



Exogenous chondroitin sulfate glycosaminoglycan associate with arginine-rich peptide–DNA complexes to alter their intracellular processing and gene delivery efficiency

Rangeetha J. Naik, Rajpal Sharma, Daniel Nisakar, Gunjan Purohit, Munia Ganguli *

CSIR-Institute of Genomics and Integrative Biology, Mathura Road, Delhi 110020, India

ARTICLE INFO

Article history:

Received 13 September 2014
Received in revised form 7 January 2015
Accepted 20 January 2015
Available online 28 January 2015

Keywords:

Arginine-rich peptide
Gene delivery
Glycosaminoglycan
Trafficking

ABSTRACT

Arginine-rich peptides have been used extensively as efficient cellular transporters. However, gene delivery with such peptides requires development of strategies to improve their efficiency. We had earlier demonstrated that addition of small amounts of exogenous glycosaminoglycans (GAGs) like heparan sulfate or chondroitin sulfate to different arginine-rich peptide–DNA complexes (polyplexes) led to an increase in their gene delivery efficiency. This was possibly due to the formation of a ‘GAG coat’ on the polyplex surface through electrostatic interactions which improved their extracellular stability and subsequent cellular entry. In this report, we have attempted to elucidate the differences in intracellular processing of the chondroitin sulfate (CS)-coated polyplexes in comparison to the native polyplexes by using a combination of endocytic inhibitors and co-localization with endosomal markers in various cell lines. We observed that both the native and CS-coated polyplexes are internalized by multiple endocytic pathways although in some cell lines, the coated polyplexes are taken up primarily by caveolae mediated endocytosis. In addition, the CS-coat improves the endosomal escape of the polyplexes as compared to the native polyplexes. Interestingly, during these intracellular events, exogenous CS is retained with the polyplexes until their accumulation near the nucleus. Thus we show for the first time that exogenous GAGs in small amounts improve intracellular routing and nuclear accumulation of arginine-based polyplexes. Therefore, addition of exogenous GAGs is a promising strategy to enhance the transfection efficiency of cationic arginine-rich peptides in multiple cell types.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Arginine-rich peptides constitute one of the most widely explored classes of cell penetrating peptides. They have been used in delivery of multiple types of therapeutic cargo including nucleic acids, proteins, liposomes and nanoparticles [1–3]. Arginine-rich peptides form complexes with plasmid DNA through electrostatic interactions and these nano-sized polyplexes have been used for gene delivery to different cell types in vitro and in vivo [4–8]. However, the mechanism of internalization of these peptides is not conclusively understood, especially when they are complexed to cargos like nucleic acids. This impedes the development of these peptides as effective carriers of nucleic acid therapeutics. Negatively charged cell surface molecules, particularly the proteoglycans, have been implicated in the literature as possible partners in the entry process of cationic cell penetrating peptides [9–11]. However, their role in the entry (and the subsequent intracellular events) of arginine-rich peptides complexed with cargos

is ambiguous. Different factors like peptide length, sequence, arginine content and nature of cargo seem to be important in this process. In a previous report, we had demonstrated that 16-mer homopeptide of arginine (R_{16}) enters cells independent of the cell surface proteoglycans [12]. This was true for both the free peptide as well as the peptide–DNA complex (polyplex). Other recent reports have also made similar observations for arginine-rich peptides [13–15] and it is now believed that the cell surface proteoglycans, in spite of their negative charge density, may be dispensable for the cellular uptake of these peptides or their complexes with cargo. It is also possible that the arginine-rich peptides interact with the negatively charged membrane lipids such as phospholipids for initiating their entry into the cells [16–18].

We had also reported that addition of small amounts of soluble glycosaminoglycans (GAGs) like heparan sulfate and chondroitin sulfate to arginine polyplexes enhanced their transfection efficiency [12]. We demonstrated that the exogenous GAGs used in small amounts could improve the stability of arginine polyplexes possibly through formation of a “GAG coat” and provide better protection of the compacted DNA from nucleases. This resulted in higher uptake in both the wild-type and cell surface GAG-deficient Chinese hamster ovary (CHO) cells and enhanced transfection efficiency as compared to the native polyplexes. This was an unusual observation since addition of

* Corresponding author at: CSIR-Institute of Genomics and Integrative Biology, Mathura Road (near Sukhdev Vihar), Delhi 110020, India. Tel.: +91 11 29879 225; fax: +91 11 27667471.

E-mail addresses: mganguli@igib.res.in, mganguli@igib.in (M. Ganguli).

exogenous GAGs, particularly at high concentrations, had earlier been reported to inhibit the cell entry and cargo delivery of peptides, polymers and liposomes [19–22]. However, it was not clear whether in addition to the enhanced stability there were differences in the intracellular processing between the native polyplexes and those coated with GAGs and whether such differences, if any, had a role to play in the increase in transfection efficiency.

