Inhibition of the cellular uptake of positively charged liposomes by HeLa cells and the different uptake mechanisms involved

Philip Kooi
s3674541
Anna Salvati and Catharina Reker-Smit
Nanomedicine and drug targeting department
24th June 2022

Abstract:

Aim: The aim of this study is to get a better understanding of the uptake by HeLa cells of positively charged liposomes, how can these pathways be inhibited and to what extent are they involved in the uptake. **Methods and materials:** 5 inhibitors were selected and exposed over time to the HeLa cells and measured for liposomal uptake using flow cytometry. Drug efficacy was measured by the measuring fluorescence. **Results and conclusion:** The results were interpreted using FlowJo and it was demonstrated that the uptake is more actin-dependent than microtubule-dependent and for macropinocytosis and clathrin-mediated endocytosis no clear conclusion could be drawn.

Introduction:

Nanomedicine is a new outstanding area in drug development and relatively new in the pharmaceutical development branch. Liposomes are one of the most prominent biological vesicles for drug delivery into human cells but difficult to deliver to specific cell organelles. Liposomes are composed of biological lipid vesicles containing multiple amphipathic lipids with hydrophilic ''heads'' and long hydrophobic carbon tails. The lipids are alternated with cholesterol which increases the stability of liposomes in plasma, by reducing passive water diffusion into the liposomes.

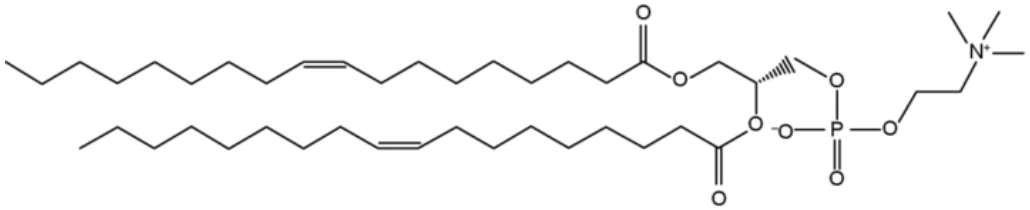


Figure 1: Lipid used in the experiments, DOPC : cholesterol 20:1 ratio singular lipid.

The main advantages of using liposomes as drug carriers is the reduced immunogenicity and toxicity compared to conventional drugs as well as being biodegradable, lowering liver toxicity. Liposomes also decrease metabolization by enzymes, protecting the drug when administered parentally, increasing the half-life of the drug. Pegylated nanoparticles are especially useful since the half time is drastically enhanced by escaping phagocytosis.**[1],[2],[12]**
Coupling ligands on the surface makes it easy to change the directional site of the drug carrier and its metabolism pathway(liver metabolites or renal excretion).**[1],[2]** Coupling amino acids or antibodies to the liposome bilayer allows for specific receptor binding, increasing the accuracy and precision of the drug delivery and reducing side effects.**[20]**
The two most prominent areas of drug administration as a result of liposomal formulations include DNA vaccination as well as improved gene-therapy**[20]**.
Some disadvantages include a low half-life for non-pegylated lipids and a low water solubility. Low water solubility also makes parental administration(most common method for liposome administration) difficult**[19]**. From an economical view this increases the production cost, making cost-effectiveness difficult to achieve for liposomal formulations of drugs.

One class of nanoparticles is the cationic lipids(cationic liposomes), holding positive charge. It is generally believed that cationic liposomes are taken up via multiple pathways including passive membrane fusion and active endocytic pathways.
These are some of the difficulties and changes in environment that influence the liposomal uptake. Therefore it is necessary to research how and to what extent different drugs influence liposomal uptake and via what pathways. Especially the interaction of commonly used drugs is important because drug-drug interactions as a result of one drug influencing a liposomal formulation can have major health consequences, leading to under/over dosing.
Research question: How can the multiple uptake mechanisms of positive liposomes by HeLa cells be inhibited and to what extent are the different uptake mechanisms involved.
To be able to research this the pathways of cellular liposomal uptake must first be known.
The major pathways that contribute to liposomal uptake are summarized in the following paragraphs.

Passive diffusion:

Passive diffusion for positively as well as negatively charged liposomes is negligible due to the size of the liposomes being >100 nm, ruling out passive diffusion.**[13]**
Membrane fusion however is possible depending on the carbon tail length as shown by Takikawa et al.**[3]** It was found that longer carbon tails(even only carbons) of the lipids majorly increase membrane fusion with HeLa cells as a delivery route.

Clathrin-mediated endocytosis:

Clathrin-mediated endocytosis is one of the major cellular liposomal uptake mechanisms. When the receptor is activated adapter proteins start to accumulate in the cytosol(mainly AP2) and the receptors also cluster together on the membrane. Clathrin then binds to AP2 and forms a coating layer around the clustered receptors and forms a small endosome. The clustered proteins and Clathrin then disconnect from the endosome. The substance that bound to the receptor now in the endosome in the cell. Positively charged liposomes can also be taken up via this way.**[13],[14]**

Macropinocytosis:

Macropinocytosis is the process with which the cell takes up extracellular fluid in small vesicles called macropinosomes. The main trigger for macropinocytosis is the growth hormone receptor**[18]**, this activated actin polymerization, which then encloses the macropinosomes. The macropinosomes then fuses together with the lysosome and the content of the macropinosomes is digested by the enzymes of the lysosome.
[16],[17]

Corona protein formation:

cMEM contains serum compounds which can form a corona-complex by binding to the cationic liposome bilayer. This changes the net charge of the liposome to negative value and change electrostatic interactions. Since cMEM was used in this experiment it can influence the results. This was also seen in the DLS measurements(see methods)**[26].**

To research the contribution of passive/active pathways to cationic liposome uptake multiple inhibitors(see table 1) were selected to block active uptake pathways by HeLa cells.
HeLa cells were the cells of choice for this research for their convenience, being able to replicate in only 24 hours and are an accurate tool to test pharmacodynamics/kinetics in vitro.**[4]**

Table 1: selected inhibitors to research cationic liposome uptake by HeLa cells	
Sodium azide(NaN_3) **[6],[7]**	Crystalline compound used to deplete ATP in in vitro testing
Chlorpromazine	commonly used antipsychotic affecting chlathrin mediated endocytosis
N-ethyl N isopropyl(EIPA) **[5]**	Antiarrhythmic drug that blocks H^+/Na^+ channel in the membrane
Cytochalasin	Agent used to block microtubule polymerization
Nocodazole	commonly used antineoplastic agent

To be able to understand the effect of the inhibitors and relate the measurements to the uptake pathways it is necessary to understand how the inhibitors work.
The inhibitory mechanisms are summarized and what pathway they inhibit

Sodium azide(NaN_3):
Sodium azide depletes the adenosine tri-phosphate(ATP) storage in the HeLa cells by inhibiting cytochrome oxidase which is necessary for the oxidative phosphorylation involved in the electron transport chain, which ultimately synthesizes ATP in cells. Sodium azide binds into the oxidase pocket of the mitochondrial membrane protein IV preventing the conversion of oxygen into water, therefore depleting ATP. Since sodium azide blocks ATP, addition of this compound to the cells will deplete ATP and decrease active transport uptake mechanisms of the cationic liposomes by the HeLa cells. Sodium azide is used to study the contribution of active ATP-dependent cellular liposome uptake.**[6],[7]**

Chlorpromazine:
Chlorpromazine blocks chlathrin-mediated endocytosis via 2 pathways, one by inhibiting the AP2 protein. Chlorpromazine binds directly to the AP2 protein. Chlorpromazine blocks chlathrin-coated pits in the membrane which cells use to cluster receptors, therefore trapping receptors inside endosomes, this decreases endosome recycling. Chlorpromazine in this study is used to study the contribution of chlathrin-mediated endocytosis in the liposome uptake by HeLa cells.**[9]**

EIPA(N-ethyl N isopropyl):
EIPA specifically blocks the macro-pinocytosis uptake pathway by non-covalently binding the Na^+/H^+ exchanger pump. This in turn leads to the blockage of the Rac1 and cdc42 signaling pathways. This is due to the decrease in pH because of an increase in protons leading to a more acidic cytosol, close to the membrane blocking macro-pinocytosis**[23]**. EIPA in this study is used to study the contribution of macro-pinocytosis uptake pathway in the liposome uptake of the cells.**[22],[23]**

Cytochalasin(cyto-D):
Cytochalasin blocks microtubule polymerization by inhibiting F-actin of binding to the barbed end of actin, this stops the polymerization of microtubules needed for the cytoskeleton which in turn leads to a decreased cell stability. Multiple uptake pathways are dependent on actin and therefore cytochalasin is used in this study to check the effect of actin dependent pathway blockages on the liposome uptake compared to only 1 pathway blocked as with other inhibitors. Cytochalasin blocks chlathrin-mediated endocytosis as well as macro-pinocytosis. Cytochalasin is prominent in blocking macro-pinocytosis, more than nocodazole(see next paragraph).**[24]**

Nocodazole:

Nocodazole has a similar effect as cytochalasin, namely, the blockage of polymerization of microtubules and weaking the cytoskeleton of the cell and actin blockage. Nocodazole achieves this effect by binding directly to tubulin. Therefore nocodazole is also used in this study to determine the effect of the microtubule dependent pathways blocked at the same time on liposome uptake.

