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Characterization of the uptake mechanisms for internalization of negatively charged liposomes in HeLa cells

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Abstract

Aim: In this report we try to give a clear view of what uptake mechanisms are involved in internalization of negatively charged liposomes in HeLa cells. **Materials and methods:** Liposomes were exposed to six different pharmacological inhibitors that all have a different mechanism of action when inhibiting internalization pathways. Uptake of liposomes was measured by staining them with a fluorescent dye and performing flow cytometry. **Results & conclusion:** The different experiments that we performed showed that macropinocytosis might be the main pathway of internalization for the negatively charged liposomes. However, all known pathways of liposomal internalization seemed to be involved in uptake. Finally, liposomal internalization depends on a lot of parameters varying from cell type to liposome characteristics making. Combination of different methods and testing of more parameters in the future will clear up how liposomes exactly are internalized into the cells.

Introduction and Theory

Nanotechnology is a revolutionary technology that has been applied in multiple fields. Application of nanotechnology for medical purposes has been deemed as nanomedicine. This uses nanomaterials for treatment and prevention of diseases, diagnosis, monitoring and control (Soares et al., 2018). Liposomes are a type of well-established nanoparticles that is commercially used to deliver drugs and vaccines within the cell. These particles resemble the cell membrane and consists of a lipid bilayer. This layer provides solubilization and protection of both hydrophobic and hydrophilic agents. Because of this, several therapeutic drugs can be encapsulated and delivered to cells. (Cheung & Al-Jamal, 2018). To this date, over eighteen liposomal drugs are approved by the FDA for cancer treatment, infectious diseases and pain management (Kim & Jeong, 2021).

Liposomes

Even though liposomes are the ideal way to transfer encapsulated drugs directly into the cell, they do carry some drawbacks. These consist of a short half-life, low stability and high production costs. Along with that, the drugs or molecules that are encapsulated could leak out through the membrane. Through size and charge the nanoparticles could also cause major toxicity to the cells (Amornwachirabodee et al., 2018; Maja et al., 2020). However, these flaws can be helped by modification of the liposomes. The physical and chemical properties of liposomes can be genetically altered to improve their workings. This means that the genetic material is modified in such a way which is not normal. Some of these modifications include change in particle size, surface charges and attachment of polyethylene glycol polymer chains (PEGylation) (Ren et al., 2019). When looking at charge, it has been found that nanoparticles that contain a negative charge are preferentially internalized, in contrast to positively charged nanoparticles. This is the case, because positively charged nanoparticles cause more disruption of the plasma membrane, liposomal/mitochondrial damage and a higher number of autophagosomes when compared to nanoparticles with a negative charge (Fröhlich, 2012).

Internalization pathways

It has also come to attention that liposomes are internalized via several different pathways inside the cell, which will be delved into later. (Alshehri et al., 2018). The earlier mentioned liposomal characteristics (surface charge, particle size, PEGylation etc.) have an effect on the liposomal uptake as well (Montizaan et al., 2020; Verma, 2003). It is possible for nanomedicines to pass the cell membrane through passive uptake, however this mainly plays a role after long-time exposure to low nanoparticle concentration. The way in which surface properties of nanoparticles behave in passive uptake is not yet fully characterized (Treuel et al., 2013). Thus, most uptake is done through active processes via different pathways. As the field of nanomedicine tries to improve features of the nanoparticles for detection and treatment of several pathologies, better understanding of how these pathways work at a cellular level could enable man to further improve the characteristics of the liposomes (Sabourian et al., 2020). There are many methods that are available to study how liposomes are taken up in cells. One method is blocking of a certain entry point for the liposomes to enter the cell. Many pathways are involved in liposomal internalization, however not all pathways are regulated in the same way. Blocking one entry point gives information about the importance of that specific pathway for the uptake of the liposomes. Properties of the liposomes that are important for transport through that pathway are then allowed to be modified, to further optime liposomal internalization. The liposomes mainly enter the cell through endocytosis. This process can be classified into two categories: pinocytosis and phagocytosis. Phagocytosis is mainly viewed at as a process of cleaning up debris. Pinocytosis, otherwise known as “cellular drinking” can be further subcategorized into micropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis (Foroozandeh & Aziz, 2018). Blockade of these uptake mechanisms by pharmacological inhibitors is the most common way to determine its involvement in uptake. Another way is to block synthesis of proteins that are involved in certain pathways. These inhibitors either block transcription of mRNA or translation of the proteins. (Laham-Karam et al., 2020). Both these methods can be used to study nanoparticle uptake in the cell, however there remain drawbacks. When one pathway or protein is blocked, the cell might compensate for this by upregulation of other mechanisms. With this in mind, the study was performed using several pharmacological inhibitors. This method is preferred due to a relatively fast onset of action to hopefully limit

the possibility for cells to adapt. Along with that they are easy to use (dos Santos et al., 2011; Francia et al., 2019). However, pharmacological inhibitors do contain limits as some show low specificity and efficacy can strongly vary depending on what type of cells are used an can be cell-type specific (Niepel et al., 2017).

Inhibitors & mechanism of action

With this knowledge six pharmacological inhibitors have been chosen which are applied in nanomedicine uptake studies. These inhibitors have been derived from the article: *"Limits and challenges in using transport inhibitors to characterize how nano-sized drug carriers enter cells"* (Francia et al., 2019). The concentration per inhibitor was optimized in this study by testing the effect of different concentrations on the cell viability. The compounds all work on different mechanisms so that differences in cell uptake can be closer studied. The following compounds were chosen:

- Chlorpromazine, inhibitor of chlathrin-mediated endocytosis;
- Cytochalasin D, inhibitor of actin polymerization;
- Nocodazole, inhibitor of microtubule polymerization;
- EIPA (N-ethyl-N-isoprpylamiloride), inhibitor of macropinocytosis;
- Dynasore, inhibitor of dynamin;
- MBCD (Methyl-ß-cyclodextrin), cholesterol depletion.