In this manuscript, we report our observations on the intracellular processing of the polyplexes of two different arginine-rich peptides in the presence and absence of exogenous chondroitin sulfate (CS) with an aim to delineate the role of the added GAGs in the enhancement of transfection efficiency. We chose the 16-mer arginine homopeptide (R_{16}), and an arginine-rich peptide (32 AR; sequence (ARRRAARA)₄) which contains a heparan sulfate-binding motif, where the arginine residues are clustered with each cluster separated by neutral alanine residues [23]. We also used different CHO cell lines with varying density of cell surface proteoglycans to understand whether exogenous GAGs play a universal role in enhancing transfection efficiency of polyplexes made with arginine-rich peptides. We observed that irrespective of the GAG density on the cell surface or the nature of the arginine-based peptide used, a small amount of exogenously added CS always increases the cellular uptake and transfection efficiencies of the polyplexes. Further, we found differences in the uptake pathways between the native polyplexes and the CS-coated polyplexes which could be playing a role in the enhanced transfection efficiencies of the latter. The most interesting conclusion in this study pertains to our observation that CS GAGs remain associated with the polyplexes during intracellular processing, improve their endosomal escape and aid in better accumulation of the polyplexes at the nuclear periphery as compared to the native polyplexes. Thus, we show for the first time that exogenous GAGs (CS in particular) in small amounts confer not only extracellular stability but also allow improved intracellular routing and localization of arginine-based polyplexes. Such insights would not only shed light on the role of exogenous GAGs in gene delivery by arginine-based peptides but will also help in development of an effective strategy for efficient delivery of therapeutic nucleic acids in a wide range of cell types.

2. Experimental section

2.1. Materials

Peptides used in this study were custom synthesized by GL Biochem (Shanghai) Ltd (>95% purity). pMIR-REPORT™ Luciferase (Ambion) was used for the transfection experiments and pEGFP-C1 (Clontech) labeled with fluorescein or tetramethyl rhodamine using Label IT® Tracker kit (Mirus Bio Corporation, U.S.A.) was used for the flow cytometry and microscopy studies. Both plasmids were amplified in *Escherichia coli* DH5- α and purified using GenElute HP Endotoxin-Free Plasmid MaxiPrep Kit (Sigma).

2.2. Cell culture

Parental Chinese Hamster Ovary cells (CHO-K1) and the glycosaminoglycan mutant cell lines pgsA-745 and pgsD-677 were maintained in Ham's F12K medium (Sigma). All media were supplemented with 10% (v/v) fetal bovine serum (Life Technologies, U.S.A.) and cells were kept in a humidified 5% CO₂, 37 °C incubator.

2.3. Transfection and luciferase gene expression assay

Cells were seeded in 24-well plates to achieve a confluency of 70% after 24 h. Peptide–plasmid DNA complexes (polyplexes) were prepared at Z(+/–) of 10 with final DNA concentration of 20 ng/ μ l and incubated for 1 h at room temperature. Z(+/–) refers to charge ratio or positive charge of peptide side chain per negative charge of DNA phosphate. Indicated amounts of chondroitin sulfate A (Sigma)

were added to the polyplexes after 30 min of incubation where required. 100 μ l of polyplex (2 μ g DNA/well) was added to cells in serum free media (OptiMEM, Invitrogen). After 5 h of incubation at 37 °C, cells were replenished with 500 μ l complete growth medium. After 24 h of transfection, luciferase expression was measured as previously described [12].

For evaluating the role of endocytic pathway in the transfection efficiency, cells were pre-treated with the chemical inhibitors for 1 h: chlorpromazine (5 μ g/ml), genistein (200 μ M), dimethylamiloride (200 μ M), cytochalasin D (20 μ M) or nocodazole (20 μ M) in Opti-MEM. Cells were then incubated with the polyplexes along with the inhibitors for 5 h at 37 °C and luciferase expression was assessed after 24 h as mentioned above.

To assess the endosomal escape of the polyplexes, either chloroquine (100 μ M) or bafilomycin A1 (100 nM) was added during the incubation of cells with the polyplexes. In case of the latter, cells were treated with bafilomycin A1 (100 nM) in Opti-MEM for 30 min prior to polyplex addition.

2.4. Flow cytometry

Cells were treated with the polyplexes as described in the transfection protocol except that the plasmid was labeled with fluorescein. After 4 h of incubation with the polyplexes at 37 °C, cells were washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4) containing 1 mg/ml heparin to remove the extracellular bound polyplex, and with 0.4% trypan blue in PBS to quench any extracellular fluorescence. Cells were collected by trypsinization and resuspended in PBS and placed on ice. Flow cytometry measurements were carried out on Guava® EasyCyte™ System (Guava Technologies) using CytoSoft™ software. 10,000 live cells were used for each analysis.

For evaluating the endocytic pathway, cells were pre-treated with the chemical inhibitors for 1 h as mentioned under transfection assay. Cells were then incubated with the polyplexes along with the inhibitors for 4 h at 37 °C and processed as mentioned above.

