Efficient polyethylenimine-mediated gene delivery proceeds via a caveolar pathway in HeLa cells.

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SUMMARY

Most in vivo gene therapies will require cell-specific targeting. Although vector targeting through ligand attachment has met with success in generating gene delivery particles that are capable of specific cellular interactions, little attention has been given to the possible effects of such ligands on subsequent intracellular processing. In this study, we examine the impact of targeting two distinct endocytic routes—the caveolar and clathrin pathways—on polyethylenimine-mediated gene delivery in HeLa cells. Targeting complexes to the caveolar pathway with folic acid and the clathrin pathway with transferrin yields enhanced gene delivery relative to unmodified polyethylenimine. Colocalization studies with caveolin-1 and clathrin heavy chain indicate that the ligands successfully deliver their cargo to the intended pathways. However, inhibition of only the caveolar pathway—whether through the use of small molecule drugs or RNA interference—reduces gene delivery efficiency, suggesting that successful polyethylenimine-mediated gene delivery proceeds via a caveolar pathway in HeLa cells. Transfections in the presence of chloroquine and pH tracking studies suggest that a contributing factor to the success of the caveolar pathway is avoidance of lysosomes. Collectively, these data demonstrate that uptake mechanism and subsequent endocytic processing are important design parameters for gene delivery materials.

Keywords: polyethylenimine, caveolae, clathrin, endocytosis, folate, transferrin

INTRODUCTION

The most effective gene delivery strategy for a given application is often dictated by particular disease characteristics. For systemic diseases like hemophilia, treating any of a number of tissues or cell types may be adequate provided that sufficient levels of the transgene product are expressed and have access to the circulatory system.[1] However, for localized disorders like cancer or cystic fibrosis, it is often beneficial and sometimes critical to target gene delivery vectors to specific diseased cell types.[2,3] It has been reported that the "receptors" for polycationic gene delivery vectors are clusters of negatively charged proteoglycans on the cell surface. Unfortunately, these "receptors" do not afford cell specificity.[4] To direct gene delivery vehicles to particular cells, peptides, proteins or small molecule ligands may be attached to the surface of polyplexes.[5-8] Ideally, the ligand chosen would target a receptor found on only one particular type of diseased cell, thereby ensuring that healthy cells are unaffected. While such targeting methods have met with success in generating particles that are capable of specific cellular interactions, little attention has been given to the possible effects on the intracellular trafficking of targeted gene delivery complexes.

Transferrin and folic acid are commonly used ligands for drug and gene targeting due to the overexpression of their respective receptors on many cancer cells.[9-11] Notably, these ligands utilize different uptake mechanisms which ultimately result in distinct intracellular processing. Transferrin is internalized by clathrin-coated pits and follows the traditional endolysosomal pathway.[12] More specifically, upon ligand binding, clathrin is recruited to receptor sites, forming a cage-like structure that stabilizes the vesicle as it buds from the membrane. As the endocytic

vesicle moves through the cell, proton pumps anchored within its membrane gradually acidify the vesicle contents from approximately pH 7.2 to pH 6. At this pH, the vesicle is termed an early endosome. Through continued acidification, the early endosome eventually reaches pH 5 at which point it is considered a late endosome. Finally, when the endosome has fully matured, it fuses with lysosomes and reaches a pH as low as 4.5 where degradative enzymes are activated.

Folic acid, in contrast, is internalized by caveolae—flask-like invaginations on the cell surface that bud from membrane microdomains rich in cholesterol and the protein caveolin.[13] Upon receptor/ligand binding, the cell membrane pinches off to form a vesicle that is directed towards caveosomes. Endocytosis through caveolae and the fate of caveosomes are less well understood than its clathrin-mediated counterpart. There is speculation that caveosomes may fuse with early endosomes through the action of the GTPase Rab5 or proceed through the cell by an independent path.[14] Unlike the clathrin pathway, however, caveosomes avoid trafficking to lysosomes. In the case of the cholera toxin binding subunit (CTxB), one of the better studied ligands utilizing caveolar uptake, the ligand is believed to be transported to the Golgi complex via early endosomes.[15,16] Simian virus 40 (SV40), which also utilizes caveolar uptake, is thought to avoid the harsh pH and enzymatic environment of the endosomal-lysosomal pathway and remain entirely in pH-neutral caveosomes at it travels through the cell.[17,18]

Polyethylenimine (PEI) is an efficient off-the-shelf gene delivery material. It is believed that the relatively high gene delivery efficiency of PEI is attributable to its ability to act as a proton sponge.[19] According to the proton sponge hypothesis, these polyplexes are sequestered in endocytic vesicles that are acidified by V-type ATPases within the endosomal membrane. PEI,

with its abundance of primary, secondary and tertiary amines, is thought to effectively buffer the vesicle lumen. As increasing numbers of protons and counter ions are pumped in to acidify the buffered compartment, water is osmotically driven into the lumen. The excess water is believed to cause swelling and eventual lysis of the endosome, releasing polyplexes into the cytosol. This proposed mechanism for the endosomal escape of proton sponge-containing polyplexes has been met with both corroborating and conflicting data.[20-23] Recently, we demonstrated that acetylation of PEI resulting in decreased buffering capacity—as well as weaker interactions with nucleic acids—significantly improved gene delivery efficiency, suggesting that the proton-sponge effect may not be a complete representation of the intracellular processing of such polyplexes.[24,25] The study presented here examines the importance of the caveolar uptake pathway that potentially avoids vesicle acidification and thus precludes the proton sponge effect, compared to the clathrin-mediated uptake pathway that is inherently tied with endosome acidification, on PEI-mediated transfection of HeLa cells.

RESULTS

Generation of folate-targeted PEI

NHS-folate was conjugated to 25-kDa branched polyethylenimine at 1.1, 3.2 and 7.2 folate molecules per PEI (PEI-Fol). When subsequently used to transfect the HeLa cervical cancer cell line, known to overexpress folate receptors, PEI-Fol with 1.1 folate moieties per polymer chain [PEI-Fol(1.1)] yielded gene expression levels roughly five-fold greater than unmodified PEI, albeit only at the sub-optimal polymer:DNA weight ratio of 0.5:1 ($p < 0.1$, **Figure 1a**). This is likely due to non-specific electrostatic interactions between the negatively charged cell

membrane and positively charged polyplexes at higher weight ratios. For subsequent experiments, transfections were performed with complexes formed at the polymer:DNA weight ratio of 0.5:1 using PEI-Fol(1.1). The uptake of targeted and non-targeted polyplexes was similar at times up to one hour post-transfection. Corresponding with the increased transfection efficiency, PEI-Fol(1.1)/DNA polyplexes showed a 1.5-fold increase in uptake in HeLa cells relative to unmodified PEI polyplexes at two and four hours post-transfection. ($p < 0.05$, **Figure 1b**). The delayed uptake of PEI-Fol(1.1)/DNA complexes is likely due to the slow kinetics of caveolae-mediated uptake.[9, 26, 27]. To verify that folate-targeted complexes entered cells through folate receptors, a competitive inhibition assay was performed. Addition of 100 to 300 μM free folic acid to the transfection medium reduced PEI-Fol(1.1)/DNA-mediated luciferase expression by roughly four-fold relative to folate-free conditions ($p < 0.05$, **Figure 1c**). Free folic acid also reduced the uptake of PEI-Fol(1.1)/DNA by 25% relative to uptake in the absence of folic acid ($p < 0.05$, **Figure 1d**). Combined, the data indicate that at least a portion of the folate-targeted complexes entered cells via the folate receptor.

Generation of transferrin-targeted PEI

HeLa cells were transfected with transferrin-targeted PEI complexes formed at a total polymer:DNA weight ratio of 0.5:1, based on the results described above, to limit non-specific electrostatic interactions with cell membrane components. Following the procedure of Kircheis et al., the complexes were formed with varying ratios of unmodified PEI to transferrin-targeted PEI.[8] Polyplexes composed of between 10% and 50% PEI-Tf yielded gene expression levels between two- and eight-fold higher than unmodified PEI ($p < 0.05$, **Figure 2a**). Polyplexes composed of 25% PEI-Tf and 75% unmodified PEI [PEI-Tf(25%)] were used for subsequent

experiments. Corresponding with the increased transfection efficiency, uptake of PEI-Tf(25%)/DNA increased two-fold relative to unmodified PEI at one ($p < 0.1$), two and four ($p < 0.05$) hours post-transfection (**Figure 2b**). The more rapid uptake of transferrin-targeted complexes relative to untargeted complexes—observable even after 30 minutes—is likely attributable to the faster kinetics of clathrin-mediated uptake.[27-29] To verify the targeting of transferrin receptors by PEI-Tf(25%), transfection experiments were performed in the presence of free transferrin. The presence of 0.5 μM transferrin in the transfection medium reduced the PEI-Tf(25%)/DNA-mediated luciferase expression by approximately 60% relative to transferrin-free conditions, but the difference was not statistically significant ($p < 0.15$, **Figure 2c**). Further inhibition was possible at higher concentrations of free transferrin, but the specificity of the inhibition decreased—the electrostatic interactions between negatively charged transferrin and the cationic PEI substantially reduced the gene delivery efficiency of targeted as well as untargeted complexes (data not shown). The presence of 0.5 μM transferrin also reduced the uptake of PEI-Tf(25%)/DNA by 30% relative to transferrin-free conditions, but again the difference was not statistically significant ($p < 0.15$, **Figure 2d**).

Investigation of complex colocalization with caveolin-1 and clathrin heavy chain

As mentioned previously, folic acid is endocytosed via a caveolar pathway while transferrin enters cells in a clathrin-mediated fashion. To verify that folate- and transferrin-targeted PEI complexes indeed enter HeLa cells through their intended pathways, confocal microscopy was performed to investigate the colocalization between fluorescently-labeled polyplexes and the proteins caveolin-1 and clathrin heavy chain. PEI-Fol(1.1)/DNA colocalized primarily with caveolin-1 at 30 minutes post-transfection, suggesting that the complexes are predominantly

endocytosed via a caveolar process (**Figure 3)**. Correspondingly, PEI-Tf(25%)/DNA colocalized primarily with the clathrin heavy chain at 30 minutes post-transfection, suggesting that they enter cells via clathrin-mediated endocytosis. Unmodified PEI/DNAcan be seen colocalized with both caveolin-1 and the clathrin heavy chain, indicating that these polyplexes likely enter cells through a nonspecific combination of both pathways.