Clathrin-mediated endocytosis depends on a protein called clathrin which interacts with other proteins to form clathrin-coated vesicles. Adaptor proteins, like AP2, are important for linkage between the clathrin coated vesicles and the membrane-bound cargo. Along with that, AP2 also has interaction with other adaptor proteins which in turn further facilitates endocytosis (Smith et al., 2017). Chlorpromazine inhibits AP2, leading to inhibition of clathrin-mediated endocytosis on numeral levels. Cytochalasin D blocks actin polymerization. Actin plays an essential role in endocytosis as it creates protrusions that could encompass extracellular materials (Mooren et al., 2012). Cytochalasin D acts as an inhibitor of the interaction between cofilin, a regulator needed for actin filament dynamics, and G-actin. To add to this, cytochalasin D also inhibits binding of cofilin to F-actin leading to a decreased rate of polymerization. Cytochalasin D is also well known for its binding to the barbed end of the F-actin (Shoji et al., 2012). Because actin is involved in several pathways, interpretations should be taken with caution as actin is involved in macropinocytosis, clathrin-mediated endocytosis and clathrin-independent macropinocytosis (Francia et al., 2019). As actin is the major cytoskeletal protein for most cells, inhibition of its polymerization might also lead to change in cell shape (’Cooper, 2000). Just like actin, microtubules are cytoskeletal elements that are also involved in several pathways. These microtubules are composed of Þ-tubulin and ß-tubulin heterodimers (Logan & Menko, 2019). Nocodazole is a compound which binds to tubulin and therefore inhibits microtubule polymerization. Microtubules have been found to be essential in clathrin-mediated endocytosis (Day et al., 2015), macropinocytosis through disturbance of between F-actin and microtubules (Li et al., 2020) and several other mechanisms (Tsuchiya et al., 2021). EIPA is used as inhibitor for macropinocytosis. This is a form of endocytosis which is initiated by activation of several growth factor signaling pathways. After a biochemical signal, the cells starts with internalizing extracellular particles into certain endocytic vesicles, which are known as macropinosomes. These deliver the particles into lysosomes for degradation (Xiao et al., 2021). Macropinocytosis has a unique relationship with Na^+/H^+ exchanger pump. These Na^+/H^+ exchanger pumps are known to regulate the activity of pH sensitive signaling molecules. EIPA inhibits this pump leading to a change in sub membranous pH, close where the macropinosomes are formed. This leads to inhibition of macropinocytosis (Koivusalo et al., 2010; Lin et al., 2018) . Dynasore is a compound that inhibits dynamin. Dynamin is a GTPase which plays a great role in clathrin-dependent endocytosis and other vesicular trafficking processes. It acts as a pair of molecular scissors and plays a role in cleavage of clathrin coated vesicles (Singh et al., 2017). Along with that dynamin is heavily involved in membrane fission, which is highly involved in endocytosis. Dynamin activity is therefore found in multiple pathways correlated with endocytosis (Antonny et al., 2016). MBCD is a compound which causes acute cholesterol depletion. Mostly caveolae-mediated endocytosis is affected. Caveolae-mediated endocytosis leads to nanoparticles being trafficked to caveolae invaginations on membrane of the cells which internalizes the particle. Caveolae-mediated endocytosis has a complicated signaling pathway (Rees, 2013). Caveolin-mediated endocytosis is known to be dependent on

cholesterol-rich domains or so called lipid rafts. These are plasma membrane microdomains that are enriched in cholesterol and sphingolipids. These are involved in lateral compartmentalization of particles at the membrane surface. MBCD removes cholesterol therefore inhibiting both caveolae-mediated endocytosis and formation of lipid rafts (Hao et al., 2012; Lajoie & Nabi, 2010).

Cytochalasin D, nocodazole, dynasore and MBCD all show to have an involvement with several pathways, as they all affect mechanisms for multiple pathways. Therefor interpretation of the results obtained with these compounds is complex, making it difficult to conclude on what is the leading mechanism in liposome uptake. Nevertheless, these inhibitors do give us more insight about the pathways and eventual upregulation. With this in mind, the uptake mechanisms of negatively charged liposomes was investigated. First, the internalization of the negative liposomes was studied using flow cytometry. Next, it was tested whether these liposomes were taken up through active or passive processes by depleting the ATP in HeLa cells using sodium azide (NaAz). Finally, the different transport inhibitors described above were tested on how they affected the liposome uptake. Overall, all results obtained could give a clearer view on liposome uptake mechanisms and could further help improving liposome formulations.

Materials and Methods

Cell culture

For all experiments HeLa cells (ATCC) were used. These cells were grown in MEM (Gibco™) cell culture medium (cMEM) which were supplemented with 10% v/v fetal bovine serum (FBS, Gibco™) . The cells were grown in T75 flasks under standard conditions at 37 °C and 5% CO_2 until cell culture contained $10*10^6$ cells. Cells between passage four and twenty were used. Cells at higher passage numbers could become less sensitive to the compounds used. When splitting cells, they were first washed twice with 5 ml phosphate buffer solution (PBS). 3 ml TEP was added to the flask to free the cells and incubated in the stove for 5 minutes at 37 °C. After incubation cMEM was added to the cells, transferred to a tube and centrifuged for 5 minutes at 300 rpm. After removing the medium without removing the cell pellet, it was resuspended in 10 ml medium and a cell count was performed. Cells were counted using a hemocytometer (counting plate). Through this count a new T75 flask could be prepared for upcoming experiments. For each experiment wells plates were plated out at least 24 hours before conducting the experiment. Each well contained 50.000 cells. Each well was plated out with 500 µl of cell solution.

Liposomes

Negatively charged liposomes were used composed of DOPC : DOPG : cholesterol in the ratios 10 : 6,67 : 1, respectively. The structures are found in **Figure 1.** The zeta potential of the liposomes in PBS was -30 mV. Two different batches of liposomes were used, however the composition remained the same. The liposome concentration in each well containing liposomes was 50 µg/ml. The liposomes were stained with DiL (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate ('DiI'; DiIC18(3))) with excitation and emission wavelengths of 549 and 565 nm. These were measured in the 585/42 BP channel in the flow cytometer.

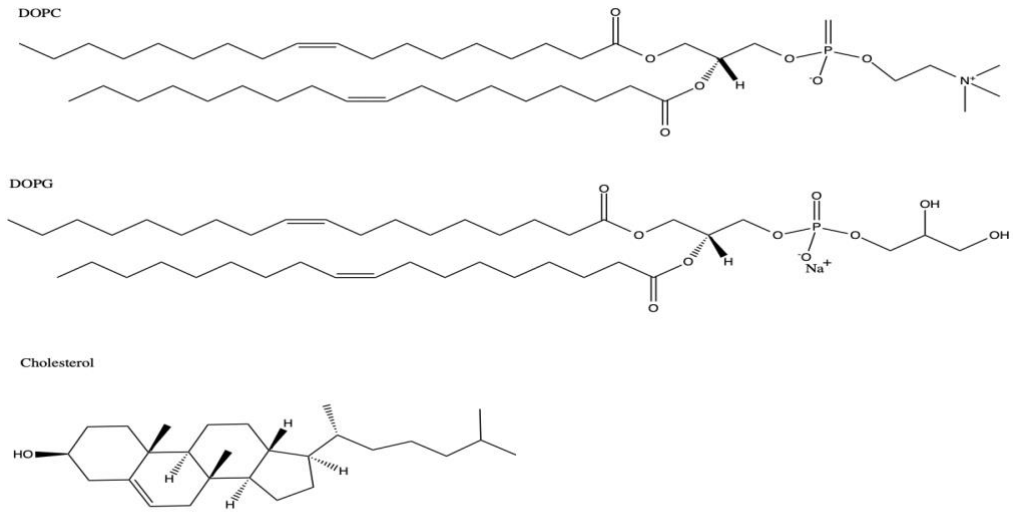


Figure 1. Structural formulas of DOPC, DOPG and cholesterol, respectively. These structures were drawn with ChemDraw.