2.5. Microscopy

Cells were seeded in 35-mm μ -dishes (ibidi, Germany) and incubated for 24 h to achieve 70–75% confluency. Polyplexes at Z(+/–) 10.0 containing rhodamine labeled DNA (2 μ g per dish) were added to the cells in serum-free media (Opti-MEM) and incubated at 37 °C for 4 h. Chondroitin sulfate, either unlabeled (Sigma) or fluorescein labeled (Cosmo Bio Co., Ltd., Japan), at the indicated concentrations, was added to polyplexes when required. Hoechst 33342 (0.3 μ g/ml, Invitrogen) was added during the final 30 min of incubation to stain the cell nucleus. Cells were washed three times with ice-cold PBS(+) containing heparin (1 mg/ml) and kept in Opti-MEM. Imaging was done using a Nikon TiE motorized inverted fluorescence microscope equipped with a 60 \times , 1.4 N.A. plan apochromat oil immersion objective. Filter sets corresponding to DAPI (Ex330–380 nm, Em435–485 nm), FITC (Ex465–495 nm, Em515–555 nm) and TRITC (Ex540/25 nm, Em605/55 nm) were used. NIS viewer element (NIS-AR) software was used for analysis of images. For co-localization with BODIPY FL C₅-lactosylceramide (Invitrogen), polyplexes were prepared as above and incubated with the cells for either 20 min or 1 h at 37 °C. Lactosylceramide (0.7 μ M) was added along with the polyplexes or during the final 30 min of incubation respectively. Cells were washed extensively with ice cold PBS(+) containing 5% defatted BSA [24] and heparin (1 mg/ml) respectively. For co-localization with LysoTracker Green DND-26/LysoTracker Red DND-99 (Invitrogen), polyplexes were incubated with cells for 2 h/4 h at 37 °C, and LysoTracker (150 nM/350 nM) was added during the final 5 min/30 min of incubation respectively. Cells were washed with ice-cold PBS(+) containing heparin (1 mg/ml) and kept in Opti-MEM. Fixing was done wherever required. Imaging was done on an inverted LSM 510 META laser

scanning microscope (Carl Zeiss) using a plan-apochromat 63 \times 1.4 N.A. lens. The 488 nm line of an argon laser was used to excite the endosomal markers and 543 nm helium neon laser was used for rhodamine label on DNA. Leica Confocal Microscope (Leica TCS SP5) with 60 \times 1.2 N.A., water immersion using LAS AF 2.6.0 software with filter sets: DAPI (Ex405 nm, Em420–480 nm), FITC (Ex488, Em500–550 nm) and LysoTracker Red (Ex532 nm, Em550–680 nm) was used for Fig. S8. For co-localization with Alexa Fluor 488 transferrin (Invitrogen), cells were treated with the polyplexes and transferrin (20 μ g/ml) for 1.5 h at 37 °C. Cells were then washed with ice-cold PBS(+) containing heparin (1 mg/ml), followed by ice-cold acid wash buffer [24] and PBS(+). Cells were imaged on a Nikon TiE motorized inverted fluorescence microscope equipped with a 60 \times , 1.4 N.A. plan apochromat oil immersion objective.

Minimal processing of the microscopy images for enhancing brightness and contrast was done using ImageJ software [25]. Pearson's coefficient and Mander's coefficient for co-localization were calculated using the JACoP plug-in [26] for ImageJ.

2.6. Immunostaining

CHO-K1 cells were seeded on 22 mm coverslip 24 h prior to the experiment. At 70–80% confluency, cells were treated with native R₁₆ polyplexes or R₁₆ polyplexes with CS at charge ratio 10 in Opti-MEM. After 1 h of incubation, cells were washed with PBS containing heparin (1 mg/ml) and with normal PBS twice each. Cells were fixed using 4% paraformaldehyde for 10 min at 37 °C and washed with PBS thrice for 5 min after which cells were permeabilized using 0.25% Triton-X100 for 15 min. Cells were further washed with PBS and blocked using 1% BSA in PBS for 1 h at room temperature. Cells were then incubated with primary antibody for clathrin (anti-clathrin heavy chain antibody ab 21679), caveolin (anti-caveolin-1 antibody ab2910) at 1:500 dilution for overnight at 4 °C and giantin (anti-giantin antibody ab 24586) for 2 h at room temperature. Cells were then washed thrice with PBS containing 1% BSA for 5 min each and further incubated with secondary antibody (Goat Anti-Rabbit) labeled with Alexa Fluor 488 (ab 150081) at a dilution of 1:500 for 2 h. Cells were again washed thrice with PBS containing 1% BSA and further incubated with 500 μ g/ml of Hoechst 33342 solution for 10 min. Cells were again washed with PBS and water and mounted with anti-fade mounting media. Imaging was done on an inverted LSM 510 META laser scanning microscope (Carl Zeiss) using a plan-apochromat 63 \times 1.4 N.A. lens and 405 nm, 488 nm and 543 laser lines. All antibodies used were purchased from Abcam.

2.7. Statistical analysis

Fold change of transfection was calculated as treatment/control and converted to log₂ scale (log FC). Single sample t-test was used to calculate significance of difference using R software. p-Value < 0.05 was considered as statistically significant.