To measure the effects of the inhibitors flow cytometry and dynamic light scattering offer possibilities. The fluorescently labelled liposomes are measured to see the total uptake of the liposomes by HeLa cells. Dynamic light scattering will offer more insight into the stability, size and zeta potential of the liposomes. Generally seen, none of the inhibitors will offer insight in 1 specific pathway since multiple pathways are present and possibly upregulated, but it does offer useful information for future studies.**[27],[28]**

A small summary of the mechanisms behind flow-cytometry and dynamic-light scattering

Flow-cytometry:

Flow cytometry is a technique using lasers to measure the size(forward scatter) as well as the complexity(sideward scatter) of single cells or particles. The sideward scatter is on a 90^{o} degree angle compared to the forward scatter. Forward scatter shows the size of the single cells, while sideward scatter reveals information about the complexity of the internal organelles of the single cells. Using a laser the measured single cells/particles are excited under a set wavelength, emitting photons corresponding with the excitation wavelength. **[10],[11]**

Dynamic-light scattering:

Dynamic-light scattering is used to determine the size of particles well as the zeta potential in a solution and suspensions and also reveals information about the stability, looking at the size distribution and zeta potential. Light of a set wavelength is shot at the particles, which scatter the light in all directions, due to Brownian motion in solution the scattering will differ slightly. The difference in scatter per time interval gives information about the size of the particle, since its Brownian motion is determined by size and behavior in suspension**[21]**. The intensity of the scattered light is measured which differs due to constructive and destructive interference of waves.**[21]**

Comparing the cellular liposomal uptake in the presence of the a inhibitor and without an inhibitor, combined with flow-cytometry and dynamic light-scattering will reveal information about the uptake mechanisms of HeLa cells and the effect of the inhibitors on these mechanisms.

Methods and materials:

HeLa cells and cMEM medium

The HeLa cell line(CCL-2^{tm}) was used in the experiments. The cells were cultured in MEM(supplied by Gibco) with the addition of 10% FBS(Gibco) (supplied by Gibco), incubated in 500 µl cMEM at 37 ^{o}C with 5% CO_2. For the experiments, cell passages between 4 and 18 passages only were used. Water was stored at the bottom of the incubator and replaced when needed. T75 flasks were used for the incubation, the caps were turned a quarter open during incubation for ventilation.

Liposomes:

The liposomes used have a 20:1 DOPC(see figure 1) : cholesterol ration respectively.
Two batches of liposomes were used. For the second batch of liposomes the size/zeta potential were measured using DLS in the different solvents used.
For water as a solvent the theoretical size was 100 nm on which they were filtered, but the measured

diameter with DLS was 113,7 ± 1,1 nm for the solvent water. The measured zeta potential in the solvent water was 27,2 ± 0.8 mV However the size increased for cMEM to 124,3 nm(see figure 10). The net zeta potential in cMEM becomes negative due to the corona-complex being formed by proteins sticking to the positively charged lipid surface. DLS was performed by a 3rd party using the Malvern zetasizer nano ZS, from Malvern panalytical, United Kingdom.

Flow cytometry

The fluorescence intensity was measured of the liposomes present in the single cells and produced in to a scatterplot. The fluorescent label 1,1'Di-n-octadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate was used to label the liposomes, measured with the a flow cytometer(Cytoflex, Beckman Coulter) with the excitation and emission wavelengths being respectively 550 and 564 nm. The flow cytometer measured until 20000 viable single cells were measured. The channel used for measuring was 585/42 bp.

Methods to study the used inhibitors:

Suppliers of the inhibitors: Chlorpromazine(Sigma-Aldrich, Missouri) USA),EIPA(Sigma-Aldrich, Missouri USA), cytochalasin D(Thermo Fisher scientific, USA), nocodazole(Biovision, USA)
24-well plates by(Greiner Bio-one, Austria).
The HeLa cells were preincubated with the desired inhibitor for 20 minutes in cMEM, except for EIPA uptake kinetics(see figure 6), for EIPA the preincubation time was 30 minutes. The inhibitor was then removed along with the medium and liposomes + new medium was added to reach a concentration of 50 µg/ml liposomes. The cells were left to incubate in the incubator at 37°C with 5% CO_2 until the desired harvest times. The cells were then harvested by washing with two times 500 µl PBS and loosened from the wells with 250 µl TEP. Subsequently centrifuged to collect the cells and dissolved in 100 µl PBS prior to measuring in the flow cytometer.
Certain experiments were measured after fixation the next day, these are the experiments corresponding to figures 4C, 4D, 7F, 7G and 9 Fixation was done by adding formaldehyde to kill the cells, PBS added, centrifuged to collect the cells, and were then stored in PBS in the fridge until measured with the flow cytometer.

Table 2: Inhibitor concentrations used for the (pre)incubation and duration

Inhibitor	Preincubation/incubation inhibitor concentration	Preincubation time(minutes)
Sodiumazide	5 mg/ml	20 and 90
Chlorpromazine	10 µg/ml	20
EIPA	100 µM	30
Cytochalasin	2,5 µg/ml	20
Nocodazole	5 µg/ml	20
Dextran	-	30

Results and discussion:

Processing the flow cytometry analysis

The measured median cell fluorescence intensity was used to produce the curves and tables. The results were processed using FlowJo(LLC). Scatter plots were produced with forward scatter vs sideward scatter as the gates. Experiments were measured in duplicate. Results were used if the abort percentage was <3% and the flowrate kept below 1000 single cells/second.
The measured median cell fluorescence intensity without any inhibitor in control cells was used as the 100% value. The average was calculated of all duplo values used. The percentual change in the median cell fluorescence intensity with inhibitor was divided by the measured median cell

fluorescence intensity without inhibitor * 100% to receive the percentual change. Standard deviation was not calculated since that is not possible with only 3 values per measurement.

Cellular liposomal uptake and stability in cMEM

The positive liposomes were first tested on functioning and being taken up by the HeLa cells. This was done with a concentration-uptake curve with multiple concentrations. The concentrations were chosen to be similar to future used concentrations. Figure 2 shows that the uptake of the positive liposomes increases when the concentration increases. This shows the concentration of the liposomes is indeed directly proportional to the liposome uptake by the cells. There may be a point where the cells are saturated with liposomes at a higher concentration however the used concentrations were too low to see a flattening of the curve at high concentrations. This shows that the positive liposomes were suitable to use and also indicated the concentration of 50 µg/ml liposomes was suitable to use for the research.

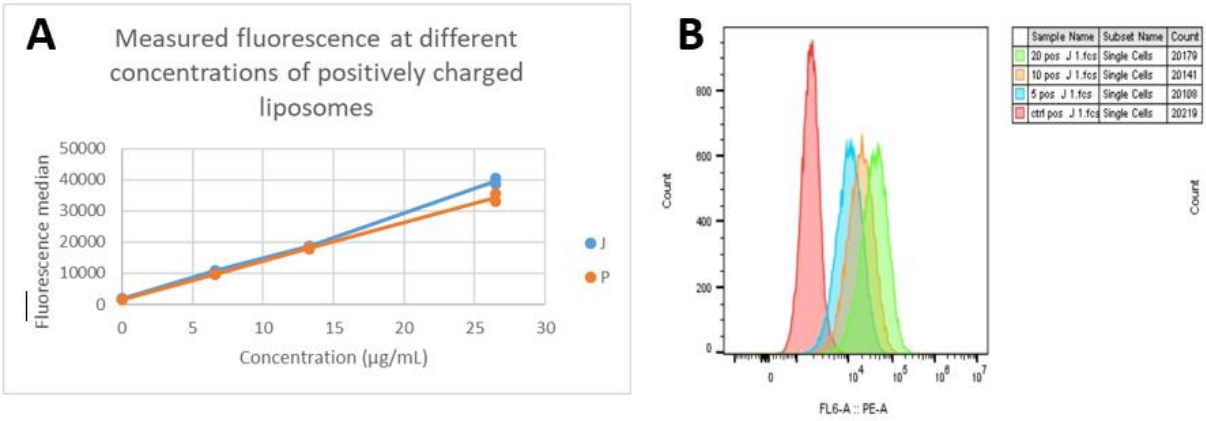


Figure 2: Effect of concentration on the uptake of positively charged liposomes by HeLa cells.

(A) Median cell fluorescence of HeLa cells exposed to different concentrations of liposomes in cMEM measured by flow cytometry. The results is a duplicate experiment(J and P, Jasper and Philip respectively) with a line going through the average of the 2 experiments.

(B) Example of the overlapped cell fluorescence distributions of the data shown in panel A. Control(red), 6,625 ug/ml(blue), 13,25 ug/ml(orange) and 26,5 ug/ml(green). FL6-A: PE-A channels used.