Inhibitor experiments

Every inhibitor was tested in the same way with the same pre-incubation of t = 20 minutes, with an exception for MBCD and Dynasore. The experiments with these inhibitors were performed with a pre-incubation time of t = 1 hour. Cells were pre-incubated with the different inhibitors at the desired concentrations. After this pre-incubation time, the wells were filled 250 µl of cMEM with the desired liposome- and/or inhibitor concentration. The optimum conditions for these inhibitors are derived from the article: *"Limits and challenges in using transport inhibitors to characterize how nano-sized drug carriers enter cells".* These conditions were as follows: chlorpromazine 10 µg/ml, cytochalasin D 2.5 µg/ml, nocodazole 5 µM, EIPA 100 µM, MBCD 2.5 mg/ml, dynasore 25 µg/ml. Efficacy of the inhibitors was assessed by measuring the uptake of the fluorescently labeled marker. 26 µg/ml of DiL (3,3'-dioctadecylindocarbocyanine-low density lipoprotein) was added to the liposomes so their uptake could be measured through fluorescence. Positive controls were used for both EIPA

(Dextran 250 µg/ml) and MBCD (LacCer 0.1 µM). These were used as a marker for macropinocytosis and caveolae-mediated and lipid raft dependent endocytosis, respectively. The cells were harvested after certain timepoints; t=1h, t=3h and t=5h. MBCD and Dynasore were measured after one timepoint t = 30 minutes, due to their inability to produce an effect in serum. The process of harvesting required washing one time with cMEM and two times with PBS. The washing step is performed because trypsin gets inactivated in presence of serum. Traces of the culture medium must therefore be completely removed. PBS is used because unlike water it prevents cells from rupturing or shriveling up by osmosis (Wangen et al., 2018). TEP (trypsin-EDTA) was added to free the cells from the bottom of the wells. After addition of medium, transferring the content of the wells to tubes and centrifuging the suspensions for 5 minutes at 300 rpm, a pellet was obtained. The cell pellet was resuspended in 100 µl PBS, and cell fluorescence measured by flow cytometry.

Flow cytometry analysis

The tubes containing the cells were analyzed under the Cytoflex Flow Cytometer (Beckman Coulter). To optimize the analyzed data, a gate was set to first filter out excess cell debris. After this a second gate was set to only analyze single cells. The y-axis contained the side scatter of the light emitted at the cells whilst the x-axis contained the forward scatter. At least 20.000 cells were acquired per sample and every sample was performed in duplicate by two students for a total of two replicate experiments. These results were then analyzed using the Flowjo software (Flowjo, LLC). From this single cell histograms were created by filtering out the leftover cell debris and the cell duplets. These histograms give an indication about the cellular uptake of the liposomes **(Supplementary figure 1).** Along with this a table was created containing the median, geometric mean and the cell count. **Figures 2-9** are expressed as median cell fluorescence intensity of the distributions obtained averaged over two duplicate samples for two replicate experiments. **Figures 3 and 4-10** also show histograms where the cells that are exposed to liposomes in the presence of a drug inhibitor have been normalized by uptake of liposomes in cells that were not exposed to the inhibitors. Error bars have been added to function as the standard deviation.

Results & Discussion

All experiments were performed in duplicates by two persons for a total of four measurements per timepoint. The results were analyzed with Flowjo. The sideward scatter 'SSC-H' was put against the forward scatter 'FSC-H' to get a clear view of all cells. Leftover cell debris was then excluded from the test results. To exclude the cell doublets from the results, 'A-FSC' was put against 'H-FSC' and a gate was set around this A histogram could then be created by putting 'cell count' on the y-axis and the settings of the fluorescence channel on the x- axis 'FL6-A :: PE-A'. The median values were extracted by creating a table using the Table editor. Transfer of this data to excel allowed us to use this the data to create the graphs related to the inhibitors.

The first step was to analyze whether the negative liposomes showed increasing uptake over time. These results can be seen in **Figure 2.** The graph shows a positive correlation between increasing time and measured intensity. This indicates that with increasing time more liposomes are internalized by cells.

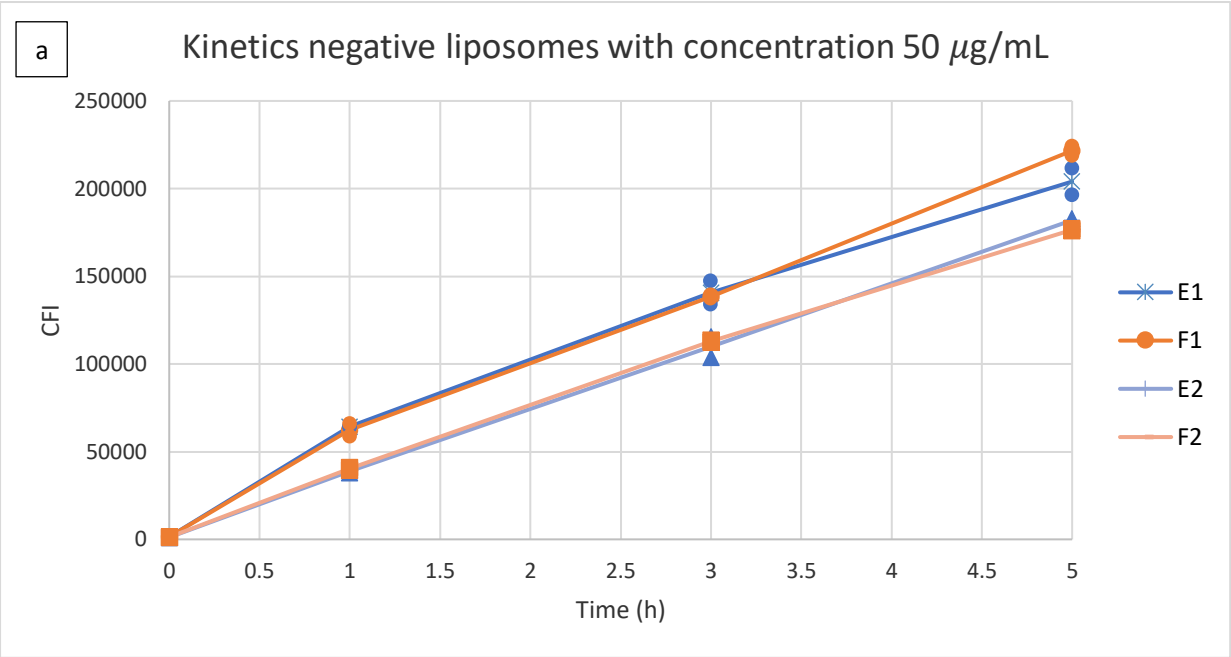


Figure 2. Kinetics negative liposomes. The measured fluorescence intensity against the time in hours. The measured intensity is proportional to the liposome uptake within the cells (a).