3. Results

3.1. Arginine-rich peptides exhibit enhanced transfection efficiency and cellular uptake in the presence of exogenous chondroitin sulfate in multiple cell lines

We had previously observed that addition of small amounts of exogenous glycosaminoglycans (e.g. heparan sulfate (HS) and chondroitin sulfate (CS)) to arginine-rich polyplexes considerably increased their transfection efficiency in wild-type CHO-K1 cell line as well as the GAG-deficient pgsA-745 cell line. We also showed that the exogenous GAGs improved the stability of the polyplexes and led to increased cellular entry [12]. In order to assess whether the presence of exogenous GAGs enhances cellular entry and

transfection efficiency across different cell types with varying density of one or more types of cell surface GAGs, we used the CHO-K1 wild-type cell line, and the CHO mutant cell lines, pgsA-745 (which lacks all types of cell surface GAGs) and pgsD-677 (which is devoid of cell surface HS but produces excess CS). We studied the effect of addition of exogenous CS and HS to arginine-based polyplexes in all the cell lines and report here the results obtained with the addition of exogenous CS only since the trend for both CS and HS is similar. Transfection efficiency of native polyplexes (no added CS) and coated polyplexes (with added CS in amounts mentioned in the earlier section) prepared with R₁₆ and 32 AR was determined using expression of the reporter gene luciferase, as shown in Fig. 1A and C. In all the cell lines used, the transfection efficiency was increased at CS: peptide w/w ratios \leq 0.5:1. In the case of R₁₆, CHO-K1 and pgsA-745 cells exhibited a maximum increase of an order of magnitude in transfection efficiency (as reported earlier by us) whereas the pgsD-677 cells showed nearly 6 times increment (Fig. 1A). The transfection efficiency achieved by the commonly used commercial transfection reagent Lipofectamine in the three cell lines is also shown for comparison in Fig. 1A. On the other hand, the increase in transfection efficiency of coated polyplexes containing 32 AR was 2.5–5 times higher as compared to the native polyplexes in all the CHO cell lines (Fig. 1C). Thus, there was an overall increase in transfection efficiency with low amount of exogenous CS in all the cases although the level of increase was dependent on the cell line and composition of the peptide. Addition of high amount of CS reduced the transfection efficiency in all the cases as seen earlier by us and others [12,22].

In order to assess whether CS also increased the cellular uptake of the polyplexes, we labeled the plasmid DNA with fluorescein and used flow cytometry to determine the cellular uptake of the labeled polyplexes. We observed polyplex uptake in more than 95% of the treated cells in all the cases after 4 h of incubation. Fig. 1B and D shows the intracellular mean fluorescence intensities of coated and native R₁₆ and 32 AR polyplexes respectively in the cell lines used. Addition of CS increased the total cellular uptake of both polyplexes across different cell lines. This indicated that the presence of exogenous GAGs increased the cellular entry of these polyplexes although the trend cannot be directly correlated to the levels of transfection efficiency in different cell lines in all the cases. This has been elaborated in the Discussion section.

3.2. Polyplexes of arginine homopeptide exhibit increased accumulation at the nuclear periphery in the presence of exogenously added chondroitin sulfate and co-localize with the chondroitin sulfate

Our next endeavor was to chart out the intracellular localization of the native and coated polyplexes. This was first carried out with the R₁₆ polyplexes. We used rhodamine-labeled plasmid DNA to prepare R₁₆ polyplexes, and added fluorescein-labeled CS (as described in the Experimental section) and observed their localization in the different cell lines using fluorescence microscopy. As seen from Fig. 2, a larger concentration of polyplexes was present at the periphery of the nucleus (stained by Hoechst) in cells treated with the CS-containing polyplexes than in cells treated with the native polyplexes after 4 h of incubation at 37 °C. This indicated that the exogenous CS enhanced the accumulation of the polyplexes near the nucleus in all the three cell lines. This phenomenon was also observed after only 1 h of incubation with CS-coated polyplexes, where the GAG was un-labeled (Fig. S1 in Supplemental information). As shown in Fig. 2, the exogenous labeled CS showed co-localization with the plasmid DNA at the nuclear periphery (as indicated by the merged yellow fluorescence around the nucleus). Pearson's coefficient, a measure of co-localization, was 0.72, 0.79, and 0.89 for the three cell lines respectively. This implied that the CS remained associated with the polyplex during its intracellular passage, at least up to this time point.

