

Shear stress increases cytotoxicity and reduces transfection efficiency of liposomal gene delivery to CHO-S cells

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Abstract Animal cells in suspension experience shear stress in different situations such as in vivo due to hemodynamics, or in vitro due to agitation in large-scale bioreactors. Shear stress is known to affect cell physiology, including binding and uptake of extracellular cargo. In adherent cells the effects of exposure to shear stress on particle binding kinetics and uptake have been studied. There are however no reports on the effect of shear stress on extracellular cargo delivery to suspension cells. In this study, we have evaluated the effect of shear stress on transfection of CHO-S cells using Lipofectamine 2000 in a simple flow apparatus. Our results show decreased cell growth and transfection efficiency upon lipoplex assisted transfection of CHO-S while being subjected to shear stress. This effect is not seen to the same extent when cells are exposed to shear stress in absence of the lipoplex complex and subsequently transfected, or if the lipoplex is subjected to shear stress and subsequently used to transfect the cells. It is also not seen to the same extent when cells are exposed to shear stress in presence of liposome alone, suggesting that the observed effect is dependent on interaction of the lipoplex with cells in the presence of shear stress. These results suggest that studies

involving liposomal DNA delivery in presence of shear stress such as large scale transient protein expression should account for the effect of shear during lipoplex assisted DNA delivery.

Keywords Shear stress · Transfection · Lipoplex · Liposome

Background

Animal cells in suspension are subjected to shear stress under diverse conditions such as due to hemodynamics in vivo or due to agitation during large scale culture of cells in bioreactors. The effect of shear stress has been largely studied in adherent cells primarily due to interest in the effect of shear stress on endothelial cells in blood vessels, and is known to affect various aspects of cell physiology including cell morphology, size and metabolism (Chatzizisis et al. 2007; Haga et al. 2007; Heo et al. 2012; Resnick et al. 2003). In addition, shear stress is also known to affect cellular uptake of extracellular cargo. Indeed, exposure to shear stress is being evaluated as a strategy for delivering macromolecules into cells. Different methods of employing shear stress have led to devices such as those made by Hallow et al. and Sharei et al. that use shear stress as the fundamental principle for intracellular delivery (Hallow et al. 2008; Sharei et al. 2013), though the exact mechanism is yet unclear.

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Exposure to shear stress has been reported to transiently increase fluid phase endocytosis and caveolae density (Davies et al. 1984; Rizzo et al. 2003; White and Frangos 2007). Endocytosis of extracellular cargo requires association of the cargo with the cell membrane as the first step. Shear stress has been widely reported to affect particle adhesion in endothelial cells, which in turn can affect uptake. These effects vary depending on the particle size and shear stress level. For example, Patil et al. report that at higher shear rates of 400–600 s⁻¹, the rate of attachment of 5–20 µm diameter microspheres coated with a recombinant PSGL-1 construct decreased with increasing size (Patil et al. 2001). Charoenphol et al. showed that binding efficiency for spherical particles of size 100 nm to 10 µm increased with increasing particle size at a shear rate of 200 s⁻¹ and for a given size the binding efficiency increased when the wall shear rate was increased from 200 to 1500 s⁻¹ (Charoenphol et al. 2010). In addition to size, particle shape affects the binding of particles under shear stress: adhesion of elongated and flattened particles was found to be significantly higher than spheres (Doshi et al. 2010). This may be of relevance to the effectiveness in the presence of shear stress of carriers such as liposomes, which can deform in the presence of shear. The duration of cellular exposure to shear stress also affects uptake: chronic exposure of human umbilical vein endothelial cells (HUVECs) to shear stress at 5 dyne/cm² led to a reduced internalization of ~180 nm diameter FITC-labeled polystyrene spheres coated with anti-PECAM, whereas acute exposure led to an increased internalization (Han et al. 2012). This was reproduced in another study where chronic shear stress led to reduced internalization of unmodified 80 nm spherical gold nanoparticles (AuNPs) in HUVEC cells (Klingberg et al. 2015). The magnitude of the shear stress obviously matters for binding/uptake: for example, nanoscale particles showed an inverse relationship between shear stress and particle uptake (Lin et al. 2010). Cell membrane properties can also be affected by shear stress, with membrane fluidity reported to increase with shear stress in endothelial cells, the mechanism of which is unclear (Butler et al. 2001; Haidekker et al. 2000). Shear stress can also induce membrane fusion, suggesting that under some conditions the interaction of liposomal carriers with cells, and hence their ability to deliver cargo, might possibly be affected by shear stress (Kogan et al. 2014).