The understanding that the liposomes were indeed taken up in the cell over time led to the next step. This was analyzing whether the liposomes were taken up in the cell through active or passive transport. The cells were exposed to sodium azide (NaAz) to test which one of the latter two was most responsible for liposome uptake. NaAz is a compound which inhibits oxidative phosphorylation through inhibition of cytochrome oxidase. This is the final enzyme in the mitochondrial electron transport chain, which results in a rapid depletion of intracellular ATP (Harvey et al., 1999). To look at this effect, NaAz was first preincubated in four wells per three plates for a total of twelve wells, for t = 20 minutes. Prei-ncubation was performed because this could enhance the inhibitory potency of NaAz (Tátrai et al., 2019). After pre-incubation the content of the wells was sucked up and they were filled up with either liposome solution, liposomes + inhibitor or a negative control. The plates were harvested and measured at three different timepoints; t = 1 hour, t = 3 hours and t = 5 hours. These results can be seen in **Figure 3**. It shows that there is indeed a decrease in uptake of the negative liposomes when NaAz is added in contrast to cells which were not exposed to the NaAz. It is peculiar that even when NaAz is present, the cell fluorescence increases, thus liposomes are still internalized, as this would indicate that the effect of the NaAz diminishes after several hours. A possible explanation for the results shown in Figure 2 could be that the pre-incubation of 20 minutes was not enough to reach a maximum inhibitor potency.

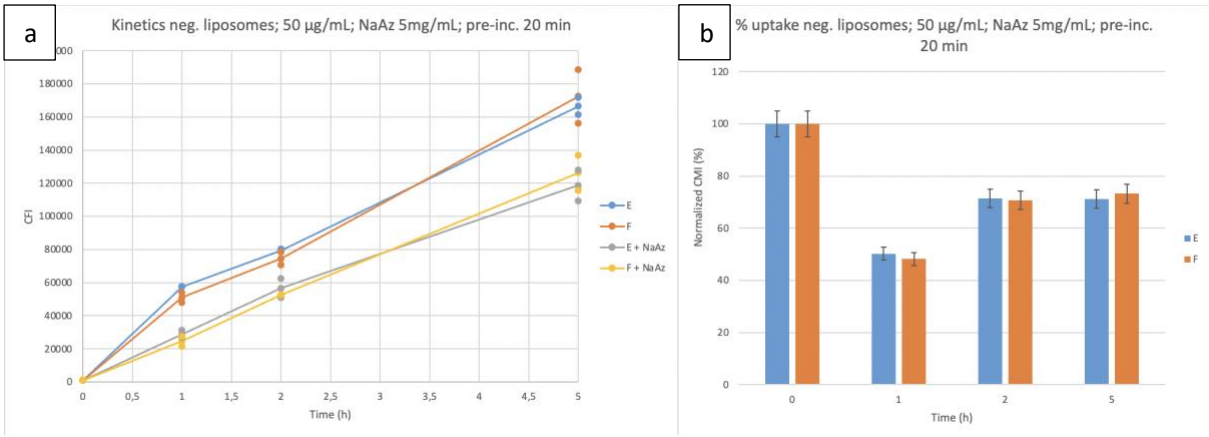


Figure 3. Sodium azide for ATP depletion with pre-incubation time t = 20min. Uptake by flow cytometry of 50 µg/ml negative liposome solution in cMEM in control cells and cells exposed to 5 mg/ml NaAz (duplicate measurements for two replicate experiments of median fluorescence intensity of 20.000 cells) *(a).* Percentage liposomal internalization normalized for uptake in control cells without NaAz *(b).*

A possible explanation for the results shown in Figure 3 could be that the pre-incubation of 20 minutes was not enough to reach a maximum inhibitor potency. It is apparent that pre-incubation times could reach up to 2 hours to reach maximum inhibitor potency (Tátrai et al., 2019). For this reason, the decision was made to redo this experiment. The only difference was that the pre-incubation time was increased from t = 20 minutes to t = 1,5 hours and one timepoint at t = 5 hours was replaced with t = 4 hours due to time reasons. The results from this experiment can be seen in **Figure 4.** As can be seen from the figure, a longer pre-incubation time with NaAz resulted in less uptake of the liposomes.

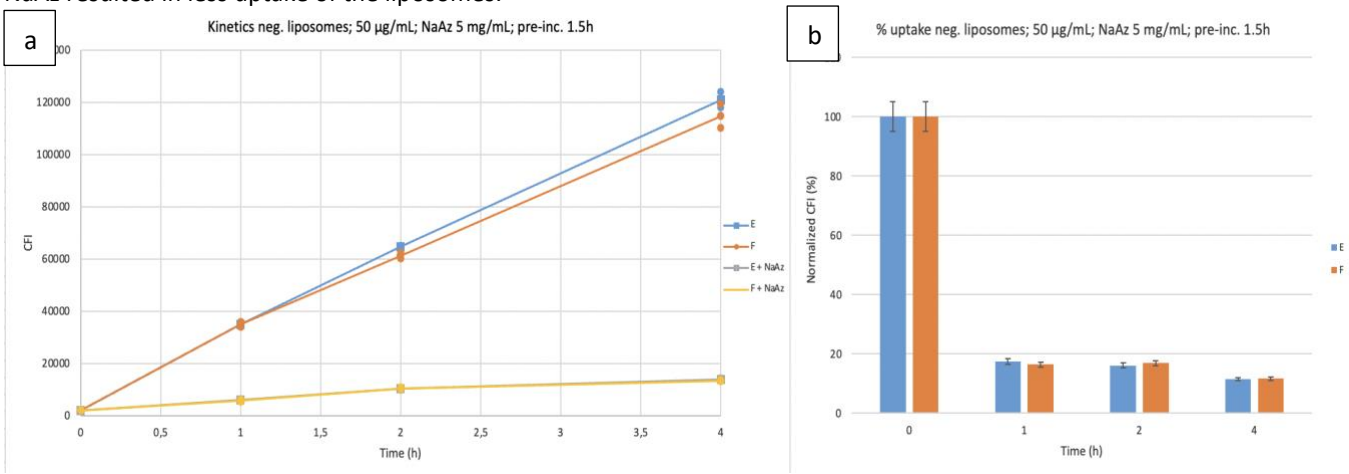


Figure 4. Sodium azide for ATP depletion with pre-incubation time t = 1.5h. Uptake by flow cytometry of 50 µg/ml negative liposome solution in cMEM in control cells and cells exposed to 5 mg/ml NaAz (duplicate measurements for two replicate experiments of median fluorescence intensity of 20.000 cells) *(a).* Percentage liposomal internalization normalized for uptake in control cells without NaAz *(b).*

By preincubating the cells with NaAz for 1.5 hours, at timepoint t = 4 hours, almost a 90% reduction in cell uptake is observed. This confirmed that the liposomes were mostly taken up through active transport, which requires ATP. Now that we confirmed that negative liposomes are mostly internalized in the cell via active transport, it was time to study the different mechanisms through which this internalization could take place. For this we used the inhibitors that were previously mentioned in the introduction.