Uptake following selective endocytic inhibition with small molecule drugs

Various drugs are capable of selectively inhibiting either clathrin- or caveolae-mediated endocytosis. The drug chlorpromazine inhibits clathrin-mediated uptake by disassociating the clathrin-coated pits that form at ligand binding sites.[30] Amantadine prevents clathrin-mediated uptake by blocking the budding of clathrin-coated vesicles into the cell.[31] Caveolae are known to bud from microdomains on the cell surface that are rich in cholesterol. The drug methyl-β-cyclodextrin (mβCD) inhibits caveolar uptake by sequestering cholesterol on the cell surface.[32] Genistein, a tyrosine kinase inhibitor, blocks caveolar uptake by preventing the phosphorylation of caveolin, a scaffolding protein that is the main component of caveolae.[33] Drug concentrations were chosen based on relevant literature and the ability of the drug to prevent the uptake of 150-nm fluorescent polystyrene nanoparticles conjugated to either folate or transferrin (data not shown). To test the effect of the inhibitor drugs on the uptake of folate- and transferrin-targeted PEI, polyplexes were labeled with the fluorescent DNA intercalator YOYO-1 and used to transfect cells that were subsequently subjected to flow cytometry (**Figure 4a**). It is evident that uptake was not completely inhibited by any of the drugs at the concentrations tested. However, the effects were more pronounced in certain targeted species than in others. Uptake of folate-targeted polyplexes was more substantially inhibited in the presence of genistein and mβCD than

chlorpromazine and amantadine as compared to PEI-Fol(1.1)/DNA uptake in the absence of drugs ($p < 0.05$). Correspondingly, uptake of transferrin-targeted polyplexes was inhibited to a greater extent by chlorpromazine and amantadine than genistein and mβCD relative to PEI-Tf(25%)/DNA uptake in the absence of drugs ($p < 0.05$). Unmodified PEI/DNA uptake was reduced by both caveolae and clathrin inhibitors as compared to drug free conditions, indicating that it likely enters cells through a combination of both pathways ($p < 0.05$). The non-specific effect of the drugs on the uptake of targeted complexes is likely a combination of their cytotoxicity (data not shown) as well as the potential for non-specific uptake of targeted complexes. Nonetheless, the data suggest that the folate- and transferrin-targeted complexes are predominantly endocytosed by caveolae- and clathrin-mediated processes, respectively.

Transfection following selective endocytic inhibition with small molecule drugs

When unmodified as well as folate- and transferrin-targeted polyplexes were used to transfect HeLa cells in the presence of the same drugs tested above, inhibitors of caveolae-mediated endocytosis drastically reduced gene expression relative to drug-free conditions ($p < 0.05$) while inhibitors of clathrin-mediated endocytosis had no deleterious effects (**Figure 4b**). Clathrin inhibition by chlorpromazine surprisingly increased the gene delivery efficiency of unmodified PEI/DNA by roughly two-fold and PEI-Tf(25%)/DNA by nearly three-fold relative to drug-free conditions ($p < 0.05$). The observed increase in gene delivery efficiency with PEI-Tf(25%)/DNA complexes suggests that efficient and successful gene delivery proceeds via a caveolae-mediated process in HeLa cells. While chlorpromazine inhibits endocytosis by blocking the formation of clathrin-coated pits on the interior of the cell, it does not necessarily prevent the binding of targeted or non-targeted complexes to the cell membrane. Thus, the

increase in transfection efficiency in the presence of chlorpromazine is likely due to increased uptake of polyplexes non-specifically bound to the cell membrane and internalized via caveolar or other uptake pathways. The same rationale explains the transfection efficiency observed in cells treated with amantadine.

Transfection following selective endocytic inhibition with siRNA

To verify the transfection results obtained through drug-mediated inhibition of caveolar and clathrin-mediated endocytosis, transfections were performed in cells in which expression of caveolin-1 (CAV-1) or clathrin heavy chain (CLTC) was silenced by siRNA. As a control, the transfection results were compared to HeLa cells treated with laminin (LAM) siRNA. Gene silencing was performed using commercially available, pre-designed siRNA duplexes and confirmed by Western blot analysis of whole cell lysates (**Figure 5a)**. Laminin siRNA had no effect on caveolin-1 or clathrin heavy chain expression. Confirming the results obtained through inhibition with small molecule drugs (**Figure 4b**), the down-regulation of caveolin-1 expression in HeLa cells decreased gene delivery by approximately 75% relative to laminin siRNA-treated cells irrespective of the presence of targeting ligands ($p < 0.05$, **Figure 5b**). Down-regulation of clathrin heavy chain expression increased gene delivery approximately two-fold relative to laminin siRNA-treated cells irrespective of targeting ($p < 0.05$). As described above, the increase is likely due to the internalization of non-specifically bound complexes by a caveolar pathway instead of a clathrin pathway.

Transfection in the presence of chloroquine and bafilomycin A1

A major distinction between clathrin and caveolae-mediated endocytosis is trafficking to lysosomes. Ligands entering the cell via the clathrin pathway are believed to ultimately enter lysosomes while ligands entering the cell via a caveolar pathway do not. To investigate whether avoidance of lysosomes in the caveolar pathway is a component in successful gene delivery, transfections were performed in the presence of the lysosomotropic drug chloroquine. When chloroquine is added to cells at 20 μM in the external medium, its intracellular concentration reaches 5 mM.[34] The localized concentration within lysosomes is likely even higher. Because of its aromatic ring structure, chloroquine is able to intercalate DNA, thereby causing a conformational change that is capable of inhibiting enzymatic DNA degradation.[35,36] The addition of 5 mM chloroquine prevents total DNA digestion, as evidenced by the faint pGL3 band seen in the corresponding lane (**Figure 6a**). The degradation inhibition is more effective at higher chloroquine concentrations, resulting in brighter, more pronounced bands.

Typically, chloroquine is used as an endosomal buffering agent with gene delivery vectors that lack the ability to buffer endosomes, such as polylysine. Since PEI is an innate buffering agent, the effect of chloroquine in cells can be attributed to its ability to prevent lysosomal DNA degradation. The addition of 20 μM chloroquine had only minimal effects on transgene expression in HeLa cells transfected with unmodified PEI/DNA and PEI-Fol(1.1)/DNA (**Figure 6b**). At least a portion of these polyplexes have entered via a caveolar pathway and thus presumably avoid lysosomes, thereby masking the potentially beneficial effects of chloroquine. However, for cells transfected with transferrin-targeted PEI, the addition of chloroquine yielded a two-fold increase in gene delivery relative to drug-free conditions ($p < 0.05$). This suggests

that trafficking to lysosomes and plasmid degradation therein is a source of inefficiency for clathrin-targeted complexes. Correspondingly, the data also suggest that avoidance of lysosomes by caveolae-targeted complexes is a contributing factor to their effective gene delivery.

When treated simultaneously with chloroquine and bafilomycin A1, a V-type ATPase inhibitor that prevents the acidification of endosomes and thus the accumulation of chloroquine in lysosomes, chloroquine loses its advantageous effects on transferrin-targeted PEI relative to drug-free conditions ($p < 0.05$). The noticeable decrease in transgene expression in cells treated with bafilomycin A1 relative to those transfected under drug-free conditions ($p < 0.05$) suggests that acidification—although not necessarily to the extent of reaching lysosomal pH—is a necessary component in PEI-mediated gene delivery regardless of uptake pathway. The acidification may be necessary in a variety of cellular processes outside of the traditional endolysosomal pathway. For example, bafilomycin A1 has been shown to affect several organelles, including parts of the Golgi complex, one of the target areas for caveolae-mediated uptake.[37,38] Correspondingly, Mineo et al. have shown that caveolin-1 colocalizes with the V-type ATPases affected by baflilomycin A1.[38] Thus, the inhibitory effect of bafilomycin A1 may be due to its ability to prevent endosome acidification outside of the clathrin pathway, or bafilomycin A1 may interfere with the caveolar pathway in currently unknown ways.

Polyplex pH measurement by ratiometric flow cytometry.

To further explore the trafficking of polyplexes to lysosome-like pH environments, ratiometric flow cytometry was performed to measure the average pH surrounding targeted and untargeted gene delivery complexes.[20] The fluorescence intensity of fluorescein decreases with pH over the

range of approximately pH 5 to 8. By labeling targeted and untargeted PEI with both fluorescein and the pH-insensitive dye Alexa Fluor 633, the fluorescence intensity ratio of fluorescein to Alexa Fluor 633 (as measured by flow cytometry) can be used to measure the pH microenvironment of the respective complexes within cells at various times post-transfection (**Figure 7**). All the tested complexes were exposed to mildly acidic environments during the first four hours post-transfection. At that time, the average pH environment of PEI-Tf(25%)/DNA was lower than that surrounding untargeted PEI/DNA and PEI-Fol(1.1)/DNA complexes ($p < 0.05$). From four to eight hours, untargeted and transferrin-targeted polyplexes continued to be acidified while folate-targeted complexes remained at a near-constant, albeit acidic, pH. Presumably, this is because a large portion of the transferrin-targeted polyplexes and some of the untargeted polyplexes were within endosomes that were continually acidified by proton pumps in the endosomal membrane. Meanwhile, the majority of folate-targeted polyplexes appeared to remain separate from the endolysosomal pathway, possibly within caveosomes that had fused with acidic early endosomes. At eight hours post-transfection, PEI-Fol(1.1)/DNA resided in less acidic environments than unmodified PEI/DNA and PEI-Tf(25%)/DNA ($p < 0.05$). As a consequence of entering cells primarily via the clathrin pathway, PEI-Tf(25%)/DNA was found at the lowest pH. Untargeted PEI/DNA is believed to utilize both clathrin- and caveolar-mediated uptake. Thus, the combination of PEI/DNA within the higher pH caveolar pathway and the lower pH clathrin pathway results in a measured pH intermediate to the two extremes. By twelve hours post-transfection, untargeted and transferrin-targeted complexes reached near-lysosomal conditions (pH 4.7) while the folate-targeted complexes did not (pH 5.1, $p < 0.1$). Only at twenty hours post-transfection did untargeted, transferrin- and folate-targeted complexes reach the same acidic pH. The time at which DNA unpackages from its condensing agent is

unknown. However, at twenty hours post-transfection it is likely that the complexes have dissociated with the free PEI being localized in lysosomes.