The effect of shear stress on the uptake of liposomal formulations has been evaluated on adherent cells. Flow can have a positive effect of improving access of the lipoplex to the adhered cell (Harris and Giorgio 2005) which can increase its uptake, but at the same time can reduce the binding affinity of the lipoplex to the cells (Fujiwara et al. 2006). Shear stress has been shown to increase transfection efficiency up to an optimal shear stress level beyond which efficiency decreases (Menesson et al. 2006; Shin et al. 2009). To our knowledge, there are no reports on the effect of shear stress on carrier-assisted delivery of cargo to suspension cells. Such scenarios are relevant to large-scale transfections carried out in bioreactors for transient expression of recombinant protein, and to liposomal gene and drug delivery to blood cells where the liposomal complex is injected into blood vessels. The primary focus of this study is on suspension adapted CHO-S cells which have been used for large scale transfection in bioreactors to transiently express recombinant protein (Baldi et al. 2007). Mardikar and Niranjana subjected various animal cells in suspension to shear stress and depending on the shear stress level report formation of pores, population and cell shrinkage (Mardikar and Niranjana 2000). It is conceivable that such formation of pores could affect uptake of extracellular cargo.

In this study we use a simple flow apparatus to evaluate the effect of shear stress on the ability of liposomal carriers to deliver DNA to suspension cells for transgene expression. We find that exposure to shear stress during transfection with lipoplex results in a decrease in transfection efficiency and reduced cell density compared to transfection carried out in the absence of shear stress, in well-plates in CHO-S cells, which are efficiently transfected by Lipofectamine 2000. This effect is not seen to the same extent when cells are exposed to shear stress in the absence of the lipoplex and subsequently transfected, or if the lipoplex is exposed to shear stress and subsequently used to transfect cells not exposed to shear.

Materials and methods

Lipofectamine 2000 (11668027) was purchased from Invitrogen Corporation (Carlsbad, CA, USA). CHO-S-SFM II and DMEM were purchased from Invitrogen Corporation (12052-114 and 12320-032, respectively).

Fetal Bovine Serum (RM1112, FBS) was purchased from Hi Media Laboratories (Mumbai, India). Cell culture compatible silicone tubing ID 2 mm × OD 4 mm was purchased from Ami Polymers (Mumbai, India). 24 well plates were purchased from Nest Scientific USA (Rahway, NJ, USA) and used for seeding K562 cells. Ultra low binding 24 well plates were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used for CHO-S cells.

CHO-S cell line was purchased from Invitrogen Corporation. Cells were seeded at a density of 0.2×10^6 cells/ml in CHO-SFM II and passaged every second day. Cells were maintained at 37 °C, 10 % CO₂ and 110 rpm, and used from passage 9–40. K562, human chronic myelogenous leukemic cell line was obtained from National Centre for Cell Sciences (NCCS, Pune, India). K562 cells were maintained in DMEM supplemented with 10 % FBS and cultured at 37 °C, 10 % CO₂ and 110 rpm. K562 cells were used from passage 39–50. Plasmid containing mCherry gene was used for transfection with fluorescent mCherry protein used as a reporter.

Flow apparatus

A flow apparatus with a reservoir for cell suspension and a peristaltic pump (Longer pumps BT100-2J Low Flow Rate Peristaltic Pump with the 6-roller pump head DG-2) was used to flow cells at a flow rate of 19 ml/min through a cell culture compatible silicone tubing (ID 2 mm × OD 4 mm, Ami polymers). For low shear stress, cells were flowed only through the silicone tube of 2 mm inner diameter. A glass capillary of 0.5 mm diameter and length of 8 cm was attached to generate moderate shear stress and a silicone tube of 0.25 mm diameter of length 1.2 cm was inserted to generate high shear stress. Under an assumption of incompressible walls and no slip at the wall, these correspond to a wall shear stress of ~2, 220 and 2000 dynes/cm² under the three conditions respectively. The experiment was repeated at least three times, and cells were seeded into two wells for each repetition. To control for any effect of shear stress due the squeezing action of the peristaltic pump head, the flow rate was kept constant under all three conditions. The pump head design allowed varying occlusion using a ratchet wheel. The extent of cell death observed when cells alone were flowed through the pump head varied for different values of this

parameter with cell death increasing at higher levels of occlusion. The highest occlusion was identified for each cell type such that there was minimal cell death immediately subsequent to exposure to low shear stress, and was then kept constant for all experiments with that cell type. The selected level of this parameter was higher for CHO-S compared to K562.