The first inhibitor tested was chlorpromazine, the AP2 inhibitor, which blocks liposome uptake through clathrin-mediated endocytosis (CME) at multiple levels. The results are seen in **Figure 5.** A peculiar observation is that between timepoint t = 1 and t = 5 the cell fluorescence increases, meaning that liposome internalization did show a slight increase over time. Why this happens is not certain, however it is known that chlorpromazine mainly blocks the CME. There is the possibility that in the early stages of liposome uptake CME is upregulated.

At a later timepoint the liposomes may enter the cells through different pathways than CME, as there are several pathways for liposomal uptake possible. It has been found that in some cases a clathrin light-chain isoform (CLCB) is upregulated in cancer cells of other tissue than the cervix. These cells showing an increased expression of CLCb exhibit an increased rate in clathrin-mediated endocytosis through dynamin. (I. Khan & Steeg, 2021). This could give the indication that at later stages clathrin-mediated endocytosis is upregulated through upregulation of other molecular mechanisms. this knowledge would be taken into consideration when experimenting with the rest of the inhibitors.

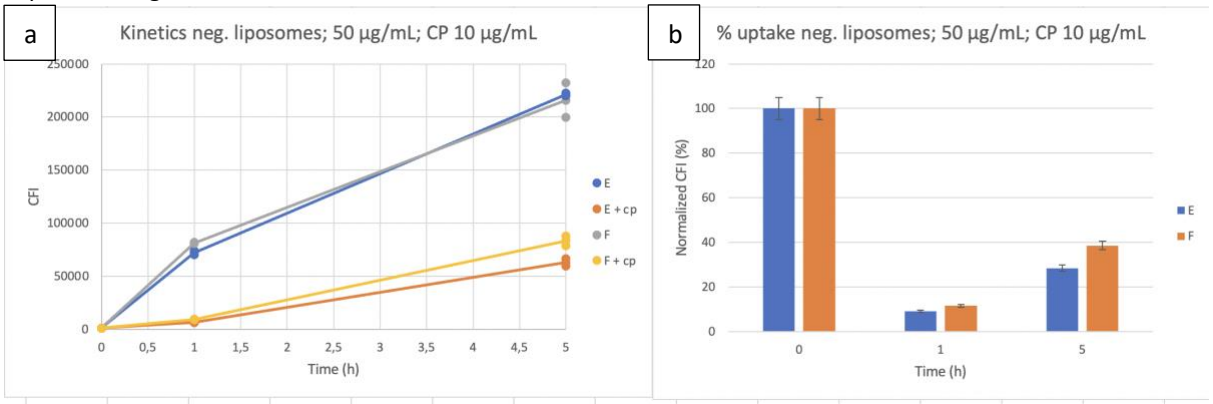
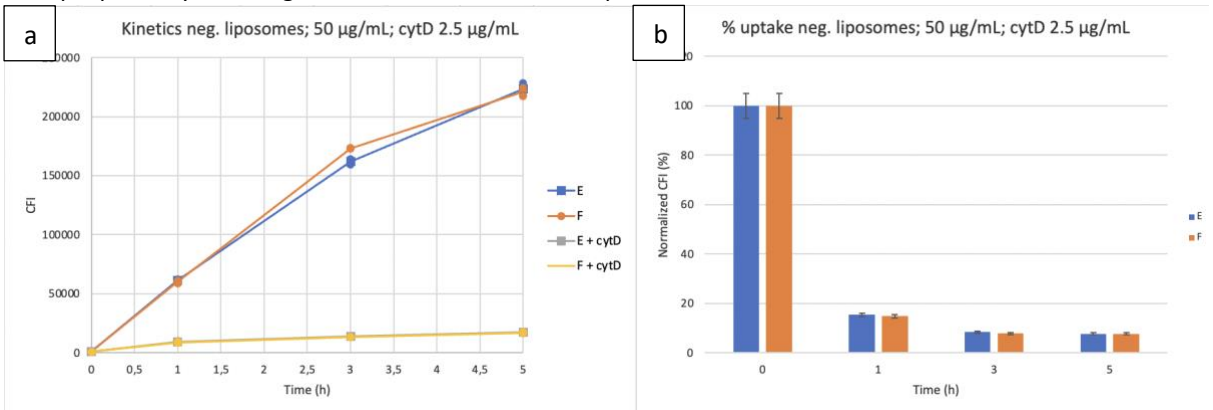


Figure 5. Chlorpromazine to block CME. Uptake by flow cytometry of 50 µg/ml negative liposome solution in cMEM in control cells and cells exposed to 10 µg/ml chlorpromazine (duplicate measurements for two replicate experiments of median fluorescence intensity of 20.000 cells) *(a).* Percentage liposomal internalization normalized for uptake in control cells without chlorpromazine *(b).*

The next experiment was with the inhibitor Cytochalasin D (CytD). CytD is a mycotoxin which binds actin and blocks its polymerization. Actin is found to be involved in several pathways, meaning that interpretation of these results might not give a clear indication about the exact pathways involved. The inhibitory effect of cytD on uptake can be found in **Figure 6.** As actin cannot be polymerized due to its inhibition the cytoskeleton also under finds changes as the filaments which provide the cells with mechanical support are not formed (Pollard & Cooper, 2009). These changes in cell form are seen in **Figure 6.** CytD is seen to have a fast onset of effect as at t = 1 hour almost 90% less liposome uptake is seen. At t = 5 hours it looks like maximum inhibition is reached. CytD generally shows the highest inhibition potency for macropinocytosis. CME and caveolae/lipid raft dependent endocytosis are also partially inhibited (Mäger et al., 2012). These results might indicate that macropinocytosis plays a major part in liposome internalization. However, as earlier mentioned cytD affects multiple pathways, making these results harder to interpret.