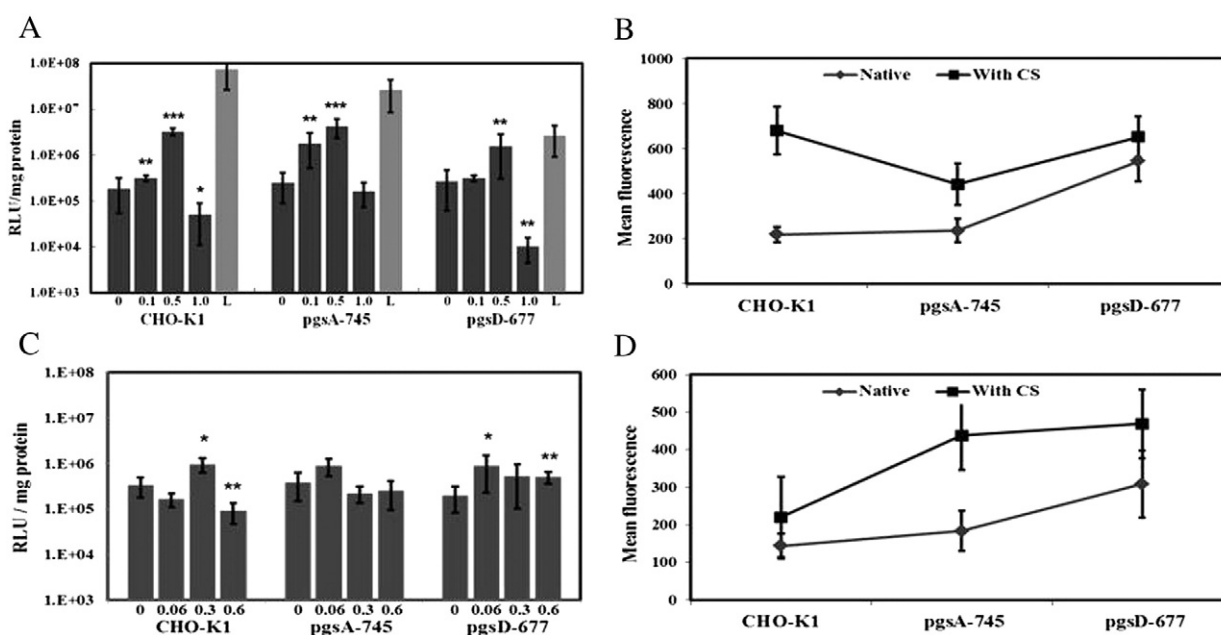


Fig. 1. Effect of exogenous chondroitin sulfate on cellular uptake and transfection efficiency of arginine-based polyplexes. A and C, R_{16} (A) and 32 AR (C) polyplexes at Z(+/–) 10.0 were treated with increasing concentrations of CS (expressed as GAG:peptide w/w ratios on x-axis) and incubated with different cell lines for 5 h. Transfection efficiency was measured from luciferase activity after 24 h. Light gray bars (named L in A) indicate transfection efficiency of Lipofectamine in the different cell lines. Values are plotted as mean \pm S.D. of three independent experiments, each in duplicate (*** $p < 0.005$, ** $p < 0.01$, * $p < 0.05$). B and D, R_{16} (B) and 32 AR (D) polyplexes at Z(+/–) 10.0 containing fluorescein-labeled plasmid DNA and those treated with 0.5 and 0.3 w/w ratio of CS respectively were added to cells. After incubation for 4 h at 37 °C, cells were washed with PBS containing heparin and trypsinized. Cellular uptake was measured using flow cytometry. Mean fluorescence intensities of the polyplexes in different cell lines are plotted as mean \pm S.D. of three independent experiments. Some of the data points in CHO-K1 and pgsA-745 cells in A and B were taken from reference [12].

3.3. Polyplexes of arginine homopeptides exhibit multiple endocytic uptake pathways

Our observations indicated enhanced cellular entry and increased accumulation of polyplexes at the nuclear periphery in the presence of CS, along with co-localization of polyplexes and CS in the case of R_{16} polyplexes. We next wanted to explore whether such increased uptake could be because of differences in the uptake pathways in the presence and absence of exogenous GAGs. We had shown earlier that the R_{16} polyplexes adopted an endocytic route of cellular entry [12]. Hence we checked their cellular uptake in the presence of chemical inhibitors which are known to selectively obstruct different endocytic pathways. Clathrin-mediated endocytosis was inhibited by chlorpromazine, which hinders clathrin-coated pit formation [27]. Caveolae-mediated endocytosis was inhibited by genistein, a specific inhibitor of tyrosine kinase involved in signal transduction [28,29]. Macropinocytosis was inhibited by dimethylamiloride, which inhibits Na^+/H^+ ion exchange in the plasma membrane and therefore, prevents membrane ruffling [30]. In addition we also used inhibitors of the cytoskeletal network such as cytochalasin D and nocodazole, which prevent polymerization of actin filaments and microtubules respectively [31,32]. Concentrations of these inhibitors were similar to those reported in the literature [33,34] and caused minimum toxicity to the different cell types (data not shown). The uptake of the polyplexes was estimated in cells treated with the indicated inhibitors using flow cytometry. The polyplex uptake in untreated cells was taken as the control. The results are shown in Fig. 3A.

In CHO wild-type and mutant cells, chlorpromazine did not inhibit the uptake of the native or coated R_{16} polyplexes (Fig. 3A). Genistein inhibited the uptake of both types of R_{16} polyplexes by 50% or more in all cell lines. Therefore caveolae-mediated pathway may be one of the major entry pathways of both the native arginine polyplexes and those with exogenous CS. Dimethylamiloride, however, had different effects on the R_{16} polyplexes with and without CS. The uptake of the

native polyplexes was reduced (by 40–50% in CHO-K1 and pgsA-745 and by 10% in pgsD-677) whereas in the case of the coated polyplexes, there was either no change or an increase. This indicated that the pathway of macropinocytosis may be operative in the case of the native R_{16} polyplexes, in addition to the caveolae-mediated endocytosis.