Transfection

Transfection for CHO-S was performed using Lipofectamine 2000 (Invitrogen Corporation) as per the manufacturer's instructions. Transfections were carried out using cells on the second day of passage. 1 µg DNA and 5 µl Lipofectamine 2000 was used per milliliter of the cell suspension. Depending on the experiment, the lipoplex complex or lipid alone were added to 4 ml of the cell suspension and flowed through the flow apparatus for 2 h. After 2 h, 500 µl of the culture was incubated in 24 well plate at 37 °C, 10 % CO₂ and 110 rpm. Samples were taken for measuring cell density immediately before and after flowing the cells, and after allowing cells to recover for one day after exposure to shear stress. Cell density was measured using a hemocytometer after appropriate dilution and viability was measured using trypan blue dye exclusion method. After 24 h of incubation, some clumping was observed for all transfected CHO-S cultures with clumps of approximately 5–20 cells. Clumps were counted as a single cell for calculation of cell density. Each experiment was repeated at least 3 times.

Transfection for K562 was performed using Lipofectamine 2000 as per a modified protocol suggested by the manufacturer. 2.4 µg DNA and 5 µl Lipofectamine 2000 was used per milliliter of the cell suspension for transfection. The complex was mixed in serum free DMEM medium and incubated for 20 min at room temperature before addition to the culture.

Calculation of transfection efficiency

Twenty-four hours post transfection, cells were harvested from the 24 well plate. After washing with PBS, the CHO-S cells were then imaged on EVOS Fluid Cell Imaging Station (Life Technologies, Carlsbad, CA, USA). Transfection efficiency for CHO-S cells was calculated by counting the percentage of cells that showed red fluorescence due to expression of

fluorescent mCherry protein. A minimum of fifteen fields per well were recorded with the Fluid Cell Imaging Station and the number of fluorescent cells and total cells was counted.

Due to the very low transfection efficiencies for K562 cells, transfection efficiency was measured using flow cytometry (BD Accuri C6 Flow Cytometer, BD Biosciences) and data were analyzed using the BD Accuriflow software (BD Biosciences, San Jose, CA, USA).

Statistical analysis

Two tailed Student's *t* test was used to determine significance of difference between all data sets. All *p*-values lower than significance level of 0.05 are denoted by asterisks in figures. Error bars indicate 95 % confidence interval.

Results

CHO-S cells were subjected to shear stress by pumping them in a closed loop at a constant flow rate using a peristaltic pump. Cells were subjected to shear stress by pumping them for 2 h either through a silicone tube of 2 mm inner diameter (henceforth referred to as 'low shear') or a silicone tube of 2 mm diameter with an attached glass capillary of 0.5 mm inner diameter and length 8 cm (referred to as 'moderate shear') or a silicone tube of 2 mm diameter with a silicone tube of 0.25 mm diameter and length 1.2 cm (referred to as 'high shear'). The use of peristaltic pump allowed aseptic continuous unidirectional flow allowing cells to be exposed to shear stress for extended periods of time.

We first evaluated the effect of shear stress on CHO-S cells to validate the use of the flow apparatus. Both, the immediate effect of shear stress on cell death measured as any decrease in viable cell density (VCD) immediately after exposure to shear stress, and a longer-term effect measured as any effect on cell growth at the end of 24 h after exposure to shear stress, were measured. The immediate decrease in VCD due to shear stress is small at low shear stress (8 % decrease) but increases at high shear stress (50 % decrease, Fig. 1a). Exposure to shear stress also affects subsequent cell growth with cells exposed to low shear stress showing an average 0.9 population doublings over 24 h, compared to growth of cells

maintained throughout in a 24 well plate showing an average 1.2 population doublings. However, cells exposed to high shear stress show a considerable decrease in VCD (average 73 % decrease in 24 h). This is similar to previous reports such as those of flow causing lysis in mouse myeloma cells at a wall shear stress of 1800 dyne/cm², validating the use of the our flow apparatus (McQueen et al. 1987; Vickroy et al. 2007). Cell viability is however not substantially reduced after exposure to shear at all levels of shear stress (Fig. 1b), indicating the decrease in viable cell density at high shear stress is likely due to cell lysis. Due to high cell death at high shear stress, the effect of shear stress on transfection of CHO-S cells using lipoplex was further evaluated at low and moderate shear stress.