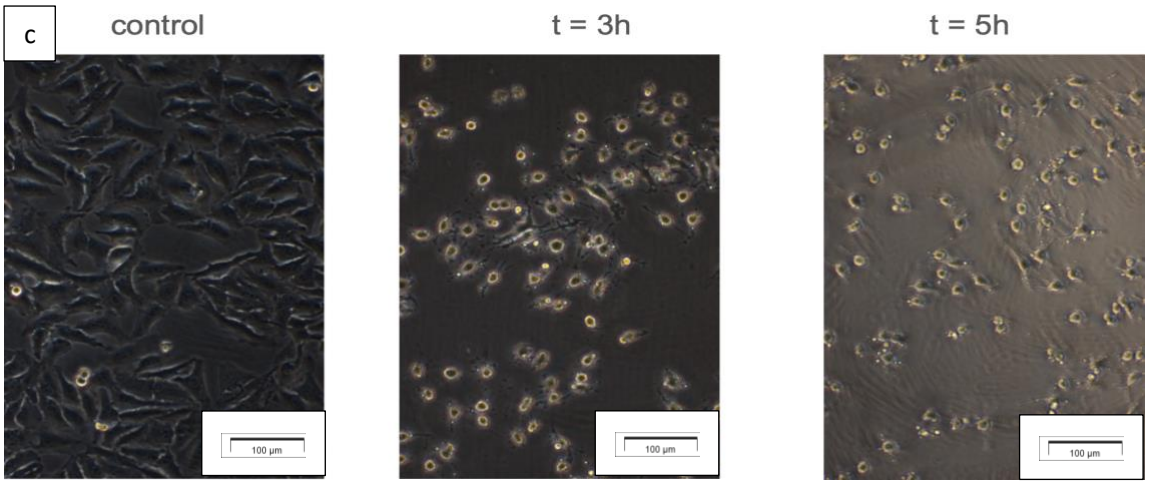
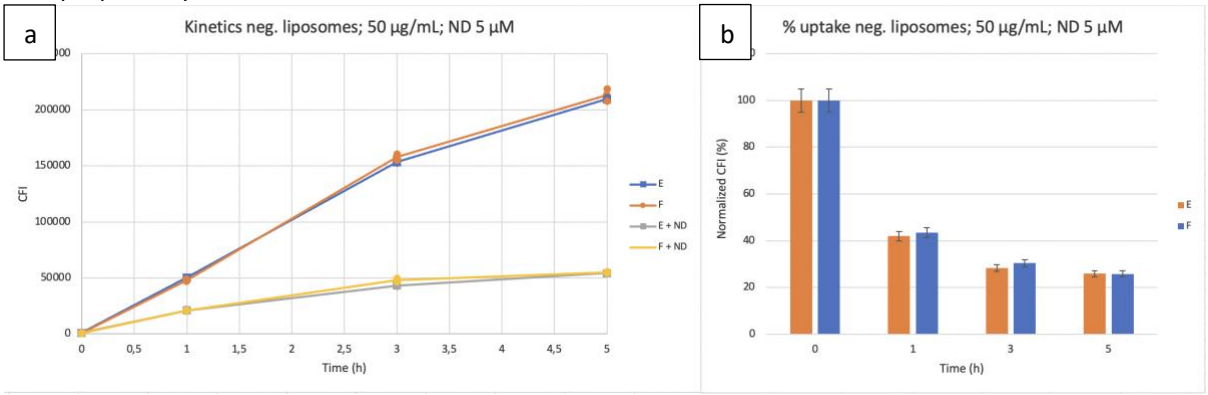


Figure 6. Cytochalasin D for inhibition of actin polymerization. Uptake by flow cytometry of 50 µg/ml negative liposome solution in cMEM in control cells and cells exposed to 2.5 µg/ml cytochalasin D (duplicate measurements for two replicate experiments of median fluorescence intensity of 20.000 cells) *(a).* Percentage liposomal internalization normalized for uptake in control cells without cytochalasin D *(b).* Light microscopy images of cells exposed to cytochalasin D in cMEM at different time stamps (scale bar: 100 µM) *(c).*

Nocodazole, like cytD, affects many pathways involved in liposomal uptake. It binds to beta-tubulin, which disrupts microtubule assembly. These microtubules are partly responsible for endocytic trafficking and are found to have a direct correlation with CME, macropinocytosis and other possible mechanisms. The inhibitory effect of nocodazole can be seen in **Figure 7**. Due to blocking of microtubule formation, the shape of the HeLa cells also changes. These changes can be seen in **Figure 7.** The results show that over time nocodazole decreases liposomal uptake. As nocodazole is an inhibitor of many different pathways these results are no surprise. Actin filaments and microtubules have been found to have several interactions with each other regarding cellular transport mechanisms. However, actin filaments are shorter and actin based transport has also been found to be slower than vesicular transport by microtubules (Apodaca, 2001; Nakase et al., 2015). This could explain the obtained results. Due to microtubules acting at a relatively higher pace, liposomal internalization could also be relatively higher compared with actin filaments. T = 1 shows a 40% total uptake for inhibition with nocodazole while figure 5 shows a 15% total uptake for this timepoint when inhibition is done by cytD. The higher mobility of microtubules compared to actin could have as result that at lower timepoints more liposomal uptake takes place when inhibiting these microtubules as the liposomes are also taken up via other mechanisms. However this remains a theory as actin and microtubules are both used in multiple pathways.



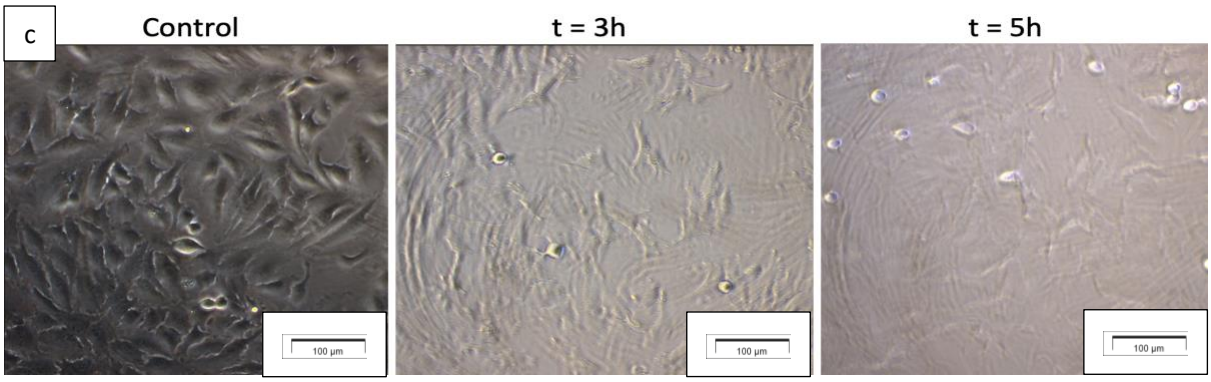


Figure 7. Nocodazole to disrupt microtubules. Uptake by flow cytometry of 50 µg/ml negative liposome solution in cMEM in control cells and cells exposed to 5 µM nocodazole (duplicate measurements for two replicate experiments of median fluorescence intensity of 20.000 cells) *(a).* Percentage liposomal internalization normalized for uptake in control cells without nocodazole *(b).* Light microscopy images of cells exposed to nocodazole in cMEM at different time stamps (scale bar: 100 µM) *(c).*

The next inhibitor that was used is the macropinocytosis inhibitor, EIPA. By blocking of the Na^+/H^+ exchanger pump, macropinocytosis is inhibited due to interference with the pH of the cytosol where the macropinosomes are formed. Dextran was used as a positive control as dextran is a common marker molecule for the macropinocytosis pathway (Nakase et al., 2015). The inhibitory effect of EIPA can be seen in **Figure 8.** This experiment is reproducible as the two values for each timepoint lie very close to each other in terms of uptake percentage. It seems that EIPA has already reached its maximum effect after 45 minutes. Along with this liposome uptake stayed at about 10% when exposed to EIPA for every timepoint. This could indicate that EIPA blocks most of the liposomal uptake and that macropinocytosis is one of the, if not, major pathways for these negatively charged liposomes.