Investigation of complex colocalization with EEA-1 and LAMP-1

Ligands within the caveolar pathway are believed to either remain in caveosomes or fuse with early endosomes en route to their intracellular destination, while ligands within the clathrin pathway are trafficked from early endosomes to late endosomes and eventually lysosomes. To investigate the effect of polyplex targeting on the intracellular pathway, fluorescently-labeled polyplexes were evaluated for colocalization with early endosome (EEA-1) and lysosome (LAMP-1) markers at ten hours post-transfection (**Figure 8**). Unmodified PEI/DNA appear to be found equally within early endosomes and lysosomes at ten hours post-transfection. However, a greater portion of folate-targeted complexes appear to be within early endosomes than lysosomes. Correspondingly, transferrin-targeted complexes appear to associate more with lysosomes than early endosomes. The colocalization data support the pH tracking data of **Figure 7**, which suggests that untargeted and transferrin-targeted complexes have entered lysosomal-like conditions (pH 4.8) at ten hours post-transfection while folate-targeted complexes are primarily still in early and late endosomes (pH 5.3).

DISCUSSION

Practical gene delivery vectors will likely utilize some form of cell-specific targeting. Because of this, it is important that studies of the intracellular behavior of gene delivery vectors include the effects of cell targeting and associated endocytic trafficking. Recent work has highlighted

the importance of caveolar uptake and processing over the clathrin pathway in successful gene delivery in multiple cell types.[27,39,40] To this end, the work presented here addresses the differences in processing of PEI gene delivery complexes targeted to clathrin and caveolar pathways using transferrin and folate, respectively.

The ability of caveolae inhibitor drugs (**Figure 4b**) and siRNA-mediated caveolin-1 down-regulation (**Figure 5b**) to dramatically reduce gene expression in HeLa cells irrespective of vector targeting suggests that caveolar processing is the efficient means of PEI-containing polyplex trafficking in HeLa cells. If both pathways were equally capable of successful gene delivery, transgene expression mediated by transferrin-targeted complexes would be expected to decrease as a result of clathrin inhibition yet be unaffected by caveolar inhibition. Since caveolar inhibition alone has a negative effect on transgene expression, the results presented here indicate that caveolar, not clathrin, processing yields successful gene delivery. This is not wholly surprising as certain viruses, such as SV40, are known to infect cells through a caveolar pathway. To understand the deficiencies in the clathrin pathway—and thus the important intracellular steps in successful gene delivery—it is useful to compare the differences in cellular processing of ligands within the two pathways. With regard to polyplex gene delivery, the most important difference between the two routes is trafficking to lysosomes. Thus, it seems likely that a portion of the efficiency of complexes internalized via the caveolar pathway is avoidance of lysosomes and degradation therein.

At elevated concentrations chloroquine is able to prevent the enzymatic degradation of plasmid DNA by affecting a conformational change in DNA that is believed to prohibit the binding of

DNAse. As a lysosomotropic agent, chloroquine accumulates preferentially in lysosomes. Thus, its ability to inhibit DNAse digestion is limited to DNA that has entered lysosomes. In the context of the work presented here, chloroquine improved the gene delivery efficiency of only transferrin-targeted polyplexes (**Figure 6b**). This suggests that lysosomal trafficking is an important barrier for complexes entering the cell through the clathrin pathway. The beneficial effects of chloroquine on PEI-Tf(25%)/DNA transfection could be elminated by preventing chloroquine accumulation in lysosomes through the use of bafilomycin A1. However, bafilomycin A1 alone reduced the gene delivery efficiency of not only transferrin-targeted polyplexes, but also unmodified and folate-targeted polyplexes. This reduction in efficiency has been interpreted to suggest that endosome acidification is an essential step in successful gene delivery.[41] However, Mineo et al. have shown that caveolin-1 colocalizes with the same V-type ATPases inhibited by bafilomycin A1.[38] Thus, the reduction in gene delivery by bafilomycin A1 may be due to effects outside of its ability to prevent endosome acidification during clathrin-mediated uptake—bafilomycin might also interfere with the caveolar pathway.

Measurement of the pH environment of PEI-based gene delivery complexes suggests that all three types of polyplexes, regardless of caveolar- or clathrin-mediated internalization, are exposed to the acidic environments of early endosomes during the first four hours post-transfection. Based on the significant decrease in transgene expression in the presence of bafilomycin A1 (**Figure 6b**), this acidification appears to be an essential step in efficient gene delivery. Evidence suggests that some caveolar vesicles fuse with early endosomes for sorting during their movement through the cell.[14,42] The acidic conditions surrounding folate-targeted complexes (**Figure 7**) support the fusion hypothesis. After four hours post-transfection, the

differences between folate-targeted, transferrin-targeted and untargeted polyplexes became evident. From four to eight hours, folate-targeted complexes remained at near constant pH (pH 5.5) while transferrin-targeted and untargeted complexes continued to be acidified. Early endosomes typically exhibit a pH of 6.0. Keeping in mind that the ratiometric assay employed measures the average pH of all polyplexes within the cells, it is likely that some complexes reside within environments at a higher or lower pH than recorded. Thus, it is conceivable that during four to eight hours post-transfection a portion of the folate-targeted complexes could be found sequestered in early endosomes while others had moved into more acidic environments. With their lower and steadily decreasing pH from four to eight hours, it appears that the transferrin-targeted and untargeted complexes were localized in late endosomes en route to lysosomes. Localization of untargeted PEI/DNA in higher pH environments than transferrin-targeted polyplexes suggests that a smaller fraction of the untargeted complexes were present in the pathway from the endosomes to lysosomes. This supports the previous data suggesting that untargeted complexes enter through a non-specific combination of both caveolar and clathrin pathways. At twelve hours post-transfection, the pH 4.7 environment surrounding transferrin-targeted and untargeted complexes suggests that they had entered lysosomes. At the same time, the bulk of folate-targeted complexes appeared to be entering the late endosome at pH 5.0. At twenty hours post-transfection, the folate-targeted complexes joined the transferrin-targeted and untargeted complexes in lysosomes. Thus, while folate-targeted complexes did not completely bypass acidic environments, they appeared to spend a greater amount of time in early endosomes and were ultimately trafficked to lysosomes at a slower rate than either transferrin-targeted or untargeted complexes.

In summary, cell targeting can influence the intracellular processing of PEI-containing gene delivery vehicles. The results presented here suggest that complexes targeted to the caveolar pathway result in efficient gene delivery in HeLa cells while those targeted to the clathrin pathway do not. While both endocytic routes involve acidification in the early endosomes, the caveolar pathway avoids rapid and direct trafficking to the more acidic late endosomes and lysosomes, suggesting that this lysosomal trafficking is a source of inefficiency with the clathrin pathway.

MATERIALS AND METHODS

Materials

Folic acid, human apo-transferrin, N-hydroxysuccinimide, N,N'-dicyclohexylcarbodiimide, 25-kDa branched polyethylenimine, genistein, chlorpromazine, amantadine and the ProteoQuest colorimetric Western blotting kit were obtained from Sigma-Aldrich (St. Louis, MO). Traut's reagent (2-iminothiolane), ninhydrin, Ellman's reagent, sulfo-KMUS and BCA protein assay kits were purchased from Pierce Chemical Company (Rockford, IL). Methyl-β-cyclodextrin was obtained from Roquette America, Inc. (Keokuk, IA). Lipofectamine 2000, the fluorescent dyes fluorescein isothiocyanate (FITC), YOYO-1, Alexa Fluor 488, Alexa Fluor 633, and the antibody labeling kits Zenon Alexa Fluor 546 Mouse IgG_1 and Zenon Alexa Fluor 647 Mouse IgG_1 were obtained from Invitrogen (Carlsbad, CA). Mouse anti-clathrin heavy chain IgG_1 and mouse anti-early endosome IgG_1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-caveolin-1 IgG_1 and mouse anti-lysosomal associated membrane protein-1 IgG_1 were obtained from BD Biosciences (Franklin Lakes, NJ). Pre-designed short interfering

RNA (siRNA) targeted towards the clathrin heavy chain was obtained from Qiagen (Valencia, CA). Pre-designed siRNAs targeted towards laminin and caveolin-1 were obtained from Dharmacon (Lafayette, CO).

Cells and plasmids

The HeLa human cervical carcinoma cell line used in this study was a gift from Dr. Sandra McMasters (University of Illinois, Urbana, IL). The cells were cultured according to their ATCC protocols at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium. The growth medium was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The 5.3-kilobase expression vector pGL3 (Promega, Madison, WI) coding for the luciferase gene driven by the SV40 promoter and enhancer, was grown in DH5α *E. coli* (Gibco BRL, Rockville, MD) and purified with a commercial plasmid purification kit (Bio-Rad, Hercules, CA).

Generation of folate-targeted PEI

Folic acid was conjugated to PEI according to the procedure of Guo et al.[43] Briefly, folic acid was dissolved in dimethylsulfoxide with a 1.1 molar excess of both N-hydroxysuccinimide (NHS) and N,N'-Dicyclohexylcarbodiimide (DCC). The reaction was allowed to stir overnight at room temperature. The following day, the insoluble dicyclohexylurea byproduct was removed by filtration. The NHS-folate product was collected, quantified by ultraviolet absorption at 363 nm and stored at 4 °C until needed. NHS-activated folate was conjugated to the primary amines of 25-kDa branched PEI by dissolving both in 0.1 M sodium bicarbonate buffer (pH 8.3) at the desired labeling ratio and stirring overnight at room temperature. The PEI-folate product was purified from reactants by gel filtration chromatography (PD-10, GE Healthcare, Uppsala,

Sweden) and characterized using a ninhydrin assay (PEI quantification) and ultraviolet absorption at 363 nm (folate quantification).

Generation of transferrin-targeted PEI

Human apo-transferrin was conjugated to PEI using the heterobifunctional crosslinker sulfo-KMUS. Briefly, 25-kDa branched PEI was first thiolated with 2-iminothiolane in PBS containing 5 mM EDTA and purified by gel filtration chromatography (PD-10, GE Healthcare, Uppsala, Sweden). The presence of thiol groups was quantified with Ellman's reagent. Sulfo-KMUS was reacted separately with the primary amines of human apo-transferrin and purified by gel filtration chromatography (PD-10, GE Healthcare, Uppsala, Sweden) to generate a thiol-reactive form of the protein. The thiol-reactive transferrin and thiolated PEI were then combined at a 1:1 molar ratio in PBS containing 5 mM EDTA and allowed to react overnight at 4 °C. The resulting PEI-transferrin conjugate was purified from reactants using a 100,000 MWCO centrifugal filter (Millipore, Temecula, CA). The concentration of PEI in the retentate was quantified using a ninhydrin assay. Transferrin covalently linked to PEI was quantified using a BCA protein assay. Prior to transfection, the apo-transferrin was loaded with iron by adding two molar equivalents of ferric citrate to the PEI-transferrin conjugate and used without further purification.