Exposure to shear stress in presence of lipoplex reduces transfection efficiency and increases cell death

We then further evaluated the effect of exposure of cells to shear stress in the presence of lipoplex (lipofectamine 2000: DNA complex). The immediate effect of shear stress on cell death was similar as in the case when cells were exposed to shear stress without the lipoplex. However surprisingly, in the presence of lipoplex, VCD at 24 h after exposure to shear stress was substantially reduced even at low shear stress (Fig. 2a). An average 25 % decrease in VCD was observed at low shear and 40 % decrease at moderate shear, compared to cell growth at an average 0.4 population doublings observed in cells transfected in the well plate. This decrease in VCD is partly caused due to greater clumping of cells in the presence of shear stress during transfection, though that alone is not sufficient to explain the difference, as verified by including the cells in clumps during cell counting. Viability was again, however, not substantially affected suggesting the decrease in VCD is due to increased cell lysis in the presence of lipoplex during exposure to shear stress (Fig. 2b). Transfection efficiency decreased significantly when cells were subjected to shear stress in the presence of lipoplex (Fig. 2c).

Higher cell death and lower transfection efficiency of the lipoplex in the presence of shear stress could possibly be due to the effect of shear on cells: for example, reduced cell growth upon exposure to shear

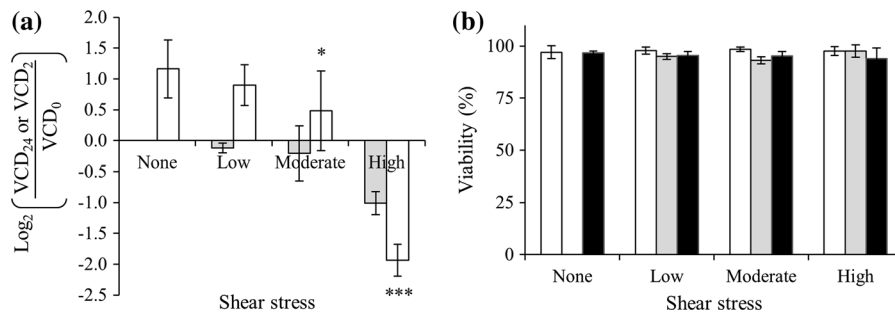


Fig. 1 The effect of shear stress on cell density and viability of CHO-S cells. CHO-S cells were subjected to shear stress (low, moderate and high, see Methods section for details on shear stress levels) for 120 min and monitored for changes in viable cell density and viability immediately after flow and after 24 h of incubation. CHO-S cells not subjected to shear stress were used as control. **a** Viable cell density (VCD) normalized to initial viable cell density. Immediately after subjecting cells to shear stress (filled bars), after 24 h (open bars). Initial viable

cell density (VCD_0), viable cell density immediately after flow (VCD_2), 24 h after flow (VCD_{24}). Log_2 transformation is used to represent cell growth in terms of population doublings, and make the data symmetric for changes in both directions (growth and death). **b** Viability. Initial (white bars), immediately after subjecting the cells to shear stress (grey bars) and after 24 h (black bars). $n \geq 3$, error bars indicate 95 % confidence interval. *** $p < 0.0005$; ** $p < 0.005$; * $p < 0.05$ for comparison to no shear condition

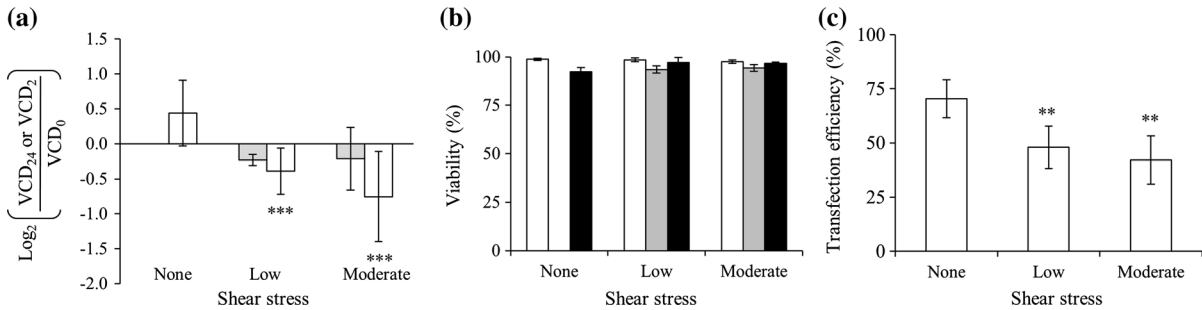


Fig. 2 Cell density, viability and transfection efficiency of CHO-S cells when exposed to shear stress in the presence of lipoplex. CHO-S cells were subjected to shear stress (low and moderate, see Methods section for details on shear stress levels) for 120 min in the presence of lipoplex and monitored for changes in cell density and viability immediately after flow and after 24 h of incubation. Control culture was not subjected to shear stress. **a** Viable cell density (VCD) normalized to initial viable cell density. Immediately after subjecting cells to shear