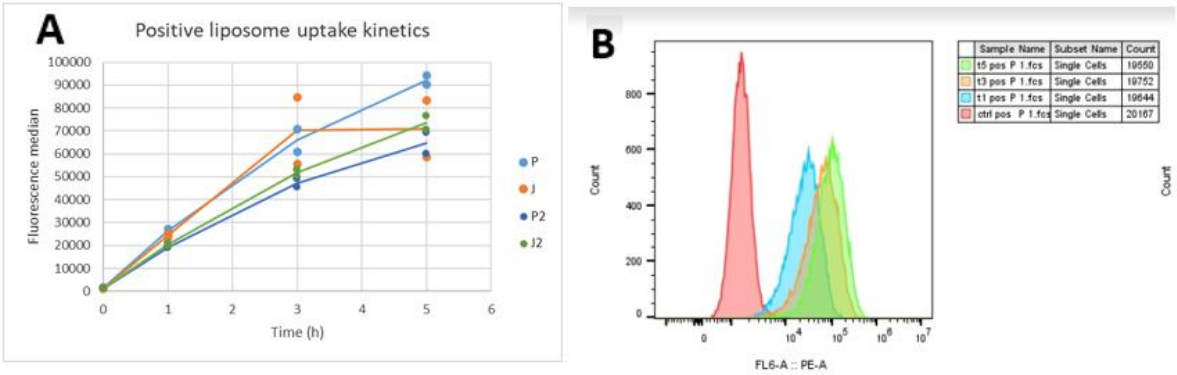


Figure 3: Effect of time on the uptake of positively charged liposomes by HeLa cells.

(A) Median cell fluorescence of HeLa cells over 5 hours exposed to 50 µg/ml liposomes in cMEM. Experiment performed in duplicate(P and J, Philip and Jasper respectively) with the line going through the average.
(B) Example of an overlapped median cell fluorescence of the data shown in panel A.

The uptake kinetics over time were measured of the positive liposomes by the cells at 3 time points that will be used, t=1, t=3 and t=5 hours. In figure 3A can be seen that the uptake of the positive liposomes increases over time when the same concentration(50 µg/ml) was used. This experiment was done without any inhibitor to quantify the basal uptake of liposomes over time. This will be compared to the uptake kinetics with an inhibitor to see the contribution of each uptake pathway. The measurements at t=3 and t=5 show strong variability, compared to t=1 for (A), this is most likely due to technical errors. In the overlay (see figure 3B) can be seen that there is no abnormality in uptake and all measurements reached close to the 20000 cells measured as intended.

In agreement with the previous results, in the repetition of the experiment the same results were found. The flattening of the curve may be due to the fact that the cells may be saturated with liposomes at a higher concentration and the curve may flatten reaching the horizontal asymptote.

Sodium azide to block active transport pathways via ATP depletion

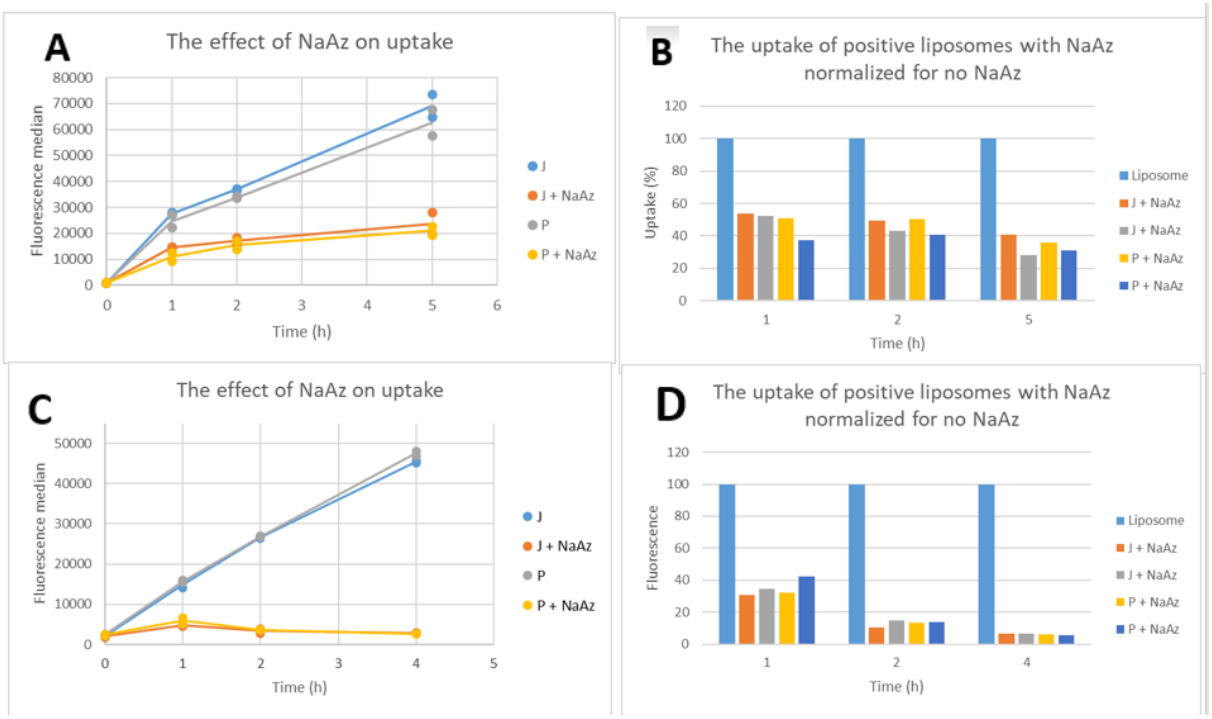


Figure 4: Effect of sodium azide on the uptake of positively charged liposomes by HeLa cells.

(A) Median cell fluorescence of HeLa cells exposed to 5 mg/ml sodium azide over 5 hours in cMEM as measured by flow cytometry. Experiment performed in duplicate(P and J, Philip and Jasper respectively) with the line going through the average of the duplicate measurements. 20 minute preincubated with 5 mg/ml sodium azide in cMEM before incubation.

(B) Percentual decrease representation of the data shown in panel A, normalized for no inhibitor present as the 100% value.

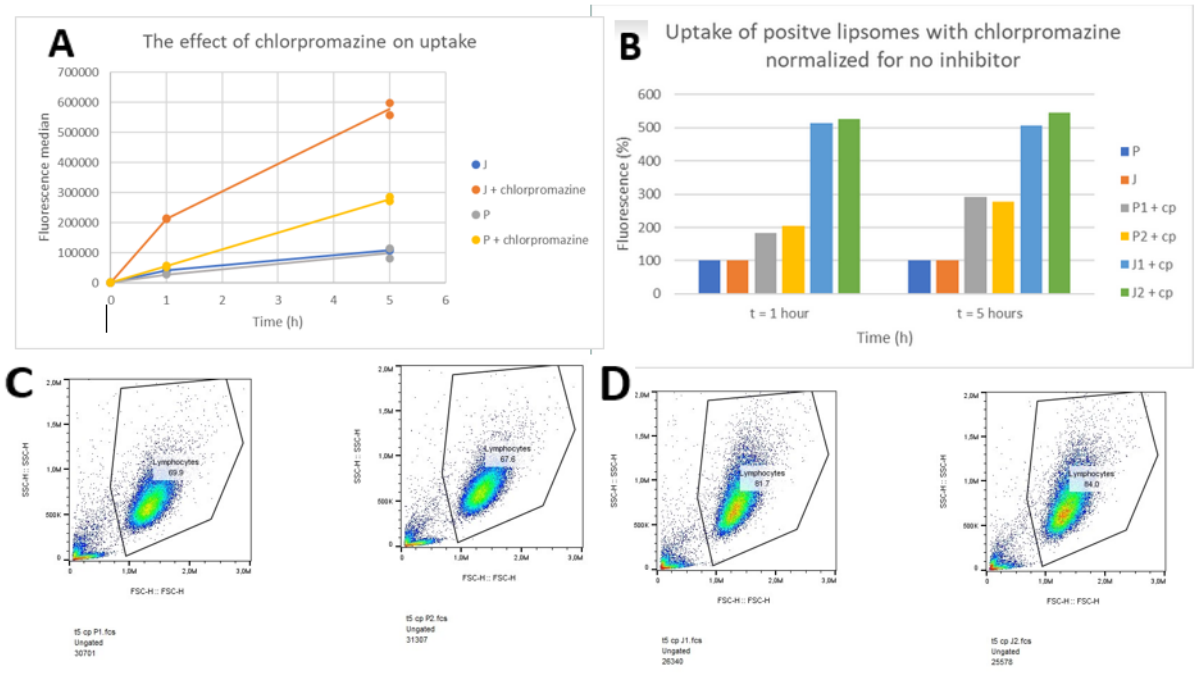
(C) Median cell fluorescence of Hela cells exposed to 5 mg/ml sodium azide over 4 hours in cMEM, measured by flow cytometry, compared to no sodium azide present. Line goes through average of

duplo, P and J, Philip and Jasper respectively. 90 minute preincubated with 5 mg/ml sodium azide in cMEM before incubation

(D) Representation of (C) in percentual decrease being normalized for no inhibitor present as the 100% mark.