Polyplex preparation and transfection

Cells were plated in 24-well tissue culture plates at 5×10^4 cells/well 24 h prior to transfection. DNA/polymer complexes were prepared at room temperature by dissolving 4 μg of DNA in approximately 100 μl of Mili-Q water and adding an equal volume of polymer solution to

achieve the desired polymer:DNA weight ratio. Complexes were then incubated at room temperature for 20 min. For transfections performed in the presence of caveolae inhibitors (50 μg/ml genistein, 10 mg/ml methyl-β-cyclodextrin) or clathrin inhibitors (5 μg/ml chlorpromazine, 1 mM amantadine), the growth medium was replaced with folate-free, serum-free medium containing the desired drug one hour prior to transfection. For transfections in the presence of 20 μM chloroquine, 10 nM bafilomycin A1 or both, the growth medium was replaced with folate-free, serum-free medium containing the desired drug immediately prior to transfection. If no drugs were present, the medium was also replaced with folate-free, serum-free medium immediately prior to transfection. At the time of transfection, 50 μl of polyplex solution was added to each well (1 μg plasmid/well). The transfection medium was replaced with serum-supplemented growth medium 2 h post-transfection. Luciferase expression was quantified 24 h post-transfection using the Promega luciferase assay system (Promega, Madison, WI). Luciferase activity was measured in relative light units (RLU) using a Lumat LB 9507 luminometer (Berthold, GmbH, Germany) and normalized to total cell protein using a BCA protein assay kit.

Down-regulation of caveolin-1 and clathrin heavy chain expression

Cells were plated in 6-well tissue culture plates at 1×10^5 cells/well 24 h prior to treatment. Twenty minutes prior to transfection, siRNA was complexed with Lipofectamine 2000 in Opti-MEM (Gibco, Rockville, MD) according to the manufacturer's protocol. Complexes were then added to cells in serum-supplemented media at a final concentration of 33 nM siRNA. The cells were washed, trypsinized and replated in 60-mm dishes 24 h post-transfection. After another 48 h, the cells were plated in 24-well tissue culture plates at 5×10^4 cells/well and allowed to grow

for 24 h before transfection and analysis as described above. Western blots of whole cell lysates were performed to verify the siRNA-mediated silencing of clathrin heavy chain (CLTC) and caveolin-1 (CAV-1) using the ProteoQuest colorimetric Western blotting kit. Caveolin-1 and clathrin heavy chain primary antibodies were used at a 1:500 dilution.

Flow cytometry

To prepare flow cytometry samples, DNA complexes were formed with either targeted or untargeted polymers as described above, save for the addition of YOYO-1 at the ratio 30 nl YOYO-1 per 1 µg of DNA (one YOYO-1 molecule per 50 DNA base pairs). Transfection was carried out as described previously. Two hours post-transfection, the cells were rinsed twice with 0.001% SDS in PBS and then PBS to remove surface-bound complexes. Next, 100 µl of 0.25% trypsin in PBS was added to each well. The cells and trypsin were allowed to incubate for five to ten minutes before 400 µl of 3.7% formaldehyde was added to each well. The cells were then collected and stored on ice. FACS analyses were performed on a BD Biosciences LSR II flow cytometer (Franklin Lakes, NJ). Data were analyzed using the FCS Express software package (De Novo Software, Los Angeles, CA).

Competitive inhibition of uptake and transfection

Unlabeled and YOYO-1 labeled DNA/polymer complexes were prepared as described previously. Thirty minutes prior to transfection, the growth medium on the HeLa cells was replaced with folate-free, serum-free medium containing the desired concentrations of either free folic acid or iron-loaded transferrin and incubated at 4 °C. At the time of transfection, 50 µl of the polyplex solution was added to each well and the cells were moved to 37 °C. The

transfection medium was replaced with serum-supplemented growth medium 30 minutes post-transfection. The cells were then subjected to either flow cytometry or allowed to grow for 24 h prior to lysis and reporter gene measurement.

Investigation of colocalization

The primary amines of unmodified or targeted PEI samples were reacted with NHS-ester functionalized Alexa Fluor 488 in 0.1 M sodium bicarbonate buffer (pH 8.3) for 1 h. Labeled PEI was purified from unreacted dye by gel filtration chromatography (PD-10, GE Healthcare, Uppsala, Sweden). Polyplexes were formed as described above using a combination of both Alexa Fluor 488-labeled and unlabeled polymers. HeLa cells, seeded at 2×10^5 cells/well and grown overnight in 6-well plates containing a coverslip in each well, were incubated at 4 °C with folate-free, serum-free medium containing fluorescent polyplexes for 30 minutes before moving the plates to 37 °C. To investigate polyplex colocalization with clathrin heavy chain (CLTC) or caveolin-1 (CAV-1), 30 min post-transfection the cells were washed with PBS, permeabilized with 0.1% Triton X-100 in PBS, stained with either mouse anti-caveolin-1 IgG_1 antibody or mouse anti-clathrin heavy chain IgG_1 antibody and fixed with 3.7% formaldehyde in PBS. The clathrin heavy chain and caveolin-1 antibodies were fluorescently labeled after addition using the Zenon Alexa Fluor 546 mouse IgG_1 labeling kit as per the manufacturer's instructions. To investigate early endosome antigen (EEA-1) and lysosomal associated membrane protein-1 (LAMP-1) colocalization, the cells were washed with PBS, permeabilized with 0.1% Triton X-100 in PBS, stained with either mouse anti-LAMP-1 IgG_1 or mouse anti-EEA-1 IgG_1 and fixed with 3.7% formaldehyde in PBS at 10 h post-transfection. The LAMP-1 and EEA-1 antibodies were fluorescently labeled after addition using the Zenon Alexa Fluor 647 mouse IgG_1 labeling

kit as per the manufacturer's instructions. Mounted cells were visualized with an Olympus Model BX60 confocal microscope equipped with a 100x oil immersion lens as well as Argon, Krypton and HeNe lasers for visualizing the Alexa Fluor 488 (λ_{ex} = 488 nm), Alexa Fluor 546 (λ_{ex} = 568 nm) and Alexa Fluor 647 (λ_{ex} = 633 nm) signals, respectively. Separate, representative images of each dye were captured and overlaid with Image J software (NIH).

DNAse/chloroquine gel electrophoresis

Plasmid DNA (pGL3) and DNAse I (Promega, Madison, WI) were mixed with various chloroquine concentrations in DNAse reaction buffer and immediately incubated at 37 °C for 10 minutes prior to DNAse deactivation. Deactivation was achieved by adding DNAse stop solution and heating at 65 °C for 10 minutes. The mixture was then subjected to agarose gel electrophoresis, stained with ethidium bromide and visualized using a Gel Doc 2000 gel documentation system (Bio-Rad, Hercules, Ca).

Ratiometric flow cytometry

Unmodified or targeted PEI samples were reacted with fluorescein isothiocyanate (FITC) and NHS-ester functionalized Alexa Fluor 633 in 0.1 M sodium bicarbonate buffer (pH 9.6) for 1 h to generate dual-labeled polymer. Labeled PEI was purified from unreacted dye by gel filtration chromatography (PD-10, GE Healthcare, Uppsala, Sweden). Targeted and untargeted complexes were formed with labeled and unlabeled polymers as described previously. HeLa cells, seeded in 24-well plates at 5×10^4 cells/well and grown overnight, were transfected with fluorescent complexes for one hour in folate-free, serum-free media before the cells were washed with 0.001% SDS in PBS and PBS and the serum-supplemented growth medium was replaced.

Transfections were staggered from 2 to 20 hours before FACS analysis to allow the cells to be harvested and analyzed simultaneously. pH standards were created in parallel with the samples by incubating cells transfected with the fluorescent polyplexes in a buffer consisting of 150 mM NaCl and either 50 mM sodium phosphate (adjusted to pH 7.6, pH 7.0 or pH 6.5), 50 mM MES (adjusted to pH 6.0 or pH 5.5) or 50 mM sodium acetate (adjusted to pH 5.0 or pH 4.5). The sodium ionophore monensin was added to the standard cells at a final concentration of 10 μM prior to analysis to equilibrate the intra- and extracellular pH.

ACKNOWLEDGEMENTS.

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REFERENCES

1. Gallo-Penn AM *et al*. Systemic delivery of an adenoviral vector encoding canine factor VIII results in short-term phenotypic correction, inhibitor development, and biphasic liver toxicity in hemophilia A dogs. *Blood 2001;* **97**: 107-113.

2. Klink D *et al*. Gene delivery systems—gene therapy vectors for cystic fibrosis. *J Cyst Fibros 2004;* **3**: 203-212.

3. Bonadio J, Smiley E, Patil P, Goldstein S. Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. *Nat Med 1999;* **5**: 753-759.

4. Kopatz I, Remy JS, Behr JP. A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin. *J Gene Med 2004;* **6**: 769-776.

5. Benns JM, Maheshwari A, Furgeson DY, Mahato RI, Kim SW. Folate-PEG-folate-graft-polyethylenimine-based gene delivery. *J Drug Target 2001;* **9**: 123-139.

6. Diebold SS, Plank C, Cotten M, Wagner E, Zenke M. Mannose receptor-mediated gene delivery into antigen presenting dendritic cells. *Somat Cell Mol Genet 2002;* **27**: 65-74.

7. Hart SL *et al*. Lipid-mediated enhancement of transfection by a nonviral integrin-targeting vector. *Hum Gene Ther 1998;* **9**: 575-585.

8. Kircheis R *et al*. Polyethylenimine/DNA complexes shielded by transferrin target gene expression to tumors after systemic application. *Gene Ther 2001;* **8**: 28-40.

9. Paulos CM, Reddy JA, Leamon CP, Turk MJ, Low PS. Ligand binding and kinetics of folate receptor recycling in vivo: impact on receptor-mediated drug delivery. *Mol Pharmacol 2004;* **66**: 1406-1414.