stress (filled bars), after 24 h (open bars). Initial viable cell density (VCD_0), viable cell density immediately after flow (VCD_2), 24 h after flow (VCD_{24}). **b** Viability. Initial (white bars), immediately after subjecting the cells to shear stress (grey bars) and after 24 h (black bars). **c** Transfection efficiency. $n \geq 3$, error bars indicate 95 % confidence interval. *** $p < 0.0005$; ** $p < 0.005$; * $p < 0.05$ for comparison to no shear condition

stress could lead to reduced transfection efficiency. On the other hand, shear stress could have an effect on the lipoplex characteristics, which in turn affect their ability to cause DNA uptake. Both these mechanisms could by themselves or in a concerted fashion produce the effect of increased toxicity and reduced transfection efficiency in the presence of shear stress. To test whether the observed effect on cell toxicity could be explained solely by the effect of shear stress on cells or on the lipoplex, we next subjected either only the lipoplex or the cells to shear stress prior to

transfection. Further experiments were only carried out at low shear stress.

Shear stress does not affect the transfectability of the lipoplex complex

The lipoplex was exposed to low shear stress for 2 h and subsequently immediately used to transfect CHO-S cells. A control culture was also transfected with lipoplex incubated for the same period of time in the absence of shear stress to control for the effect of

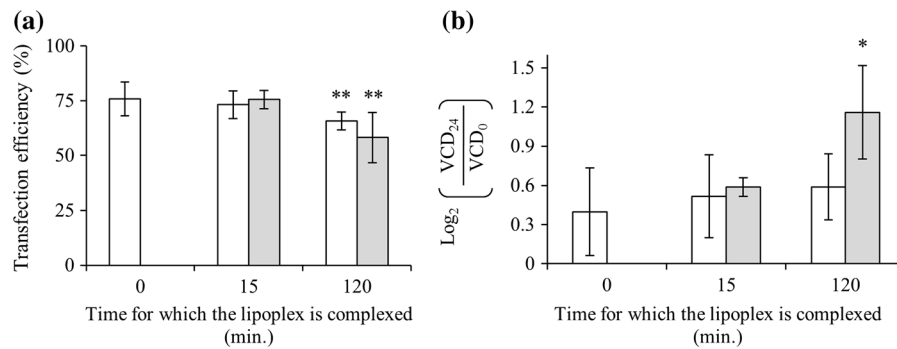


Fig. 3 Shear stress does not affect the transfectability of the lipoplex. Lipoplex subjected to low shear stress for 120 min and its unsheared control were used for transfecting CHO-S cells at different time points. **a** Transfection efficiency. Lipoplex not subjected to shear stress (*white bars*) and sheared lipoplex (*grey bar*) **b** Viable cell density (VCD) normalized to initial viable cell density. Cells transfected with unsheared lipoplex at

indicated time points after complexation (*white bars*), cells transfected with sheared lipoplex at indicated time points (sheared for 15 and 120 min) (*grey bars*). Initial viable cell density (VCD_0), 24 h after flow (VCD_{24}). The *error bars* indicate 95 % confidence interval, $n = 3$. $^{**}p < 0.005$; $^*p < 0.05$

increased incubation time on the efficacy of the complex. There is no remarkable difference in the transfection efficiency of lipoplexes sheared for 15 min (Fig. 3a). The slight decrease in transfection efficiency of lipoplex sheared for 2 h maybe attributed to the higher incubation time compared to the manufacturer's suggested optimal duration as it is also seen in the case of the lipoplex incubated for 2 h in the absence of any shear stress. Surprisingly, there is higher cell growth when cells are transfected with lipoplex sheared for 2 h, seen from the 1.2 population doublings for cells transfected with sheared lipoplex compared to the 0.6 population doublings for cells transfected with lipoplex incubated for 2 h without shear (Fig. 3b). Our data do not suggest any explanation for this observation of comparable transfectability and reduced growth inhibition by lipoplex subjected to shear stress.