Inhibition of actin polymerization by cytochalasin D resulted in reduced uptake of the native polyplexes by 20–70% in the various CHO cell lines, while the uptake of the coated polyplexes was considerably increased in most of the cases (Fig. 3A). This was also validated by imaging the CHO-K1 cells treated with the polyplexes, where the actin filaments were stained with Alexa Fluor 488 phalloidin (Fig. S2 in Supplemental information). Microtubule depolymerization by nocodazole also showed a similar pattern, wherein the drug inhibited the uptake of the native polyplexes by 30–70% but either enhanced or showed no inhibition in the uptake of the polyplexes in the presence of CS in all the cell lines (Fig. 3A).

For confirming the endocytic pathway adopted by the R_{16} polyplexes, we also studied their co-localization with a positive marker for caveolae-mediated endocytosis, C_5 -lactosylceramide [35,36], by confocal microscopy. In the wild-type and GAG-deficient CHO cell lines, both the native and coated R_{16} polyplexes co-localized with the marker after 1 h of incubation at 37 °C (Fig. 3B) (for R_{16} polyplexes Mander's coefficient for fraction of polyplex overlapping with marker was 0.437 and 0.11 in CHO-K1 and pgs A-745 respectively and for polyplex with exogenous CS, Mander's coefficient for fraction of polyplex overlapping with marker was 0.837 and 0.248 in the same cell lines respectively). The results for these experiments at a shorter time point of 20 min incubation are also shown in Fig. 3C (left panel). These results indicated that a caveolae-mediated uptake mechanism was operational in all these cases. However, co-localization of these polyplexes with the clathrin marker, transferrin, was also observed (Fig. 3C (right panel), Pearson's coefficient 0.71). A few of the polyplexes that entered CHO-K1 cells after 10 min incubation also co-localized with lactosylceramide and transferrin, as shown in Fig. S3 in Supplemental information.