10. Bleil JD, Bretscher MS. Transferrin receptor and its recycling in HeLa cells. *EMBO J 1982;* **1**: 351-355.

11. Jhaveri MS, Rait AS, Chung KN, Trepel JB, Chang EH. Antisense oligonucleotides targeted to the human alpha folate receptor inhibit breast cancer cell growth and sensitize the cells to doxorubicin treatment. *Mol Canc Therapeut 2004;* **3**: 1505-1512.

12. Dautry-Varsat A, Ciechanover A, Lodish HF. pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc Natl Acad Sci Unit States Am 1983;* **80**: 2258-2262.

13. Pelkmans L, Helenius A. Endocytosis via caveolae. *Traffic 2002;* **3**: 311-320.

14. Parton RG. Caveolae meet endosomes: a stable relationship? *Dev Cell 2004;* **7**: 458-460.

15. Parton RG, Simons, K. The multiple faces of caveolae. *Nat Rev Mol Cell Biol 2007;* **8**: 185-194.

16. Le PU, Nabi IR. Distinct caveolae-mediated endocytic pathways target the Golgi apparatus and the endoplasmic reticulum. *J Cell Sci 2003;* **116**: 10591071.

17. Pelkmans L, Kartenbeck J, Helenius A. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat Cell Biol 2001;* **3**: 473-483.

18. Ashok A, Atwood WJ. Contrasting roles of endosomal pH and the cytoskeleton in infection of human glial cells by JC virus and simian virus 40. *J Virol 2003;* **77**: 1347-1356.

19. Behr JP. The proton sponge: a trick to enter cells the viruses did not exploit. *Chimia 1997;* **51**: 34-36.

20. Forrest ML, Pack DW. On the kinetics of polyplex endocytic trafficking: implications for gene delivery vector design. *Mol Ther 2002;* **6**: 57-66.

21. Godbey WT, Wu KK, Mikos AG. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proc Natl Acad Sci Unit States Am 1999;* **96**: 5177-5182.

22. Godbey WT, Barry MA, Saggau P, Wu KK, Mikos AG. Poly(ethylenimine)-mediated transfection: a new paradigm for gene delivery. *J Biomed Mater Res 2000;* **51**: 321-328.

23. Sonawane ND, Szoka FC, Verkman AS. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *J Biol Chem 2003;* **278**: 44826-44831.

24. Gabrielson NP, Pack DW. Acetylation of polyethylenimine enhances gene delivery via weakened polymer/DNA interactions. *Biomacromolecules 2006;* **7**: 2427-2435.

25. Forrest ML, Meister GE, Koerber JT, Pack DW. Partial acetylation of polyethylenimine enhances in vitro gene delivery. *Pharmaceut Res 2004*; **21**: 365-371.

26. Peters PJ *et al*. Trafficking of prion proteins through a caveolae-mediated endosomal pathway. *J Cell Biol 2003;* **162**: 703-717.

27. Rejman J, Bragonzi A, Conese M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. *Mol Ther 2005;* **12**: 468-474.

28. Hanover JA, Willingham MC, Pastan I. Kinetics of transit of transferrin and epidermal growth factor through clathrin-coated membranes. *Cell 1984;* **39**: 283-293.

29. Ciechanover A, Schwartz AL, Dautry-Varsat A, Lodish HF. Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line. Effect of lysosomotropic agents. *J Biol Chem 1983;* **258**: 9681-9689.

30. Subtil A, Hémar A, Dautry-Varsat A. Rapid endocytosis of interleukin 2 receptors when clathrin-coated pit endocytosis is inhibited. *J Cell Sci 1994;* **107**: 3461-3468.

31. Perry DG, Daugherty GL, Martin WJ. Clathrin-coated pit-associated proteins are required for alveolar macrophage phagocytosis. *J Immunol 1999;* **162**: 380-386.

32. Shogomori H, Futerman AH. Cholesterol depletion by methyl-beta-cyclodextrin blocks cholera toxin transport from endosomes to the Golgi apparatus in hippocampal neurons. *J Neurochem 2001;* **78**: 991-999.

33. Aoki T, Nomura R, Fujimoto T. Tyrosine phosphorylation of caveolin-1 in the endothelium. *Exp Cell Res 1999;* **253**: 629-636.

34. Erbacher P, Roche AC, Monsigny M, Midoux P. Putative role of chloroquine in gene transfer into a human hepatoma cell line by DNA/lactosylated polylysine complexes. *Exp Cell Res 1996;* **225**: 186-194.

35. Allison JL, O'Brien RL, Hahn FE. DNA: reaction with chloroquine. *Science 1965;* **149**: 1111-1113.

36. Cheng J *et al*. Structure-function correlation of chloroquine and analogues as transgene expression enhancers in nonviral gene delivery. *J Med Chem 2006;* **49**: 6522-6531.

37. Palokangas H, Ying M, Väänänen K, Saraste J. Retrograde transport from the pre-Golgi intermediate compartment and the Golgi complex is affected by the vacuolar H ATPase inhibitor bafilomycin A1. *Mol Biol Cell 1998;* **9**: 3561-3578.

38. Mineo C, Anderson RGW. A vacuolar-type proton ATPase mediates acidification of plasmalemmal vesicles during potocytosis. *Exp Cell Res 1996;* **224**: 237-242.

39. van der Aa, MAEM *et al*. Cellular uptake of cationic polymer-DNA complexes via caveolae plays a pivotal role in gene transfection in COS-7 cells. *Pharmaceut Res 2007;* **24**: 1590-1598.

40. von Gersdorff K, Sanders NN, Vandenbroucke R, De Smedt SC, Wagner E, Manfred O. The internalization route resulting in successful gene expression depends on both cell line and polyethylenimine polyplex type. *Mol Ther 2006;* **14**: 745-753.

41. Kichler A, Leborgne C, Coeytaux E, Danos O. Polyethylenimine-mediated gene delivery: a mechanistic study. *J Gene Med 2001;* **3**: 135-144.

42. Pelkmans L, Bürli T, Zerial M, Helenius A. Caveolin-stabilized membrane Domains as multifunctional transport and sorting devices in endocytic membrane traffic. *Cell 2004;* **118**: 767-780.

43. Guo W, Lee RJ. Receptor-targeted gene delivery via folate-conjugated polyethylenimine. *AAPS PharmSci 1999;* **1**: 1-7.

TITLES AND LEGENDS TO FIGURES

Figure 1. (a) In vitro transfection of HeLa cells with PEI or PEI-Fol at indicated polymer:DNA weight ratios. Luciferase activity in the cell lysates is reported as relative light units (RLU) normalized by the mass of total protein in the lysate. (b) Uptake of YOYO-1 labeled pGL3 and PEI or PEI-Fol(1.1) polyplexes (polymer:DNA weight ratio of 0.5:1) in HeLa cells. (c) In vitro transfection of HeLa cells with PEI or PEI-Fol(1.1) polyplexes (polymer:DNA weight ratio of 0.5:1) in the presence of free folic acid. Luciferase activity in the cell lysates is reported as relative light units (RLU) normalized by the mass of total protein in the lysate. (d) Uptake of YOYO-1 labeled pGL3 and PEI or PEI-Fol(1.1) polyplexes (polymer:DNA weight ratio of 0.5:1) in the presence of free folic acid. Fluorescence values were normalized to the median fluorescence of cells grown in the absence of free folate. (N = 3; error bars represent standard deviation; $*$, †, ‡ = $p < 0.05$, $**$ = $p < 0.1$).

Figure 2. (a) In vitro transfection of HeLa cells with PEI or PEI-Tf at a total polymer:DNA weight ratio of 0.5:1. The percent composition of PEI-Tf in the polyplexes was varied between 10% and 100%. Luciferase activity in the cell lysates is reported as relative light units (RLU) normalized by the mass of total protein in the lysate. (b) Uptake of YOYO-1 labeled pGL3 and PEI or PEI-Tf(25%) polyplexes (polymer:DNA weight ratio of 0.5:1) in HeLa cells. (c) In vitro transfection of HeLa cells with PEI or PEI-Tf(25%) polyplexes (polymer:DNA weight ratio of 0.5:1) in the presence of free transferrin. Luciferase activity in the cell lysates is reported as relative light units (RLU) normalized by the mass of total protein in the lysate. (d) Uptake of YOYO-1 labeled pGL3 and PEI or PEI-Tf(25%) polyplexes (polymer:DNA weight ratio of

0.5:1) in the presence of free transferrin. Fluorescence values were normalized to the median fluorescence of cells grown in the absence of free transferrin. (N = 3; error bars represent standard deviation; ∗, †, ‡ = $p < 0.05$, ∗∗ = $p < 0.1$, ∗∗∗ = $p < 0.15$).

Figure 3. Confocal fluorescence micrographs of HeLa cells transfected with PEI, PEI-Fol(1.1) or PEI-Tf(25%) polyplexes (green) and immunostained for caveolin-1 or clathrin heavy chain (red) at 30 min post-transfection. Complexes were formed at a total polymer:DNA ratio of 0.5:1.

Figure 4. (a) Normalized median fluorescence of HeLa cells transfected with YOYO-1 labeled pGL3 and PEI, PEI-Fol(1.1), or PEI-Tf(25%) polyplexes (polymer:DNA weight ratio of 0.5:1) in the presence of caveolae (genistein, 50 μg/ml; methyl-β-cyclodextrin, 10 mg/ml) and clathrin (chlorpromazine, 5 μg/ml; amantadine-1 mM) inhibitors. Fluorescence values were normalized to the median fluorescence of cells grown in the absence of drugs. (b) Normalized in vitro transfection efficiency of PEI, PEI-Fol(1.1) or PEI-Tf(25%) polyplexes (polymer:DNA weight ratio of 0.5:1) in HeLa cells in the presence of caveolae (genistein, 50 μg/ml; methyl-β-cyclodextrin, 10 mg/ml) and clathrin (chlorpromazine, 5 μg/ml; amantadine, 1 mM) inhibitors. Luciferase activity in the cell lysates is reported as relative light units (RLU) normalized by the mass of total protein in the lysate. Luciferase activity of drug-treated cells was normalized to cells grown in the absence of any drugs. (N = 3; error bars represent standard deviation; ∗, †, ‡ = $p < 0.05$ compared to the same polyplexes in the absence of drug).