Shear stress affects the transfectability of CHO-S cells

Next we subjected CHO-S to low shear stress for varying durations of time and subsequently immediately transfected them. Figure 4a shows the transfection efficiency of cells transfected without exposure to shear stress or transfected after 15 and 120 min of exposure to shear stress. A short duration 15 min exposure of cells to shear stress did not decrease their transfectability significantly. The transfection efficiency however decreased significantly when the cells

were subjected to shear stress for 2 h prior to transfection. As expected from the previous results, VCD did not change significantly immediately after exposure of cells to shear stress (see gray bars in Fig. 4b). The average number of 0.9 population doublings in 24 h after exposure to shear stress was significantly higher for cells exposed to shear stress for short duration prior to transfection compared to the cells transfected without exposure to shear (average 0.5 population doublings, see white bars in Fig. 4b).

For both cases where either the lipoplex or cells are subjected to shear stress followed by transfection, VCD increases in the 24 h period post transfection. This is unlike the case when cells are exposed to shear stress in presence of lipoplex where the VCD decreases in the 24 h period after exposure to shear. A comparison of Figs. 2a, 3b and 4b thus suggests that cell growth is adversely affected by the presence of lipoplex during exposure of cells to shear stress.

Toxicity of lipoplex is not solely attributable to liposome

To understand whether the effect of shear stress during lipofection is exclusively due to the liposome, we subjected CHO-S cells to shear stress in the presence of liposome at a concentration equivalent to its concentration in the lipoplex. In the absence of shear stress, an average 1.9 and 1.2 population doublings were observed in 24 h in the absence and presence of

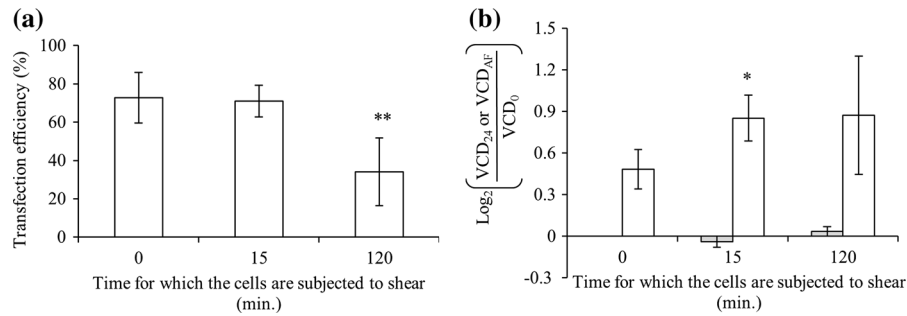


Fig. 4 Shear stress reduces transfectability of CHO-S cells. CHO-S cells were subjected to low shear stress for 120 min and subsequently transfected at different time points. **a** Transfection efficiency **b** Viable cell density (VCD) normalized to initial viable cell density. Immediately after subjecting cells to shear

stress (grey bars), 24 h after subjecting cells to shear stress for indicated time (white bars). Initial viable cell density (VCD_0), viable cell density immediately after flow for the indicated time (VCD_{AF}), 24 h after flow (VCD_{24}). The error bars indicate 95 % confidence interval, $n = 3$. ** $p < 0.005$; * $p < 0.05$

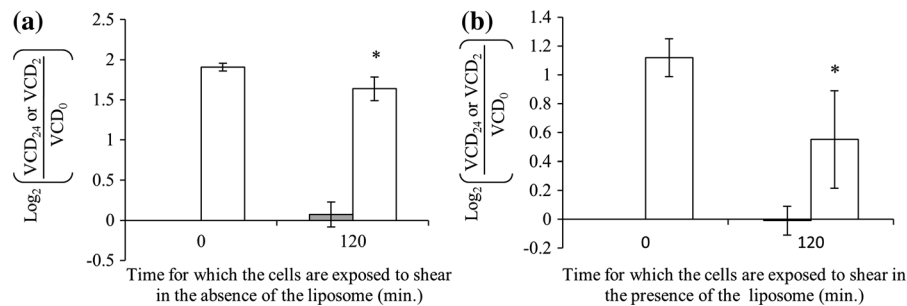


Fig. 5 Exposure of CHO-S cells to shear stress in presence of liposome causes reduction in cell growth. CHO-S cells were subjected to shear stress in the presence or absence of lipofectamine 2000 for 120 min and monitored for changes in cell density immediately after 120 min of flow and after 24 h of incubation. **a** Viable cell density (VCD) normalized to initial viable cell density for CHO-S cells subjected to a low shear stress without the liposome. **b** Viable cell density normalized to

initial viable cell density for CHO-S cells subjected to a low shear stress with the liposome. Viable cell density immediately after subjecting cells to shear stress for 120 min (grey bars), after 24 h of incubation (white bars). Initial viable cell density (VCD_0), viable cell density immediately after flow (VCD_2), 24 h after flow (VCD_{24}). The error bars indicate 95 % confidence interval, $n = 3$. * $p < 0.05$