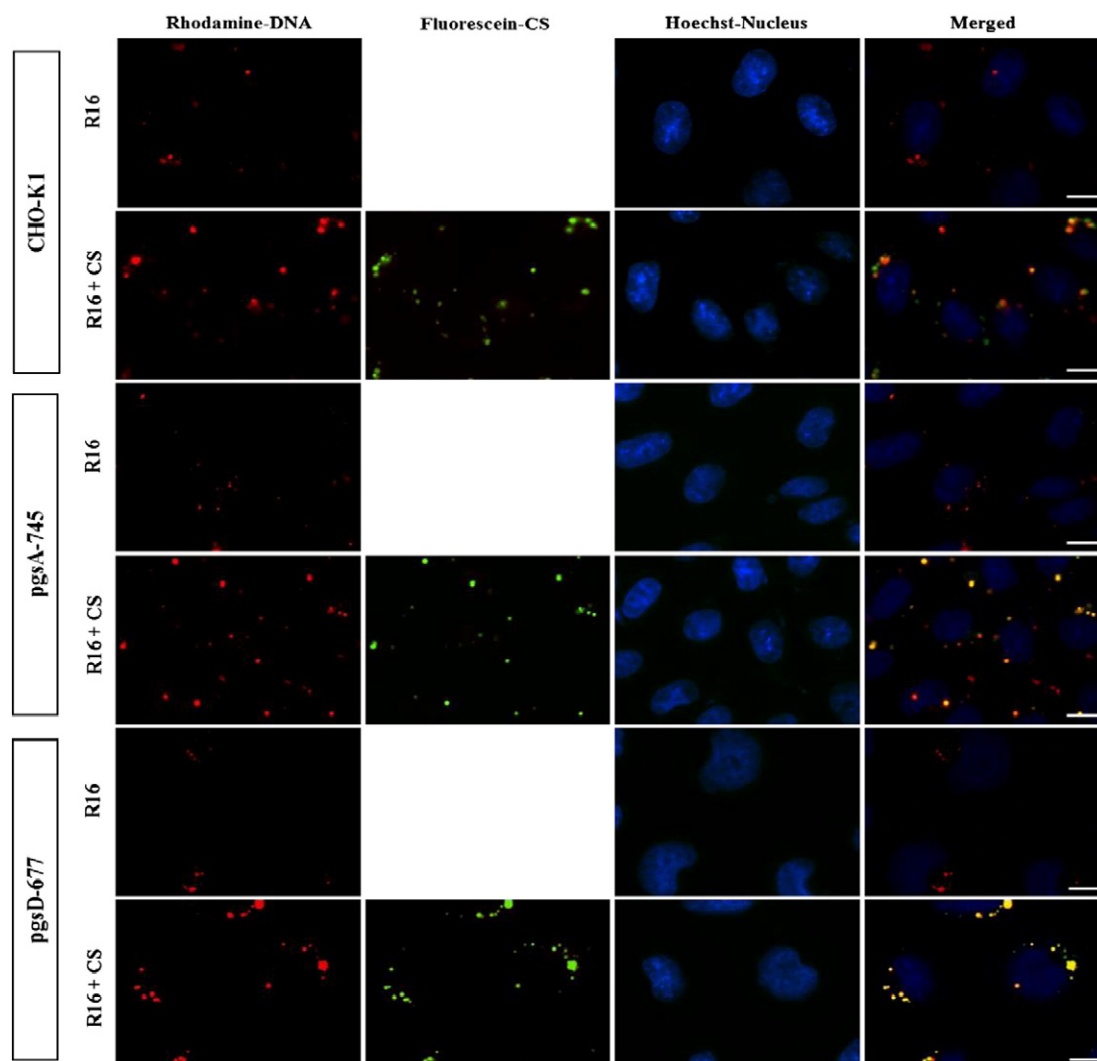


Fig. 2. Localization of R_{16} polyplexes in the presence and absence of exogenous chondroitin sulfate. R_{16} polyplexes at Z(+/–) 10.0 containing rhodamine-labeled DNA, with or without addition of fluorescein-labeled chondroitin sulfate at GAG:peptide ratio of 0.5:1, were incubated with the respective cells for 4 h at 37 °C. The cell nucleus was stained with Hoechst 33342. Cells were then washed with PBS containing heparin and imaged by fluorescence microscopy. Scale bar = 20 μ m.

In order to validate the results obtained above, we also carried out immunostaining of clathrin and caveolin after incubation with the R_{16} polyplexes in CHO-K1 cells (Fig. 4). We observed that both the native and coated R_{16} polyplexes partially co-localized with clathrin after 1 h of incubation (Fig. 4A). At the same time point, co-localization was also seen in the cells treated with either of the polyplexes and immunostained for caveolin-1 (Fig. 4B) (for R_{16} polyplexes, Mander's coefficient for fraction of polyplex overlapping with clathrin and caveolin-1 was 0.206 and 0.444 respectively and for polyplexes with exogenous CS, Mander's coefficient for fraction of polyplex overlapping with clathrin and caveolin-1 was 0.205 and 0.448).

3.4. Arginine-rich polyplexes show similar trends in cellular entry and intracellular processing as the polyplexes of the arginine homopeptides

The experiments described in the two previous sections were then carried out with the 32 AR polyplexes. The 32 AR polyplexes demonstrated a pattern of cellular entry which was comparable to that seen in case of the R_{16} polyplexes. As shown in Fig. 5A, the exogenously added CS co-localized with the 32 AR polyplex at the nuclear periphery of CHO-K1 cells (Pearson's coefficient was 0.912). A

similar trend was also seen in the other cell lines used in this study (data not shown). It appeared that the exogenous CS remained associated with the polyplex and assisted their localization around the nucleus at the time point observed.

We also elucidated the endocytic pathway adopted by the 32 AR polyplexes in all the cell lines using the chemical inhibitors mentioned above. Representative results obtained for the CHO-K1 are shown in Fig. 5B. In CHO-K1 cells, genistein significantly inhibited the uptake of both the native and coated 32 AR polyplexes by 80%, while chlorpromazine and dimethylamiloride did not inhibit their uptake. Treatment with cytochalasin D inhibited uptake of the native polyplexes but enhanced the uptake of the coated polyplexes. This trend was similar to what was observed for R_{16} polyplexes. Nocodazole treatment also increased the uptake of the coated polyplexes although it did not affect the uptake of the native polyplexes in CHO-K1 cells. Similar trends were observed in the other variants of CHO cells (data not shown).

Similar to the R_{16} polyplexes described in the previous section, we observed co-localization of the 32 AR polyplexes, with and without exogenous CS, with the markers of clathrin- and caveolae-mediated endocytosis (Fig. S4 in Supplemental information).