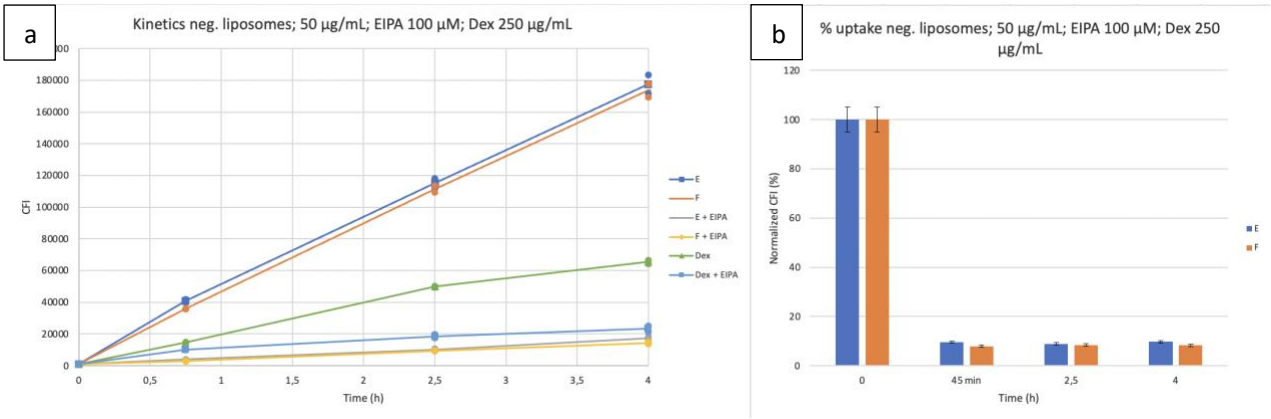


Figure 8. EIPA for inhibition of macropinocytosis. Uptake by flow cytometry of 50 µg/ml negative liposome solution in cMEM in control cells and cells exposed to 100 µM EIPA (duplicate measurements for two replicate experiments of median fluorescence intensity of 20.000 cells) *(a).* Percentage liposomal internalization normalized for uptake in control cells without EIPA *(b).*

Dynasore is a non-competitive inhibitor of dynamin GTPase activity, which blocks dynamin-dependent endocytosis. There are multiple pathways which require dynamin activity to be performed in the cell (Kirchhausen et al., 2008). MBCD is the most widely used drug for cholesterol depletion. Cholesterol is essential for formation of domains in membranes, often known as lipid rafts. Along with that cholesterol is important for membrane trafficking and cell signaling, along with lipid sorting. For this reason, application of MBCD to the HeLa cells would lead to inhibition of multiple pathways. Dynasore and MBCD are known to not have an effect when bound to serum proteins (Kirchhausen et al., 2008; Mahammad & Parmryd, 2008). Serum contains a protein source which affects liposomal uptake by both the corona composition and presence of free proteins in the solution (Yang et al., 2021). We countered this problem by preincubating the cells with dynasore and MBCD for 1 hour and then measuring one timepoint after t = 30 minutes. This timepoint was recommended as longer exposure could terminate any MBCD and dynasore activity. The results are seen in **Figure 9.** Both dynasore and MBCD showed an inhibiting effect. No changes in cell structure were observed.

Exposure to dynasore led to an 80% reduction of liposomal uptake. MBCD led only to an approximate 40% decrease, which was quite fascinating as cholesterol is an important component for multiple pathways involved in liposomal uptake, mainly being CME and caveolae-mediated endocytosis.

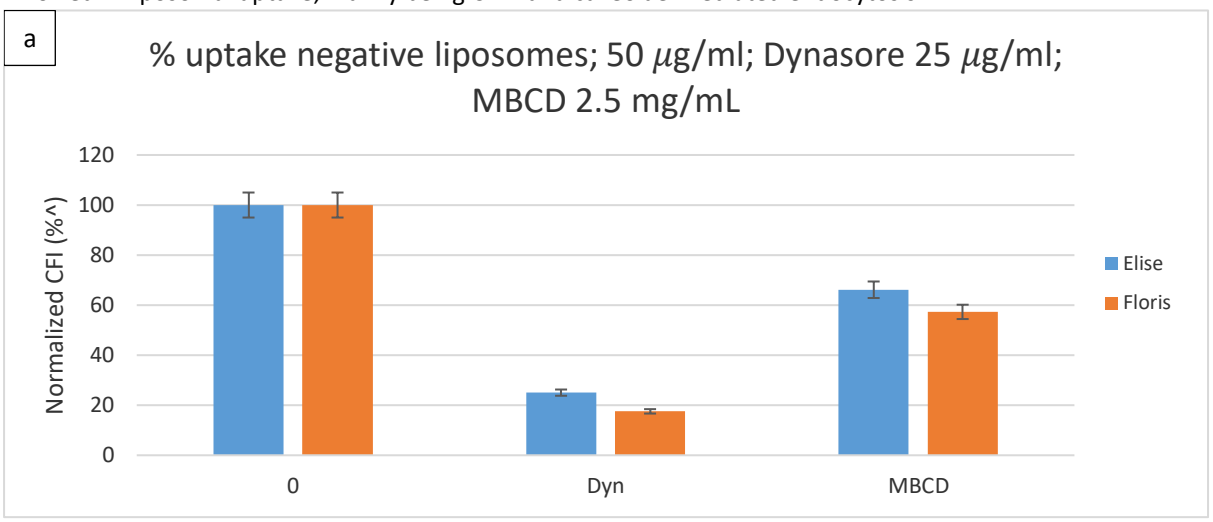


Figure 9. Dynasore for inhibition of dynamin & MBCD for cholesterol depletion. The percentage uptake of liposomes when cells are exposed to dynasore or MBCD regulated for cells not exposed to dynasore or MBCD at that timepoint *(a).* No kinetics were done as only one timepoint could be measured.

Because the results for MBCD did not show a great reduction in uptake this inhibitor was tested a second time along with a positive control LacCer, a glycosphingolipid which uptake in the cells depend on cholesterol. This LacCer was fluorescently labelled and tested in another channel of the cytoflex. The results of this second experiment can be seen in **Figure 10.** LacCer was taken up in high amounts in the cells, however when exposed to MBCD this uptake decreased significantly as expected. Quite interestingly, the uptake of liposomes in cells which were exposed to MBCD had a rather higher decrease in uptake in contrast to the first MBCD experiment. None of the variables changed between these experiments, so the exact reason for why this happened remains unknown.