Figure 5. (a) Western blot of caveolin-1 and clathrin heavy chain expression in whole HeLa cell lysates following transfection with siLAM, siCAV-1, or siCLTC. (b) Normalized in vitro

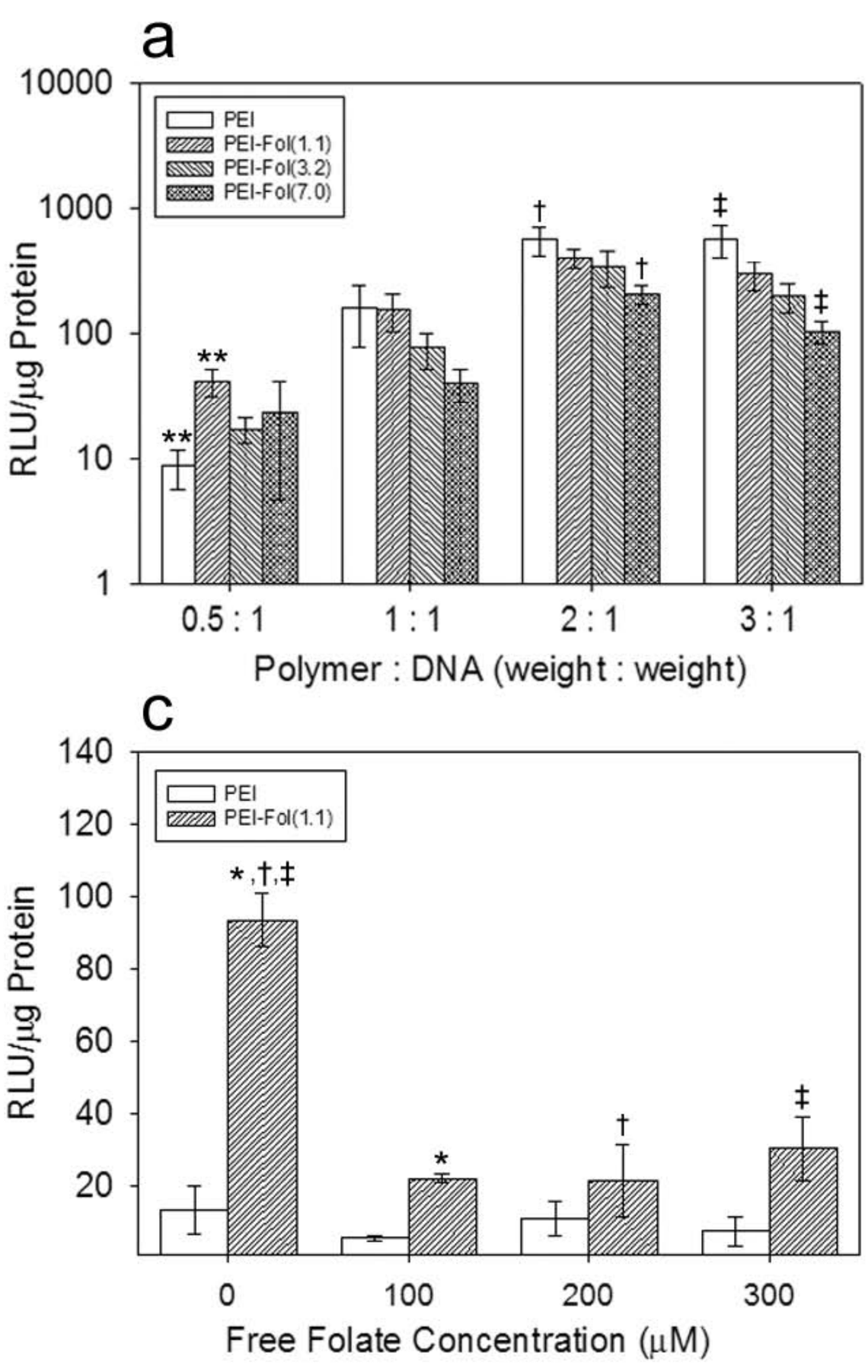
transfection efficiency of PEI, PEI-Fol(1.1) or PEI-Tf(25%) polyplexes (polymer:DNA weight ratio of 0.5:1) in HeLa cells treated with siRNAs. Luciferase activity in the cell lysates is reported as relative light units (RLU) normalized by the mass of total protein in the lysate. Luciferase activity of siRNA-treated cells was normalized to cells treated with siLAM. (N = 6; error bars represent standard deviation; ∗, †, ‡ = $p < 0.05$).

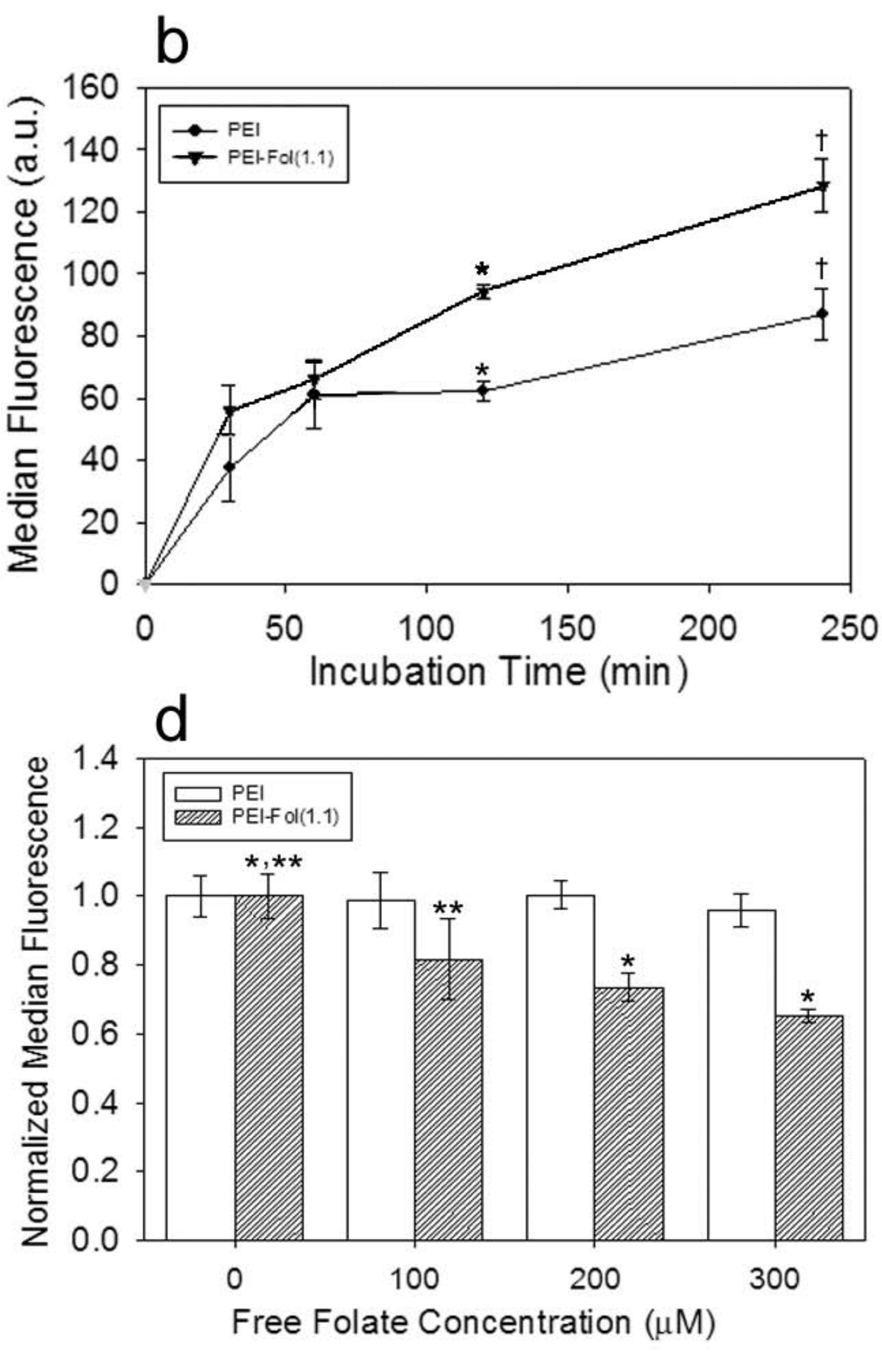
Figure 6. (a) Gel electrophoresis of pGL3 incubated with DNAse I in the presence of various chloroquine concentrations at 37 °C for 10 min. (b) Normalized in vitro transfection efficiency of HeLa cells with PEI, PEI-Fol(1.1) or PEI-Tf(25%) polyplexes (polymer:DNA weight ratio of 0.5:1) in the absence and presence of 20 μM chloroquine, 10 nM bafilomycin A1 or both. Luciferase activity in the cell lysates is reported as relative light units (RLU) normalized by the mass of total protein in the lysate. Luciferase activity of drug-treated cells was normalized to cells grown in the absence of any drug. (N = 3; error bars represent standard deviation; ∗, ∗∗, †, ‡ = $p < 0.05$, ††, ‡‡ = $p < 0.1$ compared to the same polyplexes in the absence of drug).

Figure 7. Local pH measurement of HeLa cells transfected with PEI, PEI-Fol(1.1) or PEI-Tf(25%) polyplexes (polymer:DNA weight ratio of 0.5:1) at 2, 4, 8, 12 and 24 h post-transfection. (N = 3; error bars represent standard deviation; ∗, † = $p < 0.05$, ‡‡ = $p < 0.1$ compared to the other polyplexes at the same time).