liposome respectively (Fig. 5). Thus the presence of liposome by itself adversely affects cell growth. When cells are exposed to shear stress in the absence and presence of liposome, cells show growth at a lower average of 1.6 and 0.6 population doublings. Thus the adverse effect of liposomes on cell growth is substantially enhanced in the presence of shear. This is however in contrast to the case when cells are subjected to shear stress in presence of the lipoplex, where a 25 % decrease in VCD was observed (Fig. 2a). This suggests that the adverse effect on cell growth seen when cells are exposed to shear stress in presence of lipoplex is not attributable solely to the liposome.

Shear stress does not affect toxicity of lipoplex in inefficiently transfected K562 cell line

The results above indicate that toxicity of lipoplex is increased in presence of shear stress. We next evaluated whether the same effect is seen in K562 cells, which are not as efficiently transfected by Lipofectamine 2000 as CHO-S cells. There was no substantial cell death immediately after exposure of cells to shear stress, both in absence or presence of lipoplex (gray bars in Fig. 6a, b). The cell density of K562 cells doubled 24 h post transfection irrespective of whether cells were incubated with lipoplex in the absence or presence of shear stress (white bars Fig. 6a, b). The transfection

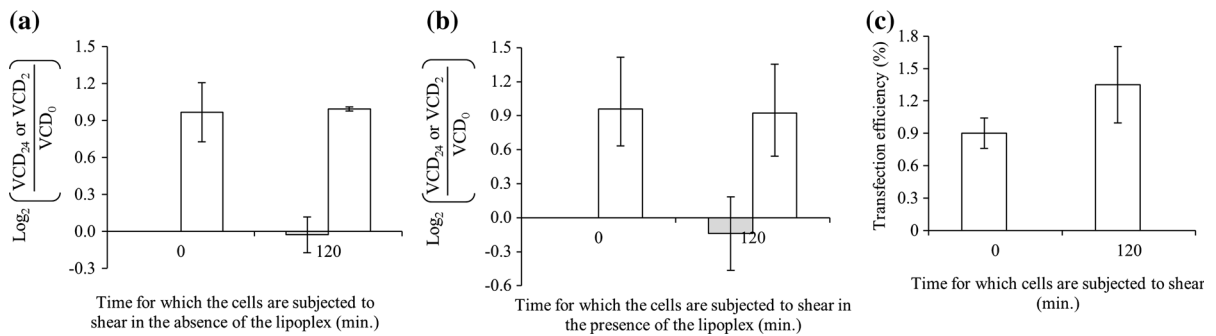


Fig. 6 Effect of shear stress on cell density and transfection efficiency of K562 cells in the presence or absence of the lipoplex. K562 cells were subjected to low shear stress for 120 min in the presence or absence of the lipoplex and monitored for changes in cell density and viability immediately after flow and after 24 h of incubation. **a** Viable cell density (VCD) normalized to initial viable cell density for K562 cells subjected to a low shear stress for 120 min. **b** Viable cell density

normalized to initial viable cell density for K562 cells subjected to a low shear stress in the presence of lipoplex for 120 min. Immediately after subjecting cells to shear stress (grey bars), after 24 h (white bars). Initial viable cell density (VCD_0), viable cell density immediately after flow (VCD_2), 24 h after flow (VCD_{24}). **c** Transfection efficiency. The error bars indicate 95 % confidence interval, $n = 2$

efficiency of K562 cells is low, and is slightly, but not significantly, increased in the presence of shear (Fig. 6c). Thus the adverse effect of lipoplex on cell growth in the presence of shear is not seen in the inefficiently transfected K562 cell line.

Discussion

Shear stress is known to affect various aspects of cell physiology like endocytosis and pinocytosis (Davies et al. 1984; Kudo et al. 1997; Rizzo et al. 2003), membrane fluidity (Butler et al. 2001; Haidekker et al. 2000), inducing membrane fusion (Kogan et al. 2014), and more recently it was shown to facilitate uptake of extracellular macromolecules (Hallow et al. 2008; Sharei et al. 2013), possibly through formation of transient pores in the cell membrane (Mardikar and Niranjan 2000). It is known that high levels of shear stress are deleterious to suspension cells. For example, McQueen et al. subjected mouse myeloma cells to high shear stress in capillary tubes and showed that flow caused lysis which was observed at a wall shear stress of 1800 dyne/cm^2 (McQueen et al. 1987). Our simple flow apparatus comprising of pumping suspension cells using a peristaltic pump also showed similar results for CHO-S cells with high levels of cell death at high wall shear stress, but not at the low and moderate shear stress levels. The use of a peristaltic pump allows long duration aseptic unidirectional flow