Next we tested if the liposome transport was ATP dependent and to what extent.
In figure 4 can be seen that sodium azide decreases the uptake, with more than 50% decrease. This showed us that active transport pathways does play a major role in the uptake of the positive liposomes and contribute the most to the total uptake as expected. There is however still some uptake even with sodium azide implying other uptake pathways that are not ATP dependent. This could be passive uptake, however positive liposomes are too big to be taken up by passive transport. Furthermore the positive charge does not decrease passive uptake by HeLa cells since the corona-complexes formed on the liposomes in cMEM change the charge to negative. Membrane fusion is not prevalent for these lipids due to the length of the carbon tail, Takikawa et al**[3].** To confirm drug efficacy the experiment was repeated.
The data in figure 4D the uptake is now almost completely diminished compared to figure 4B, in accordance to negative liposomes. This showed us that the remaining uptake seen in figure 4B was due to some ATP still being present because there was not enough preincubation. The decrease is almost 60-90% over the different time points(C) showing that the majority of the liposome uptake is due to ATP-dependent transport. Since the percentual decrease(See figure 4) increases with time due to the sodium azide incubation, this shows that there is no significant passive transport and is therefore not a relevant uptake pathway. It is worth noting a new cell line was used due to the 18 passages reached for the repeated sodium azide experiment. Since clathrin-mediated endocytosis and macropinocytosis are the main uptake pathways(see introduction), these were both tested starting with clathrin-mediated endocytosis.

Contribution of clathrin-mediated endocytosis to the liposome uptake



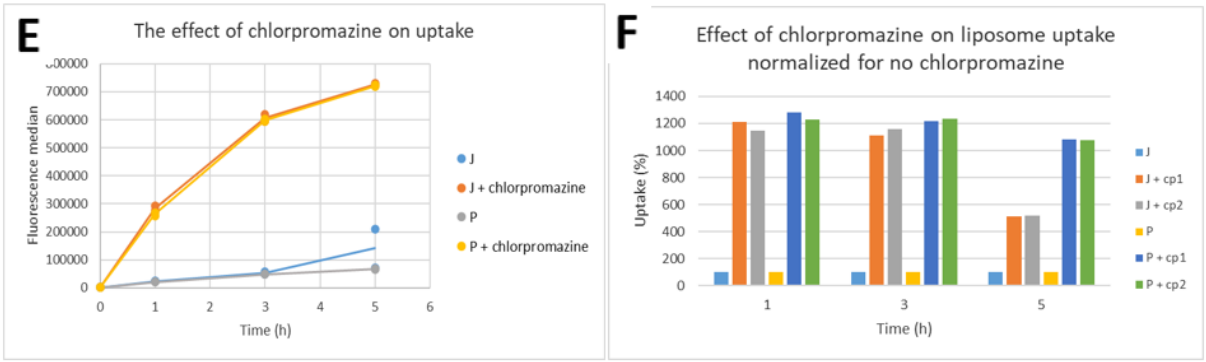


Figure 5: The effects of chlorpromazine on the uptake of the positive liposomes by HeLa cells.

- **(A)** Median cell fluorescence of HeLa cells exposed to 10 µg/ml chlorpromazine over 5 hours in cMEM as measured by flow cytometry. Experiment performed in duplicate(P and J, Philip and Jasper respectively) with the line going through the average. 20 minute preincubated with 10 µg/ml chlorpromazine in cMEM before incubation.
- **(B)** Bar chart representation of the percentual change in liposome uptake, normalized for no chlorpromazine present as the 100% mark.
- **(C)** Flow cytometry scatterplot of the t=5 hours incubation measurements with chlorpromazine of Philip. SSC-H(y-axis) and FSC-H(x-axis) used for measurement.
- **(D)** Flow cytometry scatterplot of the t=5 incubation measurement of Jasper(t5 cp1 and t5 cp2) with chlorpromazine. Side scatter vs forward scatter plotted.
- **(E)** Median cell fluorescence of HeLa cells exposed to 10 µg/ml chlorpromazine over 5 hours in cMEM, measured with flow cytometry. Experiment performed in duplicate(P and J, Philip and Jasper respectively) with the line going through the average. 20 minute preincubated with 10 µg/ml chlorpromazine in cMEM before incubation.
- **(F)** Bar chart representation of (E) showing the percentual change, normalized for no inhibitor as the 100% mark.

Chlorpromazine was used to block clathrin-mediated endocytosis which is assumed to be one of the main uptake pathways for positively charged liposomes(see introduction), this was tested to see the extent of this pathway. A decrease in uptake was expected, since one of the main uptake pathways is blocked. Figure 5 shows the uptake increased with addition of chlorpromazine ranging from 200-500 %. Unexpectedly for both Philips and Jaspers results, however the increase in cellular liposome uptake was lower for Philip than for Jaspers measurements, more than a 300%(normalized) change at t=5 hours(see figure 4B). The experiment was repeated. (Figure 5E) and (5F) show the repetition, an increase again could be seen in cellular liposome uptake in accordance with figure 5A, suggesting the results were not a fluke but reproducible. No visible change in cell viability could be seen under the microscope, nor a change in viable cell percentage was seen in the scatter plot(see figure 5C and 5D). In figure 5C and 5D a change in relative single cell placement between Philips(5C) and Jaspers(5D) was shown. This could cause the drastic change in cellular liposome uptake seen in 5B.

A possible interpretation of the results is that another pathway in the HeLa cells is upregulated as due to the clathrin-mediated endocytosis being blocked by chlorpromazine(10 µg/ml).

Liposome stability in presence of chlorpromazine is also a possibility for the increased uptake. The destabilized liposomes will range and change in size, being able to be taken up by passive uptake and membrane fusion if small enough.

It cannot be concluded from these results directly that clathrin-mediated endocytosis plays a

major role in liposomal uptake since there is no decrease visible. This issue could be technical error or one of the arguments given above.
Another plausible reason for the increased cellular liposomal uptake is that chlorpromazine becomes protonated in cMEM(pH around 7-8) since the pKa of chlorpromazine is 9,2 and therefore attaches to the liposomes since they become negatively charged due to the corona protein complexes latching on. This will cause electrostatic attraction between the negatively charged liposomes-protein complex and the positively charged conjugated acid of chlorpromazine. This would decrease the effective chlorpromazine concentration, however this would not explain the increase in uptake. From these experiments we decided to use another inhibitor involving a different uptake pathway to see if the increase in uptake was reproducible with other inhibitors.

