were exposed to just the liposomes without any inhibitor present. This was done to prevent the liposomes from coming into contact with the inhibitors. If the liposomes still show the same results as in the previous experiments, it is likely not caused by an altered stability. The experiment was also done with anionic liposomes instead of zwitterionic liposomes as an efficacy test. All three inhibitors were confirmed to decrease the anionic liposome uptake by two other students. If the inhibitors work as they should, the negative liposomes should show a clear inhibition of the uptake. The results of figure 9 do not indicate the stability of the liposomes is changed, as the liposome uptake with chlorpromazine and EIPA is still increased without the presence of the inhibitors. The negative liposome uptake is only inhibited a little by chlorpromazine and EIPA, however, meaning the effect of those inhibitors quickly vanishes.
The stability of the liposomes in the presence of the inhibitor chlorpromazine hydrochloride has been analyzed with dynamic light scattering (DLS), the results of which are shown in figure 11. Figure 11D shows the size distribution of the liposomes dissolved in cMEM in the presence of chlorpromazine at the same concentration used in the experiments. The size of the liposomes in cMEM is 124.3 nm as seen in the table of figure 11E. The size distribution in the presence of chlorpromazine has multiple peaks, which suggests the liposomes have degraded and agglomerated, resulting in the different diameters. Therefore, these results suggest the inhibitor chlorpromazine does affect the stability. These liposomes with a smaller diameter could be taken up with passive transport, which would explain the increase in uptake seen in figure 5.

Conclusions

The aim of the experiments was to determine the effects of the inhibitors sodium azide, chlorpromazine hydrochloride, EIPA, cytochalasin D and nocodazole on the zwitterionic liposome uptake by Hela cells. This was done by exposing the inhibitors to the Hela cells for different periods. This gave unexpected results for the inhibitor chlorpromazine: an increase in uptake. The results with EIPA suggest an increase in uptake as well but are not really reliable. The other inhibitors did show a decrease in liposome uptake.

The inhibitor chlorpromazine shows an increased positive liposome uptake (figure 5). The results of the liposome stability experiment (figure 9) do not indicate the liposome stability is affected by the inhibitors. However, the DLS (figure 11) that was performed shows the opposite for chlorpromazine. Therefore, it cannot be said with certainty if the liposome stability has a role in the increased uptake of the liposomes. Another explanation of the increased uptake could be another uptake mechanism takes over when the original pathway is blocked which can transport the particle much more efficiently.
The inhibitors cytochalasin D (figure 7) and nocodazole (figure 8) both show a reduced liposome uptake, which means actin and microtubules are involved in the uptake of positive liposomes. Since actin and microtubules are involved in multiple mechanisms, it cannot be clearly determined which

exact mechanisms are involved in the uptake of the particles. Based on these results, it is hard to say which uptake pathways are involved. Chlorpromazine showed an increase in uptake, which could be due to stability and therefore cannot give a clear indication whether the liposomes are taken up with clathrin-mediated endocytosis or macropinocytosis. It can be said with certainty the liposomes are mainly taken up with active transport, as the sodium azide resulted in almost full inhibition. Nocodazole and cytochalasin D did show a decrease in uptake, meaning actin and microtubules are involved in the uptake of the liposomes but not in what way.
To determine in more detail which pathways are involved in the uptake, the pathways could be inhibited using RNA interference. This would likely circumvent the effect on the liposome stability if that is indeed the problem. Furthermore, the effects of the chlorpromazine and EIPA on the zwitterionic liposomes could be looked at in an experiment using confocal microscopy. This could give a better picture of the location of the liposomes during uptake.
As the exact mechanisms involved in the liposome migration are not known, it makes it more difficult to determine the pathways involved in the uptake of liposomes.
Other studies have exposed Hela cells to zwitterionic liposomes as well. In a study by Montizaan D et al. zwitterionic liposomes with DOPC were exposed to Hela cells in the presence of chlorpromazine hydrochloride and EIPA among other inhibitors. These experiments did show a slight decrease with chlorpromazine and a moderate decrease with EIPA.