of cells. However, in this set-up, unlike systems using syringe pumps, cells are also subjected to shear stress in the peristaltic pump head, but this effect has been controlled by keeping the fluid flow rate constant and varying the diameter of the fluid path to vary wall shear stress. Though shear stress has been known to affect cell survival, and for reasons described above can be expected to affect liposomal delivery of cargo to suspension cells, to our knowledge there have been no studies explicitly analyzing the effect of shear stress on liposomal DNA delivery to suspension cells.

We report that exposure of CHO-S cells to shear stress in the presence of lipoplex reduced transfection efficiency and cell growth in the subsequent 24 h period while causing greater cell clumping. We further evaluated whether this effect could be attributed simply to the interaction of the cells with lipid in the presence of shear. The size and zeta potential of lipofectamine 2000 has been reported to change upon complexation with DNA (Son et al. 2000). In an uncomplexed state, the reported zeta potential was -4 mV and upon complexation with plasmid DNA, it was -24 mV . The hydrodynamic diameter in a complexed state was reported to increase to 488 nm from 319 nm in an uncomplexed state (Son et al. 2000). Shearing of cells in presence of liposome alone also reduces cell growth in the subsequent 24-h period, though not to an extent similar to that due to the lipoplex. This suggests that the increased toxicity is not entirely explained by interaction of the liposome

alone with the cell in the presence of shear stress. This could be due to the differences in zeta potential and/or size of the liposome and lipoplex reported in the literature. To further check if the increased toxicity due to lipoplex in the presence of shear stress might be due to long-lasting modification of the lipoplex with shear, we subjected the lipoplex to shear, subsequently using the sheared lipoplex for transfection. It was observed that there is only a slight reduction in efficiency following the 2 h incubation of lipoplex both in presence and absence of shear stress. Such slight loss of transfection efficiency was previously reported where the DNA–Lipofectamine 2000 complexes were relatively stable and continued to provide high transfection efficiency even up to 2 h after complexation (Dalby et al. 2004). This however does not preclude a change in shape and therefore increased toxicity when lipoplex is sheared along with cells. Interestingly, there is a slight increase in cell growth when cells are transfected with sheared lipoplex compared to the cell growth upon transfection with lipoplex incubated under static conditions.

The reduced transfection efficiency and increased lipoplex toxicity observed at low shear stress could also possibly be due to the effect of shear stress on cells, making the cells more susceptible to lipoplex toxicity. In that case, these effects should be seen when transfection is carried out immediately after subjecting cells to shear stress. Indeed, subjecting CHO cells to sustained low shear stress prior to transfection (for 2 h), resulted in reducing the transfectability of CHO cells, but did not increase cell death. This indicates that the observed effect of reduced transfection efficiency could be due to shear stress affecting the ability of cells to uptake and/or deliver the foreign DNA to the nucleus. This also suggests that the increased toxicity of lipoplex in presence of shear stress is a result of cellular interaction with lipoplex in the presence of shear stress and likely not due to other long-term cellular changes upon exposure to shear stress. Lipoplexes with low toxicity in vitro, when injected into the blood stream where they are subjected to shear stress, have resulted in significant toxicity to blood cells in the form of transient leukopenia and neutropenia both in animal models and human clinical trials (Freimark et al. 1998; Li et al. 1999; Ruiz et al. 2001; Tousignant et al. 2000; Zhang et al. 2005), though the cause of this is not clear. We did not observe a similar effect of higher toxicity in presence of shear stress in K562 cells. K562 cells were not efficiently transfected by the lipoplex used

in this study whereas CHO-S are efficiently transfected. It remains to be seen whether transfectability has any role in the relationship between shear stress and toxicity of the lipoplex.

In conclusion, enhanced toxicity and reduced transfection efficiency of lipoplex is observed in CHO-S cells exposed to shear stress. Further studies will be necessary to fully understand the mode of toxicity. We propose that this factor should be taken into account in the design and planning for large-scale transfections in bioreactors for transient protein expression. Our results may also be relevant to gene therapy where shear stress may contribute to performance of in vitro static cell cultures not being predictive of performance in animal models where the lipoplex is intravenously injected and evaluation of liposomal delivery agents in the presence of shear stress could be considered as an intermediate testing step before animal studies for gene therapy.

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