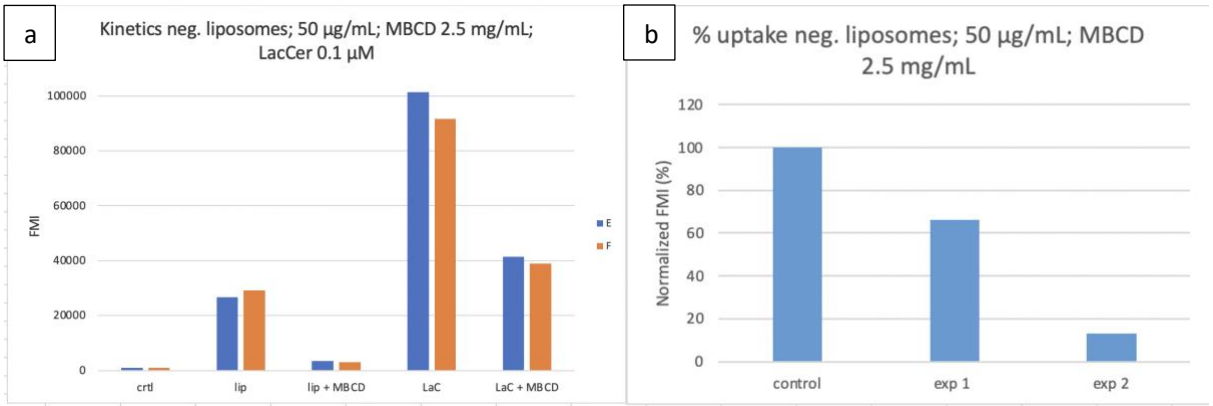


Figure 10. MBCD for cholesterol depletion. The percentage uptake of liposomes when cells are exposed to MBCD. LacCer was used as a positive control *(a).* The percentage uptake of the both experiment with MBCD shows a higher drop in the second experiment than in the first one *(b).*

Conclusion

To give insight about the mechanisms used for internalization of the negative liposomes, we first verified that liposomes were in fact taken up in the cell via active transport. The results obtained after exposure to NaAz experiment confirmed this initial claim. The inhibitor experiments were then performed to hopefully give a better view on what exact mechanisms were used by the liposomes to enter the cells. Chlorpromazine and EIPA both have a clear mechanism of action and are mainly involved in only one pathway, which makes interpretation of these results relatively clear. While chlorpromazine does show an inhibiting effect at early timepoints, this blockage seems to slowly take off with increasing time. As chlorpromazine inhibits CMEa possible interpretation is that this type of endocytosis might be more upregulated in early stages of liposome uptake. As for EIPA, the results were even more clear with almost full inhibitory effect seen after the first measurement. This could give the suggestion that macropinocytosis is the main pathway for which these liposomes are internalized. It was earlier mentioned that when looking at the inhibitors that were not specific for only one pathway (cytD, nocodazole, dynasore, MBCD), these results could be hard to interpret. That being the case, these inhibitors do give us some information regarding the uptake mechanisms.

Cytd and nocodazole mainly work on the actin filaments and microtubules, respectively. As actin and microtubules have been found to interact with each other the results obtained could be interpreted together. However, it was found that inhibition with cytD showed a significantly faster effect than inhibition with nocodazole. This was the case because cytD reached almost a full inhibitory effect after three hours. A theory was that actin based transport was slower compared with transfer through microtubules, thus explaining this difference in effect. To add to this cytD mainly inhibits macropinocytosis, while nocodazole is a main inhibitor of CME. This adds to the theory that macropinocytosis might be the main pathway in negative liposomal internalization.

It was previously shown that MBCD and dynasore were drugs that lost their efficacy when exposed to serum. For this reason pre-incubation in serum free medium was done. One timepoint was measured after half an hour in serum. The short exposure was done, to minimize the loss of efficacy of both MBCD and dynasore. Along with that, free proteins in serum seem to affect liposome uptake by the free proteins present in the serum (Jian et al., 2013). To prevent significant differences in liposome uptake when compared to other experiments, the one timepoint was measured in serum. Both showed a decrease in liposomal internalization, however when the MBCD experiment was repeated a second time along with a positive control, the percentual uptake of the liposomes was less than in the first experiment. This was a rather strange outcome as nothing was changed between experiments, other that a positive control was used.

Overall, the obtained information suggests that the negative particles are internalized in the cell via many pathways, as every inhibitor showed a significant decrease in cellular uptake. Anyhow, the results we acquired suggest that macropinocytosis is the most relevant pathway used, as inhibitors that focus on this pathway showed the most blockage of liposomal internalization. CME, CIE and caveolae-mediated endocytosis are involved as well, however the results would suggest that they are not the main pathway.

Our results may imply this, however there is no certainty whether macropinocytosis is indeed the main pathway when talking about negative liposomal uptake. Something that needs to be addressed is the reproducibility. This has been quite good for the two persons that performed these experiments. However, when redoing the MBCD experiment the results were not the similar, questioning the reproducibility of these experiments. To add to that, a maximum of three timepoints were taken for the separate experiments. More timepoints would give a more elaborate take on the workings of the mechanisms. Finally, only one analytical method in flow cytometry was performed to study these uptake mechanisms. Using other methods could give a clearer view of the applied mechanisms. Some of these techniques may include mass spectrometry or transmission electron microscopy (Drasler et al., 2017). Combination of these methods might be the best approach to get the best understanding of the uptake pathway of the liposomes.

When looking at an even broader field, we must also look at the liposome formulation. The liposomes used in this study had a zeta potential of -30 in PBS. As mentioned earlier several parameters could have an effect on liposomal internalization, such as size, charge, PEGylation etc. Other properties such as leak tightness or

stability can also be influenced by parameters like pH and temperature (Pentak, 2014). Modifications of these properties could have a positive effect an might further improve cellular uptake of liposomes as several studies have shown (A. A. Khan et al., 2020; Nguyen et al., 2016).

Finally, cellular uptake also depends on the type of cells. Not only for cancer cells, but also for regular cells like macrophages and epithelial cells (Kuhn et al., 2014). Overall, a lot of parameters determine the uptake of particles. By combining multiple methods and trying different liposome formulations we could come one step closer with, every experiment that would be performed in the future, to answer the central question of how exactly liposomes are internalized in the cell.

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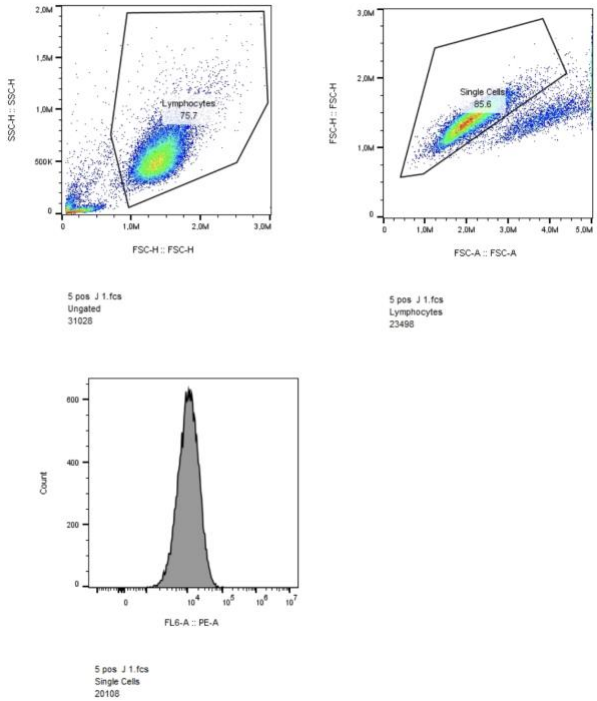
Appendices



Supplementary figure 1. Example of the gates set to separate the single cells.