Figure 8. Confocal fluorescence micrographs of HeLa cells transfected with PEI, PEI-Fol(1.1) or PEI-Tf(25%) polyplexes (green) and immunostained for early endosome marker EEA-1 or

lysosome marker LAMP-1 (red) at 10 h post-transfection. Complexes were formed at a total polymer:DNA ratio of 0.5:1.


a
PEI
PEI-Fol(1.1)
PEI-Fol(3.2)
PEI-Fol(7.0)
RLU/μg Protein
10000
1000
100
10
1
0.5 : 1
1 : 1
2 : 1
3 : 1
Polymer : DNA (weight : weight)


b
PEI
PEI-Fol(1.1)
Median Fluorescence (a.u.)
160
140
120
100
80
60
40
20
0
0
50
100
150
200
250
Incubation Time (min)

c
PEI
PEI-Fol(1.1)
RLU/μg Protein
140
120
100
80
60
40
20
0
100
200
300
Free Folate Concentration (μM)

d
PEI
PEI-Fol(1.1)
Normalized Median Fluorescence
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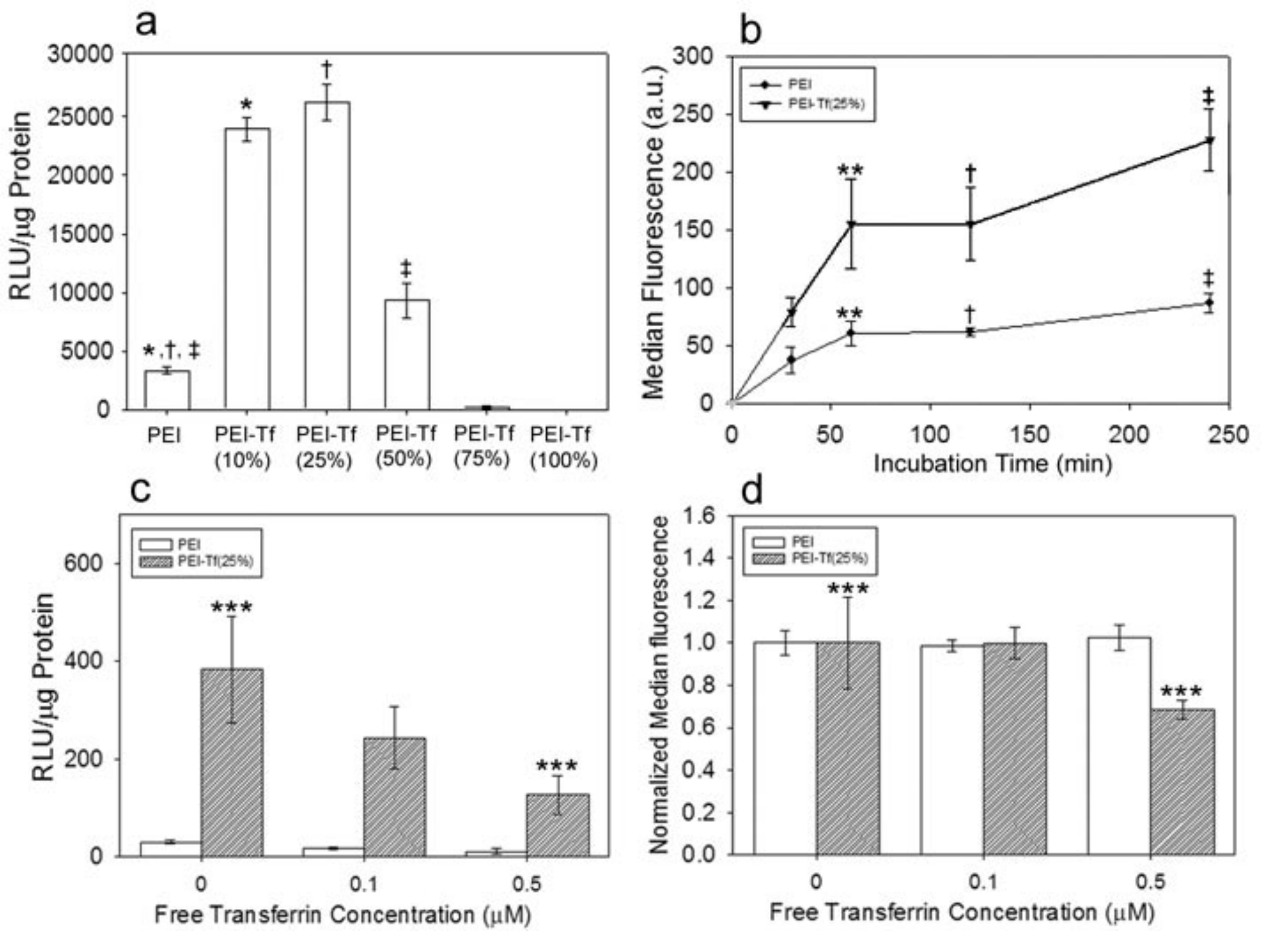
0.6
0.4
0.2
0.0
0
100
200
300
Free Folate Concentration (μM)


a
RLU/μg Protein
30000
25000
20000
15000
10000
5000
0
*,†, ‡
*
†
‡
PEI
PEI-Tf (10%)
PEI-Tf (25%)
PEI-Tf (50%)
PEI-Tf (75%)
PEI-Tf (100%)
b
Median Fluorescence (a.u.)
300
250
200
150
100
50
0
PEI
PEI-Tf(25%)
**
**
†
†
‡
‡
0
50
100
150
200
250
Incubation Time (min)
c
RLU/μg Protein
600
400
200
0
PEI
PEI-Tf(25%)
***
***
0
0.1
0.5
Free Transferrin Concentration (μM)
d
Normalized Median fluorescence
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PEI
PEI-Tf(25%)
***
***
0
0.1
0.5
Free Transferrin Concentration (μM)

Caveolin-1

Colocalization

Bright Field

Clathrin Heavy Chain

Colocalization

Bright Field

PEI

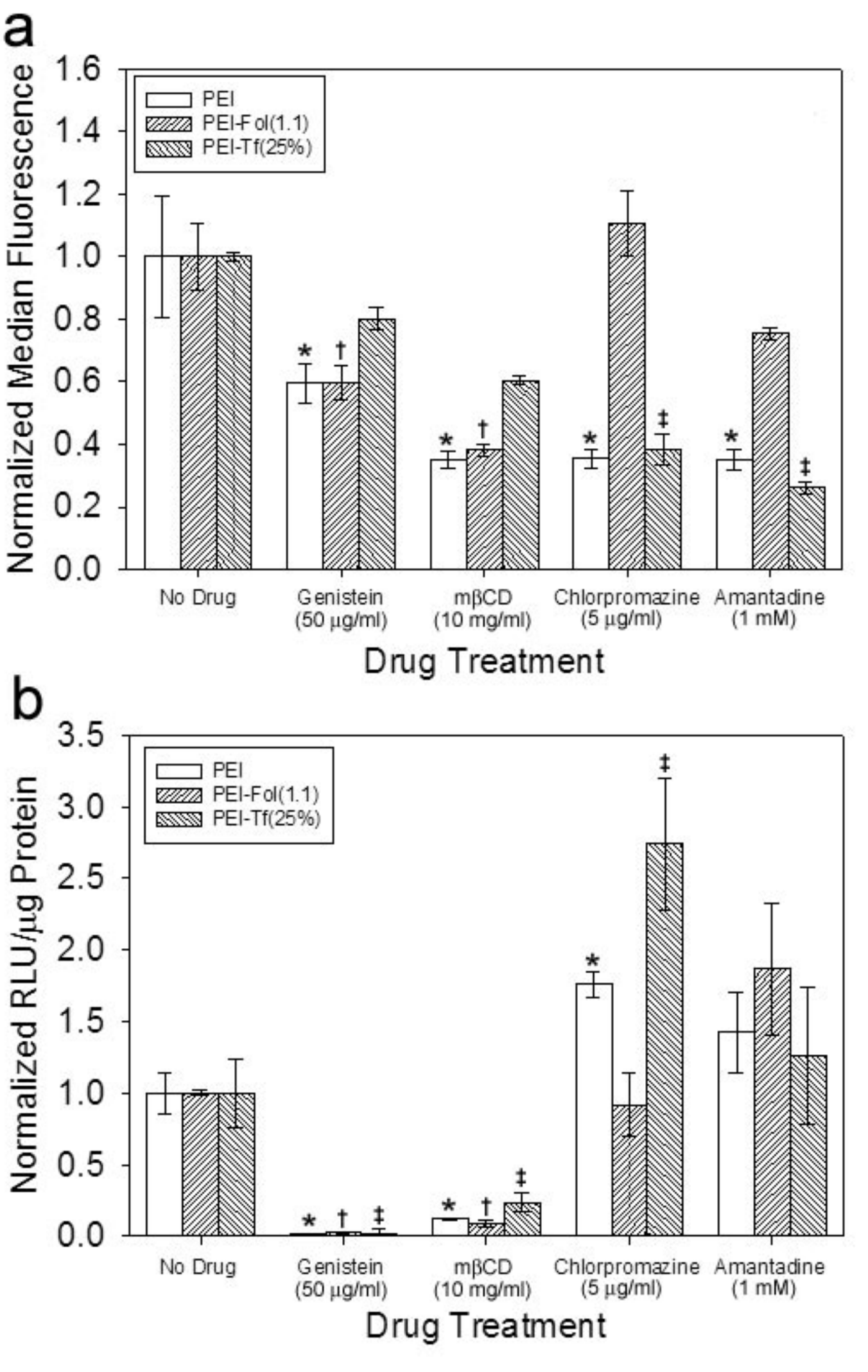
PEI-Fol(1.1)

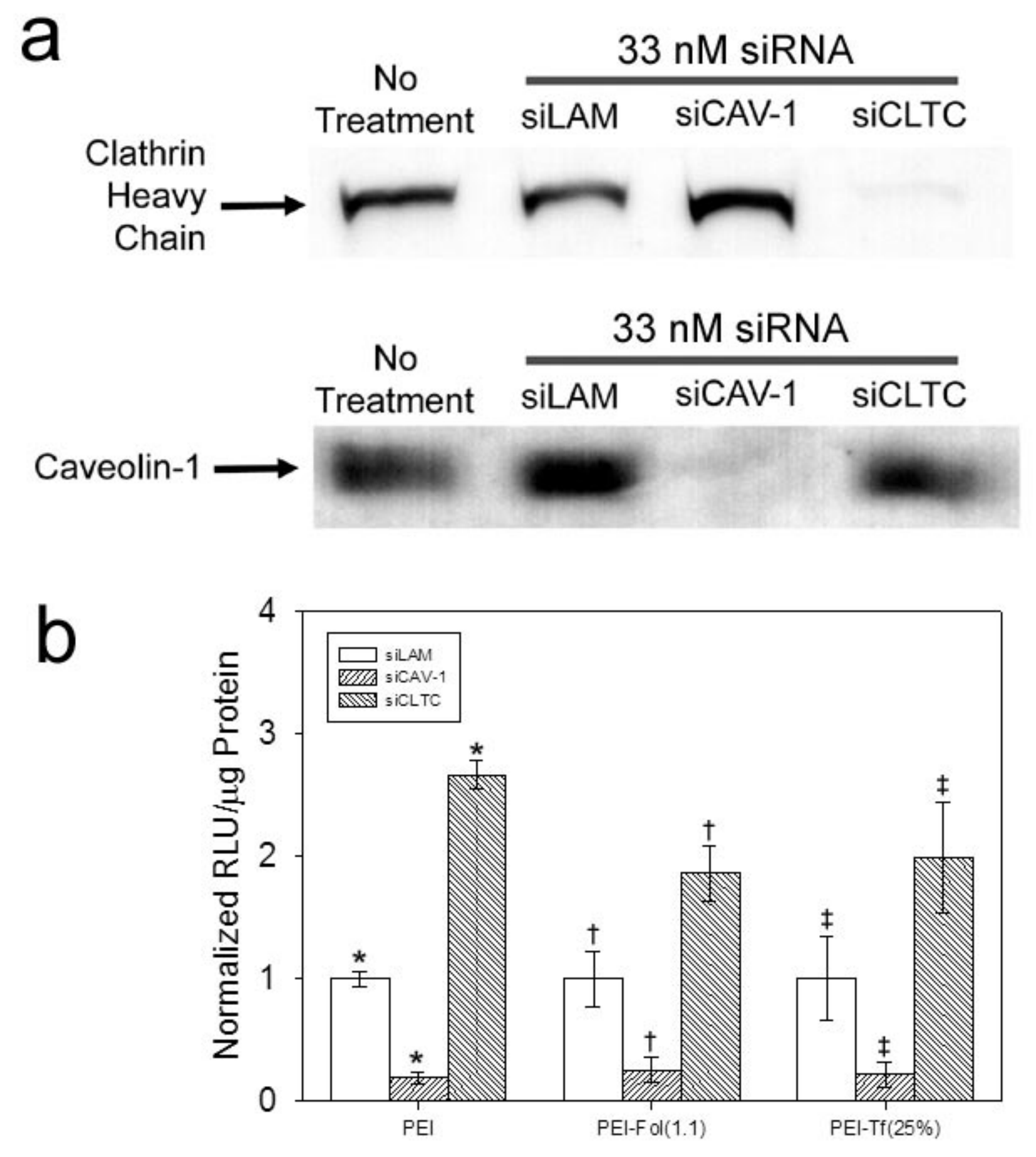
PEI-Tf(25%)