Cellular liposomal uptake in cMEM influenced by EIPA to block macropinocytosis

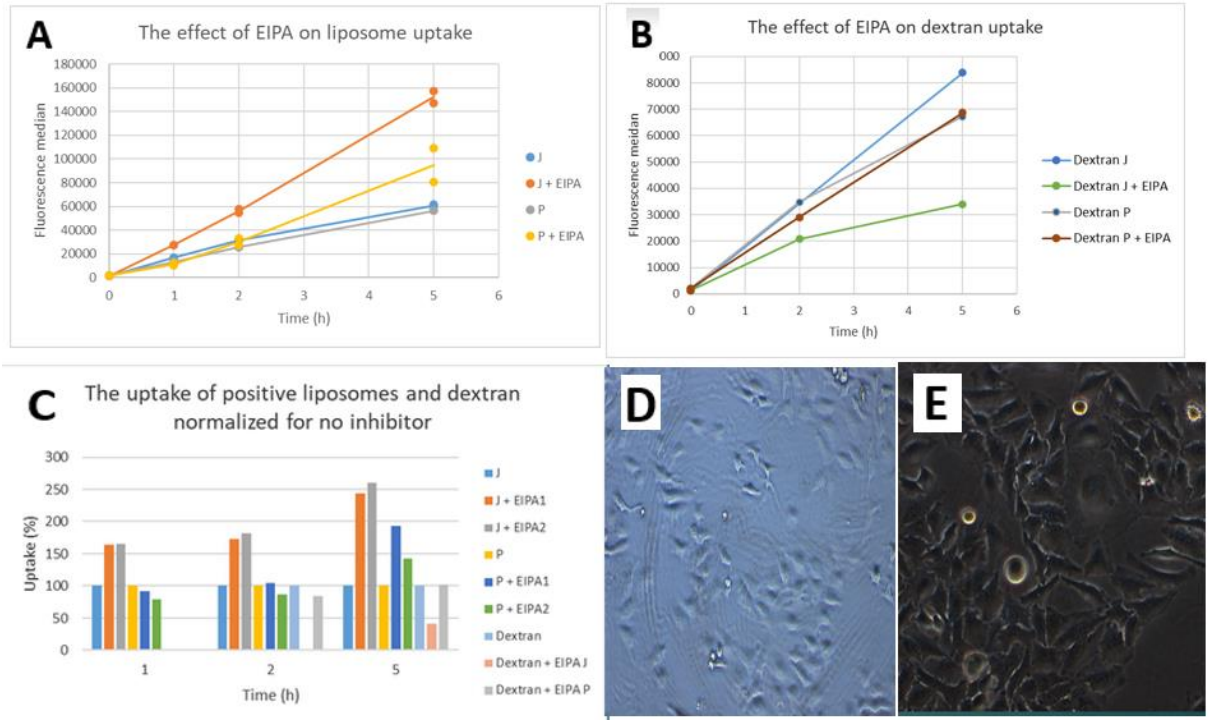


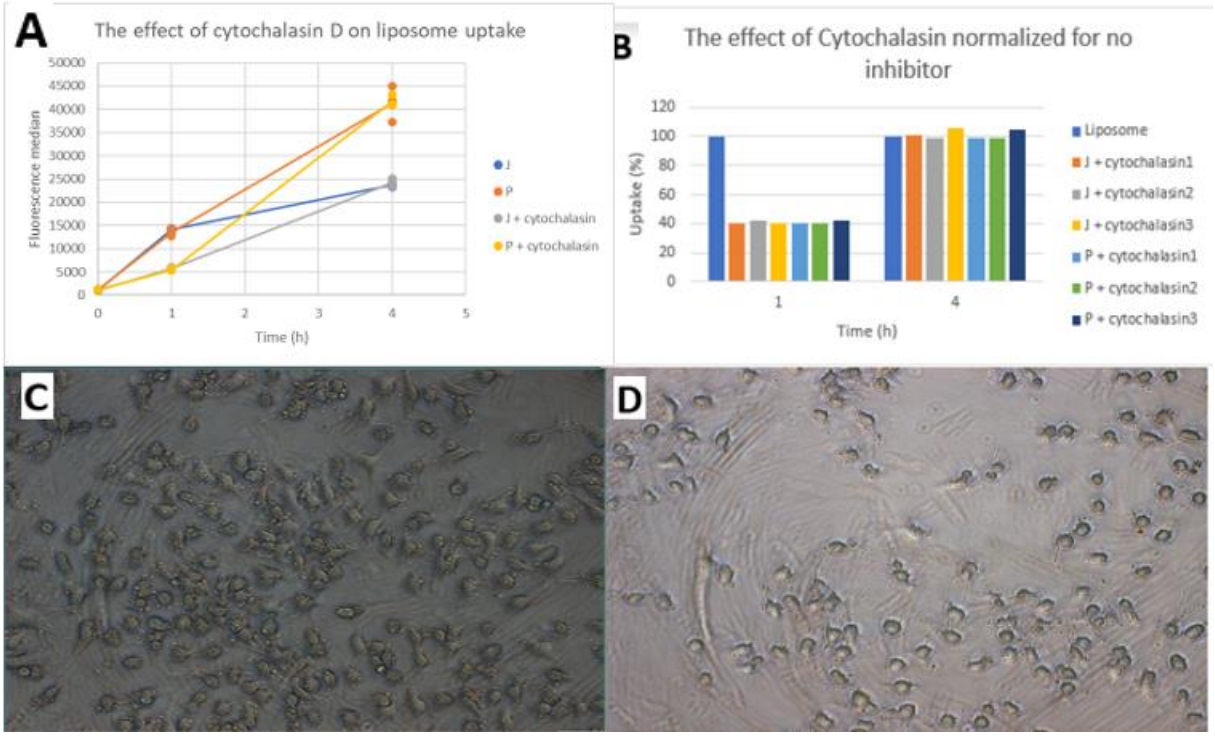
Figure 6: Effect of EIPA on the uptake of positively charged liposomes by HeLa cells.

(A) Median cell fluorescence of HeLa cells exposed to 100 µM EIPA over 5 hours in cMEM as measured by flow cytometry. Experiment performed in duplicate(P and J, Philip and Jasper respectively) with the line going through the average. 20 minute preincubated with 100 µM EIPA in cMEM before incubation.
(B) Median cell fluorescence of HeLa cells exposed to 100 µM EIPA over 5 hours in cMEM as measured by flow cytometry. Experiment performed in duplicate(P and J, Philip and Jasper respectively) with the line going through the average. 20 minute preincubated with 100 µM EIPA in cMEM before incubation. Fluorescence of dextran was measured instead of liposomes.
(C) Bar chart representation of the percentual change in liposome uptake by the HeLa cells, normalized with the normal liposome uptake without inhibitor as the 100% mark.
(D) Microscopic view of the HeLa cells after 5 hours incubated with EIPA.
(E) Microscopic view of the HeLa cells after 5 hours incubated without EIPA.

EIPA was tested to see if a decrease in uptake, expected according to the literature was seen. Figure 6A unexpectedly shows an increase in liposome uptake as a result of incubation with EIPA(100 µM) compared to no EIPA present. Also with this drug, as seen with chlorpromazine, the results showed that the uptake increases. If an decrease in uptake was seen this would have suggested the involvement of macropinocytosis. Figure 6A shows an increase, this could be interpreted that one pathway is upregulated due to macropinocytosis being blocked.

Dextran was used a positive control to check if macropinocytosis was functional in the HeLa cells in cMEM after incubation. Figure 6B shows that dextran was being up taken via macropinocytosis and EIPA decreases this in J+EIPA, since macro-pinocytosis is blocked, however in a repeated experiment(P) different results were obtained and exposure to EIPA did not affect dextran uptake. This shows that the EIPA was not effective or not present for 1 of the 2 measurements. During incubation a small decrease can be seen in uptake at t=1 and 2 hours for P but not for J. Figure 6A, 6B and 6C show very contradicting results. A negligible uptake decrease was measured and furthermore for the other experiment(J), an increase was measured. The repetition of the experiments show opposite results. Photos were taken of t=5 without and with EIPA(see figure 6D and E). No clear change in cell viability nor structure is visible, EIPA was not expected to change the cell structure or viability looking at the mechanism(see introduction). This suggests that cell viability could not have been the cause of the contradicting results.
From EIPA no clear conclusion can be made since all the results are contradicting, it was therefore decided to retry EIPA and the other inhibitors to check if liposome stability was influenced by the presence of the inhibitors during incubation.

Cytochalasin on the uptake of positively charged liposomes by HeLa cells in medium to block multiple uptake pathways.



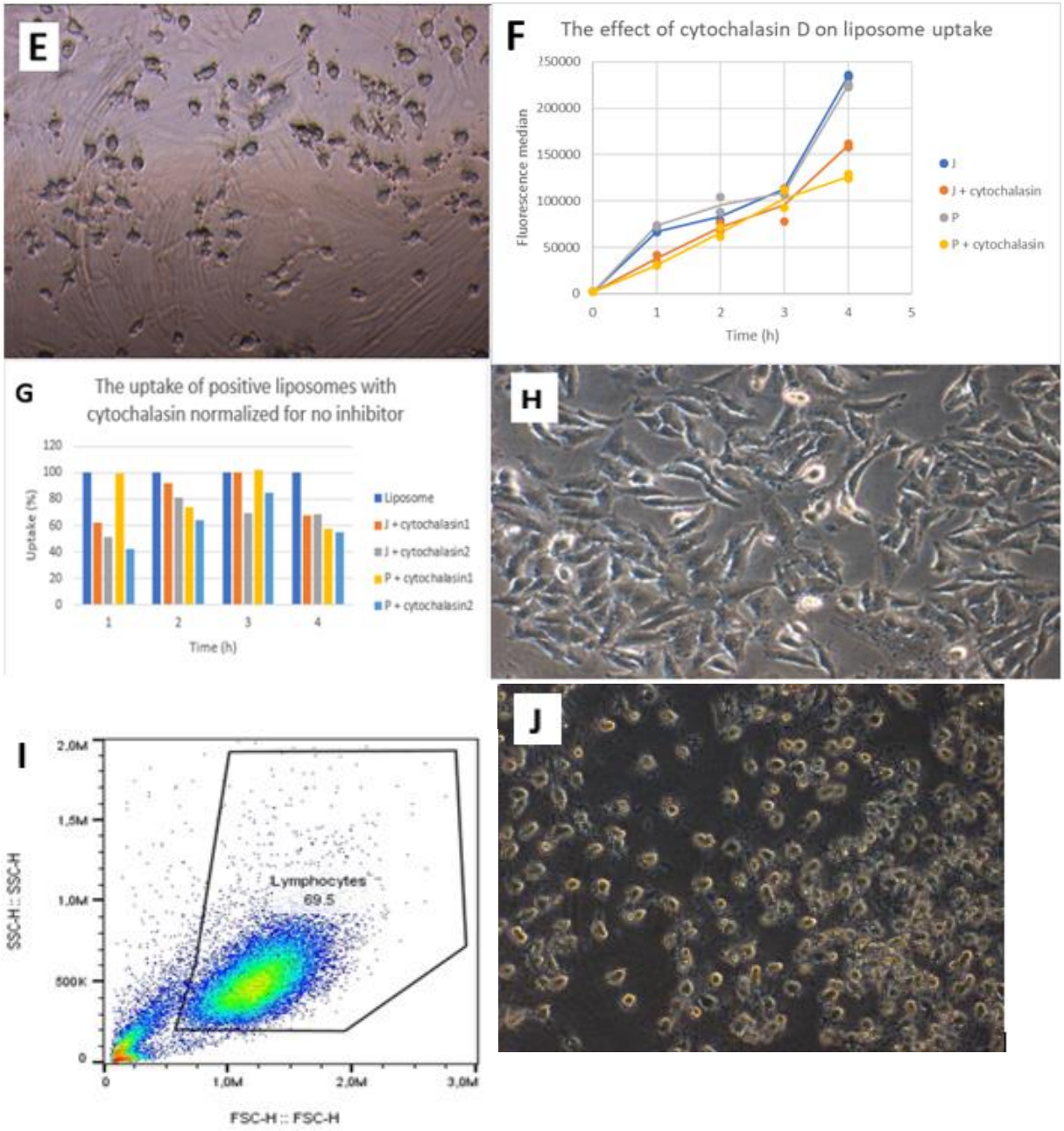


Figure 7: Effects of [cytochalasin]= 2,5 µg/ml on the liposomal uptake kinetics over time by HeLa cells.