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Appendices

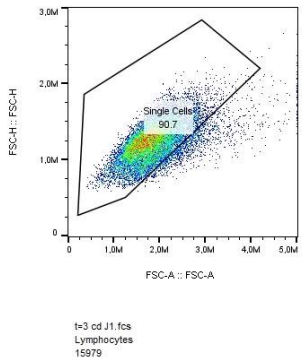
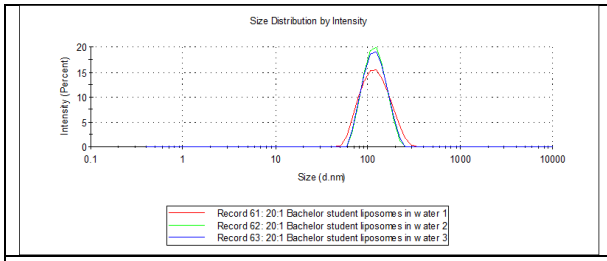
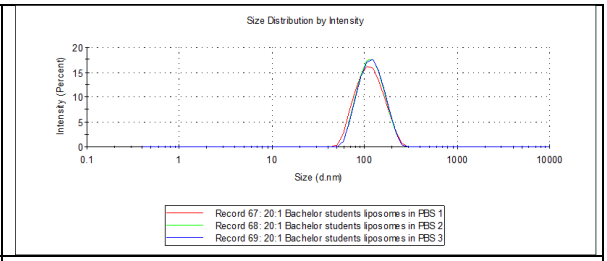


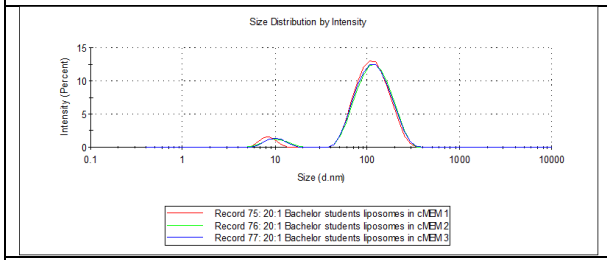
Figure 10: A light scatter plot with FSC-H on the y-axis and FSC-A on the x-axis. This figure shows the way the single cells have been gated.



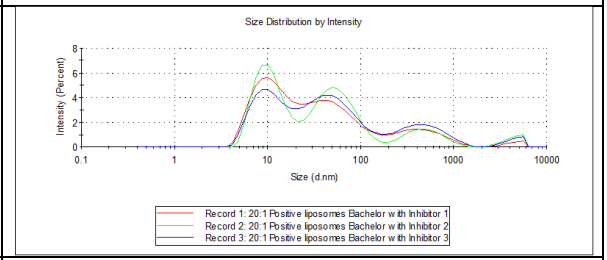
A



B



C



D

SAMPLE	Size (nm)	PdI	Zeta Potential (mV)
DOPC_DC-Chol_DiI (Bachelor) 100 nm 20:1 in Water	113,7±1,1	0,1	27,2±0,8
DOPC_DC-Chol_DiI (Bachelor) 100 nm 20:1 in PBS	110,7±3	0,1	4,7 ± 1,3
DOPC_DC-Chol_DiI (Bachelor) 100 nm 20:1 in cMEM	Peak 1→9,7 Peak 2→ 124,3	***	0
DOPC_DC-Chol_DiI (Bachelor) 100 nm 20:1 in cMEM with Inhibitor	***	***	-7,9±1,1

E

Figure 11: The dynamic light scattering (DLS) results. This experiment was performed by Roberta Bartucci. The graphs of A-D show the size distribution of zwitterionic liposomes dissolved in water (A), PBS (B), cMEM (C) or cMEM with chlorpromazine hydrochloride (D). The table of E shows the size (nm) and zeta potential (mV) of the zwitterionic liposomes in different solutions.