PEI

PEI-Fol(1.1)

PEI-Tf(25%)


a
Normalized Median Fluorescence
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PEI
PEI-Fol(1.1)
PEI-Tf(25%)
*
†
‡
No Drug
Genistein (50 μg/ml)
mβCD (10 mg/ml)
Chlorpromazine (5 μg/ml)
Amantadine (1 mM)
Drug Treatment
b
Normalized RLU/μg Protein
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
PEI
PEI-Fol(1.1)
PEI-Tf(25%)
*
†
‡
No Drug
Genistein (50 μg/ml)
mβCD (10 mg/ml)
Chlorpromazine (5 μg/ml)
Amantadine (1 mM)
Drug Treatment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33 nM siRNA
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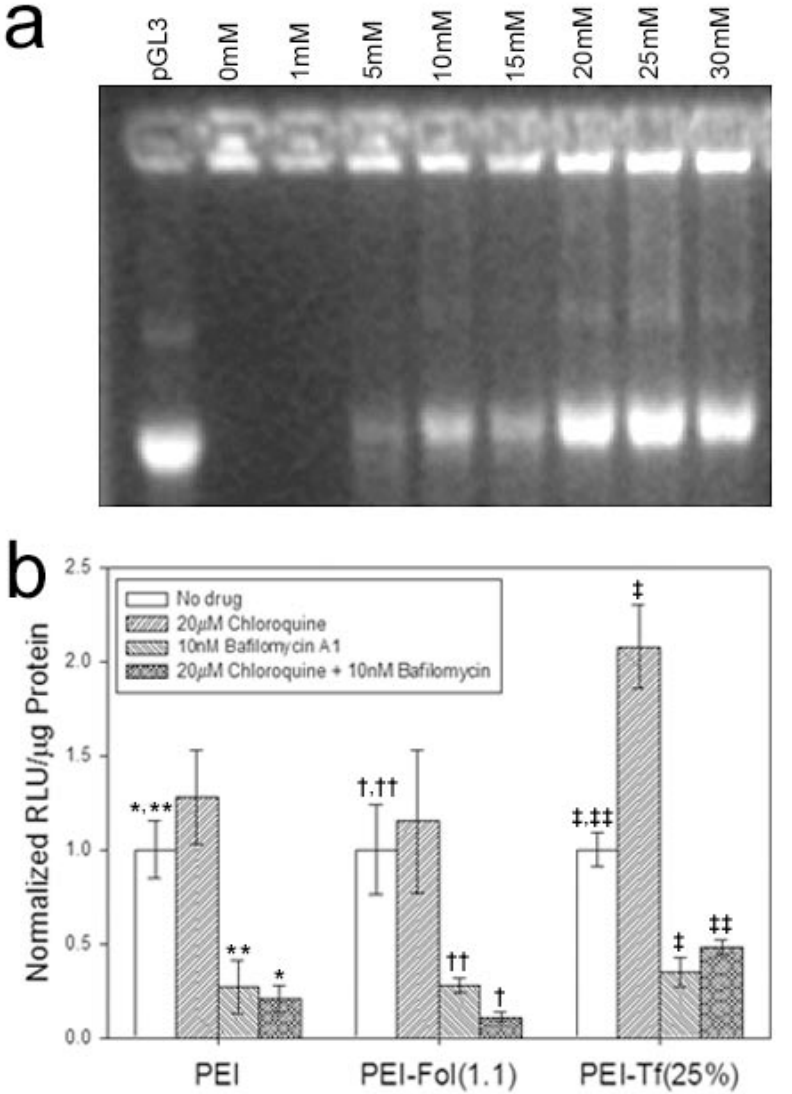
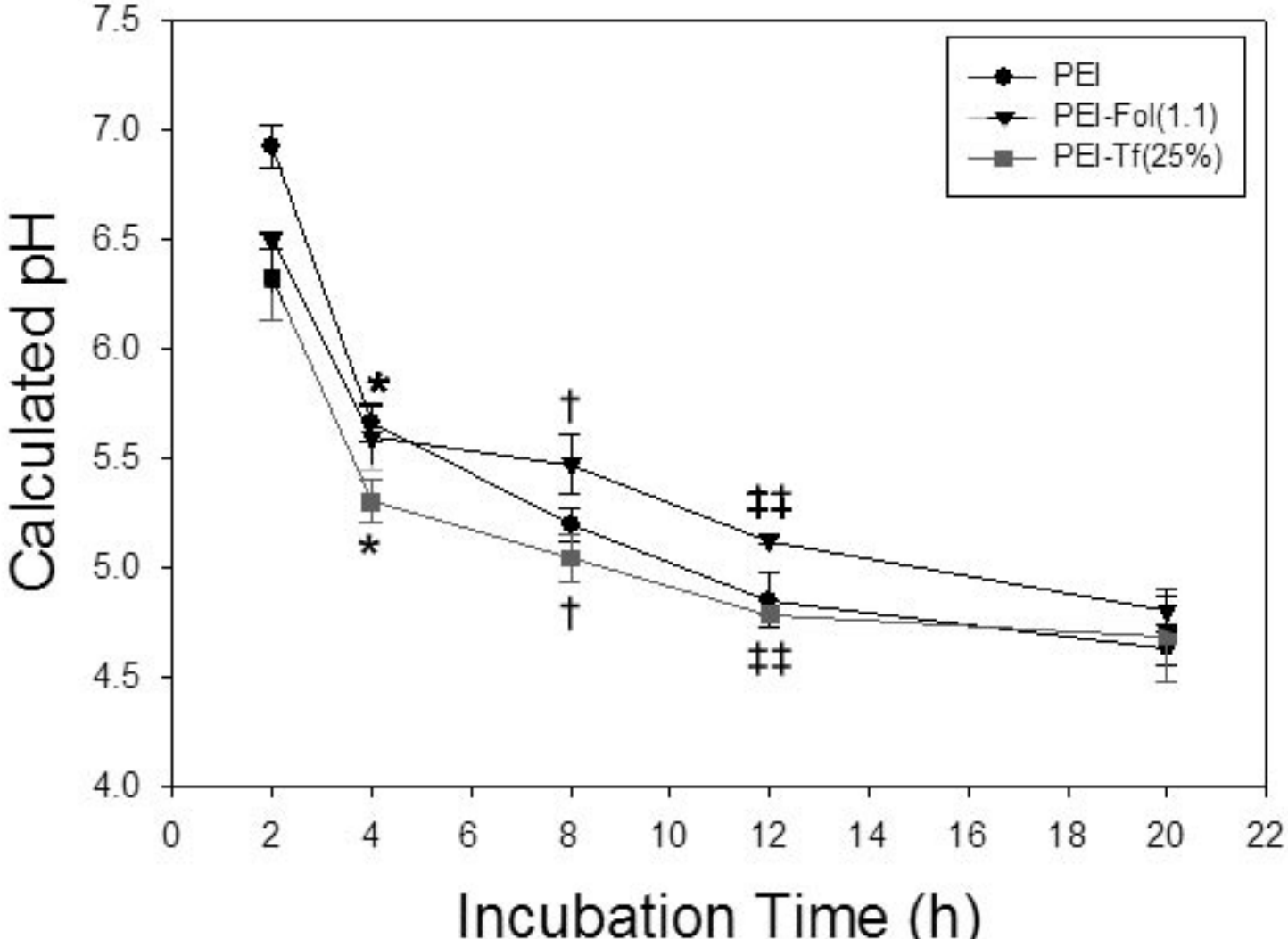
siLAM
siCAV-1
siCLTC
Clathrin Heavy Chain
33 nM siRNA
No Treatment
siLAM
siCAV-1
siCLTC
Caveolin-1
b
Normalized RLU/μg Protein
siLAM
siCAV-1
siCLTC
PEI
PEI-Fol(1.1)
PEI-Tf(25%)


a
pGL3
0mM
1mM
5mM
10mM
15mM
20mM
25mM
30mM
b
Normalized RLU/μg Protein
No drug
20μM Chloroquine
10nM Bafilomycin A1
20μM Chloroquine + 10nM Bafilomycin
PEI
PEI-Fol(1.1)
PEI-Tf(25%)


PEI
PEI-Fol(1.1)
PEI-Tf(25%)
Calculated pH
Incubation Time (h)
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
0
2
4
6
8
10
12
14
16
18
20
22
*
*
†
†
‡‡
‡‡

LAMP-1

Colocalization

Bright Field

EEA-1

Colocalization

Bright Field

PEI

PEI-Fol(1.1)

PEI-Tf(25%)

PEI

PEI-Fol(1.1)

PEI-Tf(25%)