(A) Median cell fluorescence of HeLa cells exposed to 2,5 µg/ml cytochalasin over 5 hours in cMEM as measured by flow cytometry. Experiment performed in duplicate(P and J, Philip and Jasper respectively) with the line going through the average. 20 minute preincubated with 2,5 µg/ml cytochalasin in cMEM before incubation.
(B) Bar chart representation of the data shown in (A), showing the percentual change in liposomal uptake normalized for no inhibitor present during incubation.
(C) Microscopic view of t=1 hour with 2,5 µg/ml cytochalasin, from Philips experiment(figure 7A).
(D) Microscopic view of t=4 hours with 2,5 µg/ml cytochalasin, from Philips experiment(figure 7A).
(E) T=4 hours incubated with cytochalasin 2,5 µg/ml of Jasper experiment from figure 7A.
(F) Experiment of figure 7A repeated under the exact same conditions, with more time points.
(G) Bar chart representation of (F) showing the normalized percentual decrease of the liposomal

uptake by the HeLa cells.
(H) Example microscopic photo from one of the contaminated wells experiment from figure 7G.
(I) Example of a light scatter plot of one of the contaminated wells from the experiment showed in 7G.
(J) Example of the visible effect of cytochalasin exerted on the HeLa cells after 4 hours incubation with 2,5 µg/ml cytochalasin.

Since the previous results suggests that blocking a single pathway with EIPA or chlorpromazine increases the liposomal uptake, an inhibitor that blocks multiple pathways was chosen, which is cytochalasin because it blocks actin dependent pathways. In figure 8B can be seen that there is a percentual decrease for t=1 hour incubation but this effect diminished at t=4 hours, this is visible for both the repetitions of the experiment(P and J, Philip and Jasper respectively). This may be due to technical errors since this effect was not visible in the repetition of the experiment(see figure 8G).

In figure 7C the effect of cytochalasin can be seen after 1 hour by the punctate f-actin fragments surrounding the HeLa cells showing the efficacy of cytochalasin.
At t=4 hours the f-actin punctate fragments appear decreased. This could have caused the diminished effect at t=4 hours seen in figure 7B. The experiment was repeated(figure 7G), due to the diminished effect at t=4 hours of cytochalasin.
The results seen in figure 7G show a decrease over all time points in liposomal uptake. However the wells were found to be contaminated, seen figure 7H and 7I. Figure 7I shows the single cell placement is really left shifted close to 0 M, this confirms the contamination. This makes the results less reliable, however 20000 cell counts were measured at all time points and the results appear to be correct with the expectations. The results seen in 7G show a steady repeated decrease over all time points and there is no background theory suggesting that the effect of cytochalasin in cMEM over time decreases. Furthermore the cells appear viable in figure 7D. In figure 8J the F-actin punctuates are still visible at t=4 hours but not in 7D. The diminished effect of cytochalasin at t=4 hours appears to be a technical error.
The results from cytochalasin suggest that blocking actin dependent pathways decreases cellular liposomal uptake by HeLa cells. Nocodazole was tested too for the microtubule-dependent uptake.

Nocodazole on cellular liposomal uptake kinetics by inhibiting multiple uptake pathways by blocking microtubule polymerization

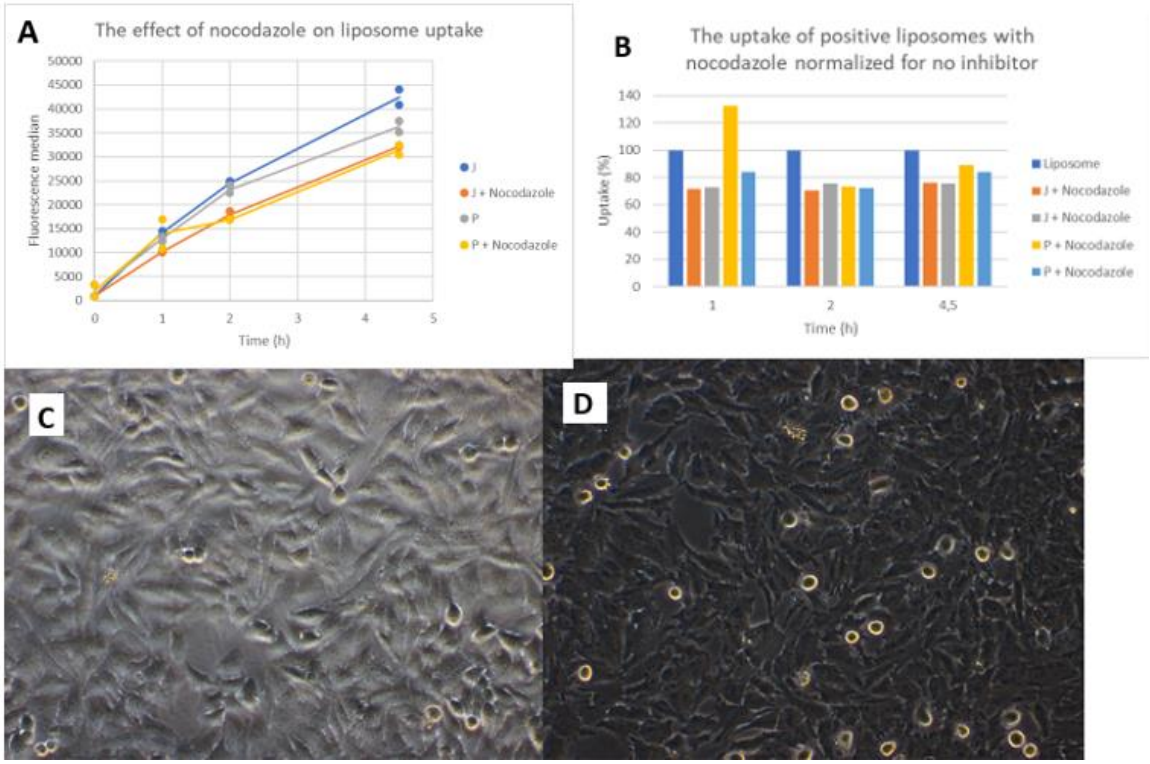


Figure 8: Nocodazole effect on cellular liposomal uptake of HeLa cells over time.

(A) Median cell fluorescence of HeLa cells exposed to 5 µg/ml nocodazole over 5 hours in cMEM as measured by flow cytometry. Experiment performed in duplicate(P and J, Philip and Jasper respectively) with the line going through the average. 20 minute preincubated with 5 µg/ml nocodazole in cMEM before incubation.
(B) Bar chart representation of (A) showing the percentual changes in the liposome uptake, normalized for no inhibitor present.
(C) Microscopic view of t=4,5 hours incubation without nocodazole.
(D) Microscopic view of t=4,5 hours P incubation with nocodazole showing the morphological changes in cell structure.

Nocodazole microtubule dependent pathways in a similar fashion to cytochalasin, therefore it was chosen to confirm the suspicion that blocking either actin or tubules dependent pathways indeed decreases liposomal uptake, contrary to blocking only one pathway. In figure 8A can be seen that the liposomal uptake decreases as a result of the incubation with nocodazole. There is one outlier for t=1 hour P + nocodazole. The microscope photos did not show any discrepancies, neither did the light scatter plot.
The decrease measured is not a significant decrease however(around 20% for all time points) as seen with cytochalasin. Cytochalasin was active as can be seen in figure 8D where the cells show slight punctuates and appear more spiky compared to 8C(no nocodazole) due to the microtubules being inhibited, which destabilizes the whole cell. Another cause for the lowered decrease could be that cytochalasin blocks macro-pinocytosis more than nocodazole which was also confirmed by Francia et al.**[25]** in the morphological changes. The results from cytochalasin and nocodazole suggest that the uptake is more dependent on actin than microtubules, also confirmed by Francia et al.**[25].**

Liposome stability in dispersion in cMEM with different inhibitors

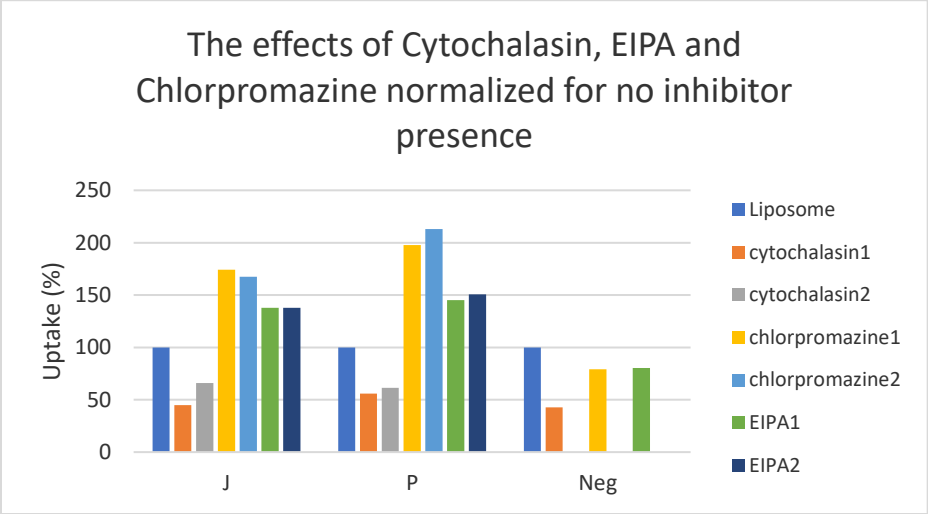


Figure 9: Stability of the liposomes tested, no drug during incubation.

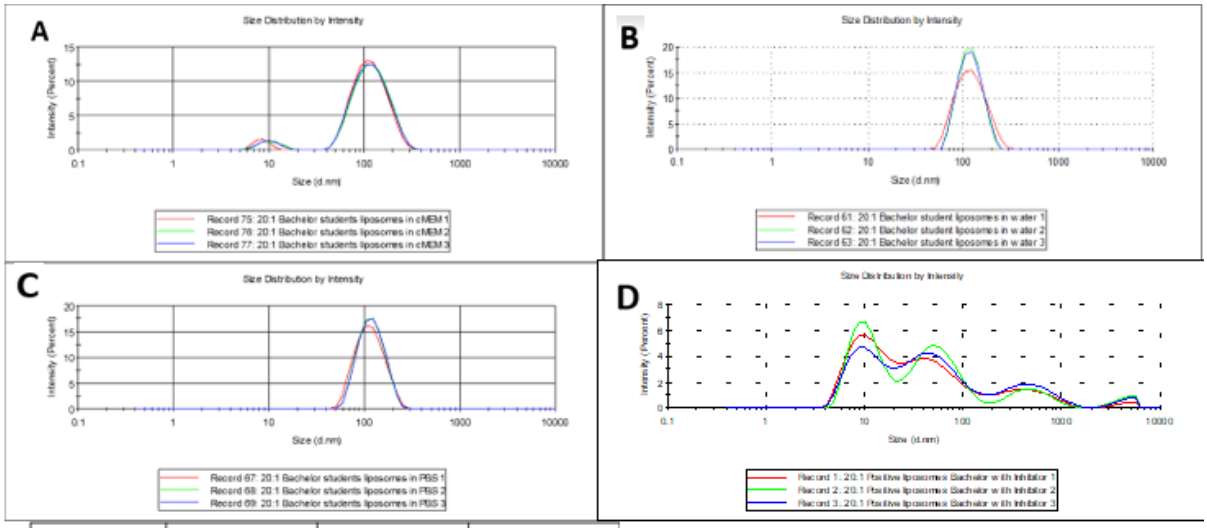
(A) Percentual change in median cell fluorescence of HeLa cells exposed separately to 2,5 µg/ml cytochalasin, 10 µg/ml chlorpromazine and 100 µM EIPA in cMEM as measured by flow cytometry. Only exposed to the drugs for 20 minutes during preincubation, afterwards incubation for 45 minutes without drug.

Since after exposure to chlorpromazine and EIPA showed an increase in cellular liposomal uptake was observed, the stability of the liposomes was tested with this experiment. In order to exclude the drugs destabilizing the liposomes, there was no incubation with the drug, but only preincubation where no liposome was present. The exposure after preincubation to liposomes was only 45 minutes to prevent the disappearance of the drug effect on the HeLa cells. If the liposomes were destabilized this could increase the uptake of them via membrane fusion. The liposomes were pre-incubated the exact same way as previously mentioned(see experimental data) with t=20 minutes and identical concentrations. Cytochalasin was also tested as an inhibitor which blocks multiple pathways by inhibiting F-actin, this causes actin to be unable to polymerize and therefore the microtubules cannot polymerize, destabilizing the cell.

Again figure 9 shows similarities with the previously obtained results about the drugs.
Chlorpromazine repeatedly shows an increase in cellular liposome uptake with EIPA having corresponding results. EIPA now shows for both duplicate experiments an increase contrary to the previous EIPA results. No change in the scatterplot or microscope photos was seen between the results of EIPA in figure 6 and figure 9.
Cytochalasin shows a decrease in cellular liposomal uptake as expected looking at the mechanism of action. It blocks microtubule polymerization which blocks multiple uptake pathways. The data suggests that only by blocking multiple pathways simultaneously decreases liposome uptake, unlike the increase in uptake seen when blocking one pathway. This also supports the idea that blockage of one specific pathway indeed upregulates other pathways and therefore increases liposome uptake.
Figure 9 shows that inhibitors destabilizing the liposomes is not likely to increase liposome uptake, suggesting that the cause is with the cells themselves.
We changed the way the cells were exposed to the drug so negative liposomes were used as a control(see figure 9). Figure 9 shows a decrease in uptake for negative liposomes, showing that chlorpromazine does work properly, under the same conditions as used for the positive liposomes.

We decided to double check the liposome stability claim using DLS on the liposomes. This was done for us by a 3rd party.

Liposome stability measured with Dynamic Light Scattering(DLS)



E

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

Figure 10: Dynamic light scattering(DLS) measurements of the positive liposomes in different solvents. DLS measurements performed by R. Bartucci.

(A) DLS measurement of the stability of the liposomes in cMEM solution.
(B) Stability of the liposomes in water as solvent. [liposome]= 50 µg/ml
(C) Stability of the used liposomes in PBS as the solvent.
(D) Stability DLS measurement of the liposomes exposed to 10 µg/ml chlorpromazine in cMEM.
(E) Table including of the size, polydispersity index and measured zeta potential of the liposomes in different solvents and chlorpromazine.

A DLS measurement was performed to double check the stability with a inhibitor present. This was tested for chlorpromazine only. From the DLS the size and zeta-potential of the liposomes can be determined and from there the stability. Figure 10 shows clear singular peaks for the size distribution of the liposomes in cMEM, PBS and water which were the main solvents and therefore due not change the stability. In cMEM there are 2 peaks, the smaller peak around 10 nm comes from the proteins that are present in cMEM and not seen in water and PBS. With chlorpromazine present however the liposomes show instability due to the large variable size distribution ranging from 10 to 10000 nm suggesting that for chlorpromazine this could be the cause of increased liposome uptake. The zeta potential also changed to negative when dispersed in cMEM in the presence of chlorpromazine, however this could also be due to cMEM as explained in the introduction. The zeta potential can also change the uptake of the liposome(see introduction), since the zeta potential

becomes negative in the presence of chlorpromazine this could cause the increased uptake.
The large size distribution variability suggests aggregation of the liposomes due to chlorpromazine.

Conclusions:
The experiment with sodium azide has shown that ATP-dependent transport plays a major role in positively charged liposome uptake, decreasing the uptake up to 90%(see figure 4).
Cytochalasin has shown a significant decrease too in liposome uptake(figure 7) showing that the uptake is also actin-dependent for positively charged liposomes.
Nocodazole has shown in figure 8 that the liposome uptake is also dependent on microtubule-dependent pathways. However the decrease in uptake from nocodazole is less than cytochalasin D suggesting that the uptake is more dependent on actin rather than microtubules. This confirms the findings from Francia et al.**[25],** however different negative liposomes were used for Francia et al so it is not completely comparable.

From the results of EIPA not much can be concluded because of the contradicting results between p and J(see figure 6). An increase in uptake is slightly visible however not really reproducible. The slight increase visible in the results of P is negligible and not reproducible so no clear conclusion can be drawn. The increase on uptake for J results were clearly visible but not for P. This could be technical error however not clearly reproduced. Macropinocytosis was however shown to be working in the experiments with dextran as the positive control(figure 6).

Chlorpromazine has shown an increase in liposome uptake as seen in figure 5. This suggests that blocking clathrin-mediated endocytosis increases another uptake pathway. However there is no data that shows that the increase in uptake is directly linked to clathrin-mediated endocytosis. More studies would have to be performed to prove the involvement of clathrin-mediated endocytosis with the increase in liposome uptake when blocked.
DLS was performed and figure 10 shows that the liposomes were not stabilized in the used solvents including cMEM, however in presence of chlorpromazine the liposomes show a wide size distribution ranging from 10-1000 nm. This may be the reason for the increased liposome uptake due to chlorpromazine seen in figure 5.

The results in figure 9 confirm the results found in the other experiments, but suggests that liposome stability was not the cause of the increased liposome uptake since there was no exposure of drugs to the liposomes, but still an increase in uptake visible.
Overall the uptake mechanisms would have to be studied more to reach clear conclusions.
Combining multiple inhibitors in one experiment could provide more information of the uptake pathways.
Circular dichroism could be used after purification using centrifugation to check if the inhibitors become charged as a result of the pH of cMEM which may influence the liposome uptake. Most errors in the experiments were technical errors, however the results can be improved by adding more and longer exposure times.
Another way to improve the research would be to use more inhibitors with more specific mode of actions that only block one specific uptake pathway.
The discovered results can be used as a guideline for future experiments or repetitions of these experiments. More accurate experiments especially about the stability of liposomes in presence of the different drugs should be studied.
Different studies can also be performed researching membrane fusion with the used DOPC 20:1 cholesterol composition as suggested by Takikawa et al.**[3].**

References:

[1] Priyanka Maurya, Samipta Singh, Shubhini A. Saraf,
Chapter 13 - Inhalable hybrid nanocarriers for respiratory disorders,
Editor(s): Kamal Dua, Philip M. Hansbro, Ridhima Wadhwa, Mehra Haghi, Lisa G. Pont, Kylie A. Williams,
Targeting Chronic Inflammatory Lung Diseases Using Advanced Drug Delivery Systems,
Academic Press,2020,Pages 281-302,
ISBN 9780128206584,
https://doi.org/10.1016/B978-0-12-820658-4.00013-3.

[2] Akbarzadeh, Abolfazl et al. "Liposome: classification, preparation, and applications." *Nanoscale research letters* vol. 8,1 102. 22 Feb. 2013, doi:10.1186/1556-276X-8-102

[3] Takikawa M, Fujisawa M, Yoshino K, Takeoka S. Intracellular Distribution of Lipids and Encapsulated Model Drugs from Cationic Liposomes with Different Uptake Pathways. *Int J Nanomedicine*. 2020;15:8401-8409
https://doi.org/10.2147/IJN.S267638

[4] Masters, J. HeLa cells 50 years on: the good, the bad and the ugly. *Nat Rev Cancer* 2, 315–319 (2002). https://doi.org/10.1038/nrc775

[5] National Center for Biotechnology Information. "PubChem Compound Summary for CID 1795, Ethylisopropylamiloride" *PubChem*, https://pubchem.ncbi.nlm.nih.gov/compound/Ethylisopropylamiloride. Accessed 19 June, 2022.

[6] Chang S, Lamm SH. Human Health Effects of Sodium Azide Exposure: A Literature Review and Analysis. *International Journal of Toxicology*. 2003;22(3):175-186. doi:10.1080/10915810305109

[7] Hall, L., Martinus, R.D. Hyperglycaemia and oxidative stress upregulate HSP60 & HSP70 expression in HeLa cells. *SpringerPlus* 2, 431 (2013). https://doi.org/10.1186/2193-1801-2-431

[8] Membrane Fusion with Cationic Liposomes: Effects of Target Membrane Lipid Composition,
Austin L. Bailey and Pieter R. Cullis
Biochemistry 1997 *36* (7), 1628-1634
DOI: 10.1021/bi961173x

[9] vanov, A.I. (2008). Pharmacological Inhibition of Endocytic Pathways: Is It Specific Enough to Be Useful?. In: Ivanov, A.I. (eds) Exocytosis and Endocytosis. Methods in Molecular Biology, vol 440. Humana Press. https://doi.org/10.1007/978-1-59745-178-9_2

[10] McKinnon KM. Flow Cytometry: An Overview. Curr Protoc Immunol. 2018 Feb 21;120:5.1.1-5.1.11. doi: 10.1002/cpim.40. PMID: 29512141; PMCID: PMC5939936.

[11] Givan AL. Flow cytometry: an introduction. Methods Mol Biol. 2011;699:1-29. doi: 10.1007/978-1-61737-950-5_1. PMID: 21116976.

[12] Correlating Corona Composition and Cell Uptake to Identify Proteins Affecting Nanoparticle Entry into Endothelial Cells
Aldy Aliyandi, Catharina Reker-Smit, Reinier Bron, Inge S. Zuhorn, and Anna Salvati
ACS Biomaterials Science & Engineering 2021 *7* (12), 5573-5584
DOI: 10.1021/acsbiomaterials.1c00804

[13] Li Y, Wang J, Gao Y, Zhu J, Wientjes MG, Au JL. Relationships between liposome properties, cell membrane binding, intracellular processing, and intracellular bioavailability. *AAPS J*. 2011;13(4):585-597. doi:10.1208/s12248-011-9298-1

[14] Kaksonen, M., Roux, A. Mechanisms of clathrin-mediated endocytosis. *Nat Rev Mol Cell Biol* **19,** 313–326 (2018). https://doi.org/10.1038/nrm.2017.132

[15] Alshehri, Abdullah et al. "Pathways of cellular internalisation of liposomes delivered siRNA and effects on siRNA engagement with target mRNA and silencing in cancer cells." *Scientific reports* vol. 8,1 3748. 28 Feb. 2018, doi:10.1038/s41598-018-22166-3

[16] J.A. Swanson, S. Yoshida, Macropinocytosis,, Editor(s): Ralph A. Bradshaw, Philip D. Stahl, Encyclopedia of Cell Biology, Academic Press, 2016, Pages 758-765, ISBN 9780123947963, https://doi.org/10.1016/B978-0-12-394447-4.20084-9.
[17] Yue-Xuan Li, Hong-Bo Pang, Macropinocytosis as a cell entry route for peptide-functionalized and bystander nanoparticles, Journal of Controlled Release, Volume 329, 2021, Pages 1222-1230, ISSN 0168-3659, https://doi.org/10.1016/j.jconrel.2020.10.049.
[18] https://www.mdpi.com/2077-0375/10/8/177/htm
[19] Savjani, Ketan T et al. "Drug solubility: importance and enhancement techniques." *ISRN pharmaceutics* vol. 2012 (2012): 195727. doi:10.5402/2012/195727
[20] Banerjee R. Liposomes: applications in medicine. J Biomater Appl. 2001 Jul;16(1):3-21. doi: 10.1106/RA7U-1V9C-RV7C-8QXL. PMID: 11475357.
[21] Stetefeld, Jörg et al. "Dynamic light scattering: a practical guide and applications in biomedical sciences." *Biophysical reviews* vol. 8,4 (2016): 409-427. doi:10.1007/s12551-016-0218-6
[22] Hosogi S, Miyazaki H, Nakajima K, -i, Ashihara E, Niisato N, Kusuzaki K, Marunaka Y: An Inhibitor of Na⁺/H⁺ Exchanger (NHE), Ethyl-Isopropyl Amiloride (EIPA), Diminishes Proliferation of MKN28 Human Gastric Cancer Cells by Decreasing the Cytosolic Cl⁻ Concentration via DIDS-Sensitive Pathways. Cell Physiol Biochem 2012;30:1241-1253. doi: 10.1159/000343315
[23] National Center for Biotechnology Information. PubChem Compound Summary for CID 1795, Ethylisopropylamiloride. https://pubchem.ncbi.nlm.nih.gov/compound/Ethylisopropylamiloride. Accessed June 23, 2022.
[24] D W Goddette, C Frieden, Actin polymerization. The mechanism of action of cytochalasin D., Journal of Biological Chemistry, Volume 261, Issue 34, 1986, Pages 15974-15980, ISSN 0021-9258, https://doi.org/10.1016/S0021-9258(18)66662-1.
[25] Francia V, Reker-Smit C, Boel G, Salvati A. Limits and challenges in using transport inhibitors to characterize how nano-sized drug carriers enter cells. Nanomedicine (Lond). 2019 Jun;14(12):1533-1549. doi: 10.2217/nnm-2018-0446. Epub 2019 Jun 18. PMID: 31208280.
[26] Francia, Valentina et al. "Corona Composition Can Affect the Mechanisms Cells Use to Internalize Nanoparticles." *ACS nano* vol. 13,10 (2019): 11107-11121. doi:10.1021/acsnano.9b03824
[27] Hasegawa, S., Hirashima, N. & Nakanishi, M. Microtubule involvement in the intracellular dynamics for gene transfection mediated by cationic liposomes. *Gene Ther* **8,** 1669–1673 (2001). https://doi.org/10.1038/sj.gt.3301573
[28] an Li, Peicai Li, Gaiping Li, Jin Wang, Erkang Wang,

The effect of nocodazole on the transfection efficiency of lipid-bilayer coated gold nanoparticles, Biomaterials, Volume 30, Issue 7, 2009, Pages 1382-1388, ISSN 0142-9612, https://doi.org/10.1016/j.biomaterials.2008.11.037.