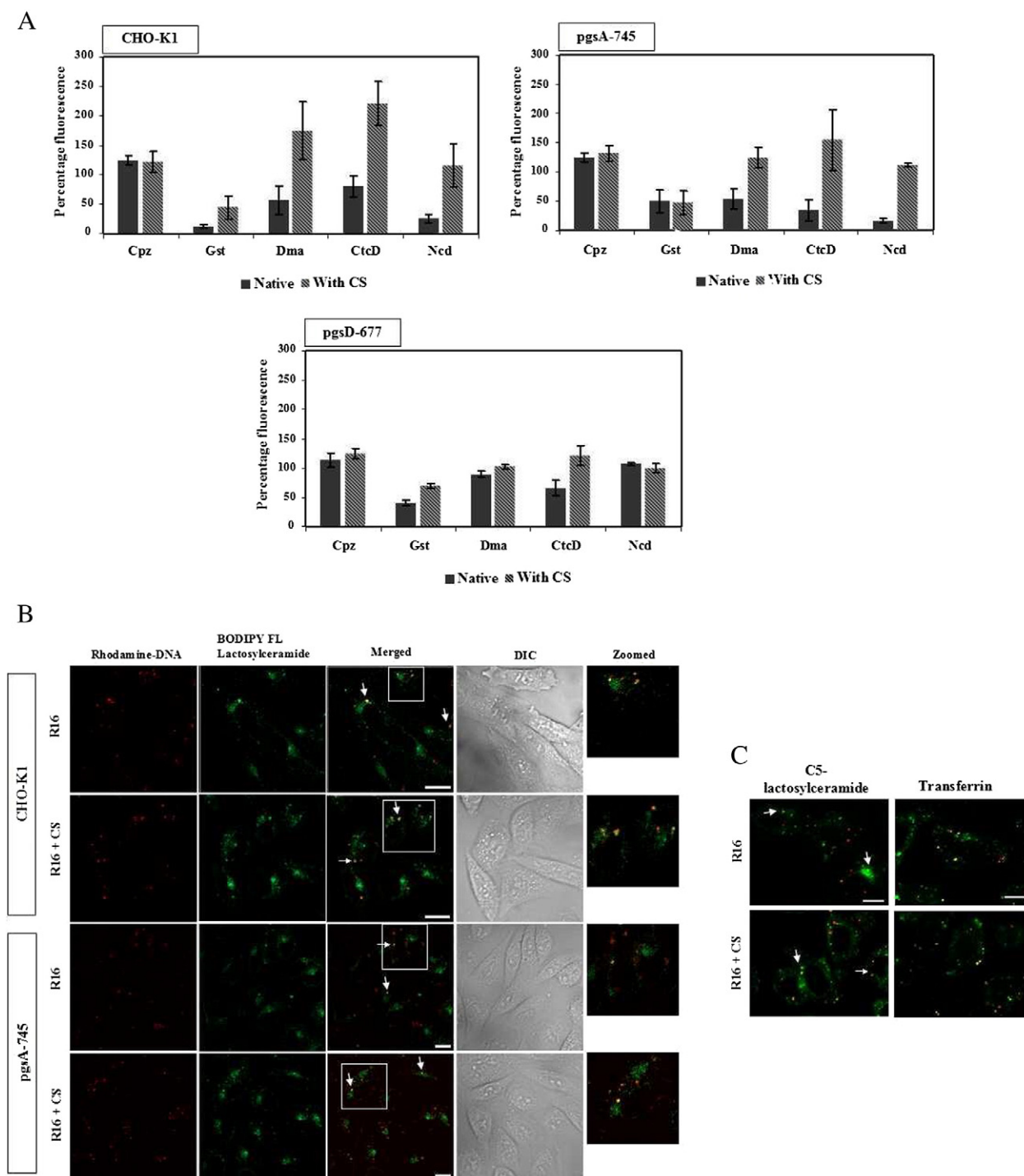


Fig. 3. Elucidation of the endocytic pathway involved in uptake of R_{16} polyplexes with and without exogenous chondroitin sulfate. **A**, Cells were treated with the indicated endocytic inhibitors for 1 h prior to the addition of R_{16} polyplexes (with fluorescein labeled DNA at Z (+/–) 10.0) with or without CS (at GAG:peptide w/w of 0.5:1). After subsequent incubation at 37 °C for 4 h, cells were washed and analyzed by flow cytometry. Results are plotted as a percentage of the uptake in untreated cells. Values are given as mean \pm S.D. of three independent experiments. Cpz, chlorpromazine; Gst, genistein; Dma, dimethylamiloride; CtcD, cytochalasin D; Ncd, nocodazole. **B**, Cells were incubated with R_{16} polyplexes (with rhodamine-labeled DNA at Z (+/–) 10.0) with or without CS (at GAG:peptide w/w of 0.5:1) and incubated for 1 h at 37 °C. BODIPY FL labeled C_5 -lactosylceramide (0.7 μ M) was added during final 30 min of incubation. Cells were washed and imaged with a laser scanning confocal microscope. Regions of co-localization are indicated by arrows and areas marked by a square are zoomed. Scale bar = 10 μ m. **C**, Cells were treated with R_{16} polyplexes (red) and BODIPY FL labeled C_5 -lactosylceramide (green), as described in **B**, and incubated for 20 min at 37 °C or Cells were treated with R_{16} polyplexes (with rhodamine-labeled DNA at Z (+/–) 10.0) with or without CS (at GAG:peptide w/w of 0.5:1) (red) and Alexa Fluor 488 labeled transferrin (green) and incubated for 1.5 h at 37 °C. Cells were washed and imaged with a fluorescence microscope. Some of the regions of co-localization are indicated by arrows. Scale bar = 20 μ m.

3.5. Exogenous chondroitin sulfate helps polyplexes of arginine homopeptide to escape the endosome

We subsequently used the R_{16} peptide to further decipher the intracellular processing of the polyplexes with CS. We determined the

transfection efficiency of the R_{16} polyplex, with and without CS, in the presence of the chemical inhibitors in order to correlate the endocytosis route with the extent of gene delivery. As shown in Fig. 6A–C, treatment with chlorpromazine increased the transfection efficiency of both the native and coated R_{16} polyplexes in the CHO cell lines. Treatment with

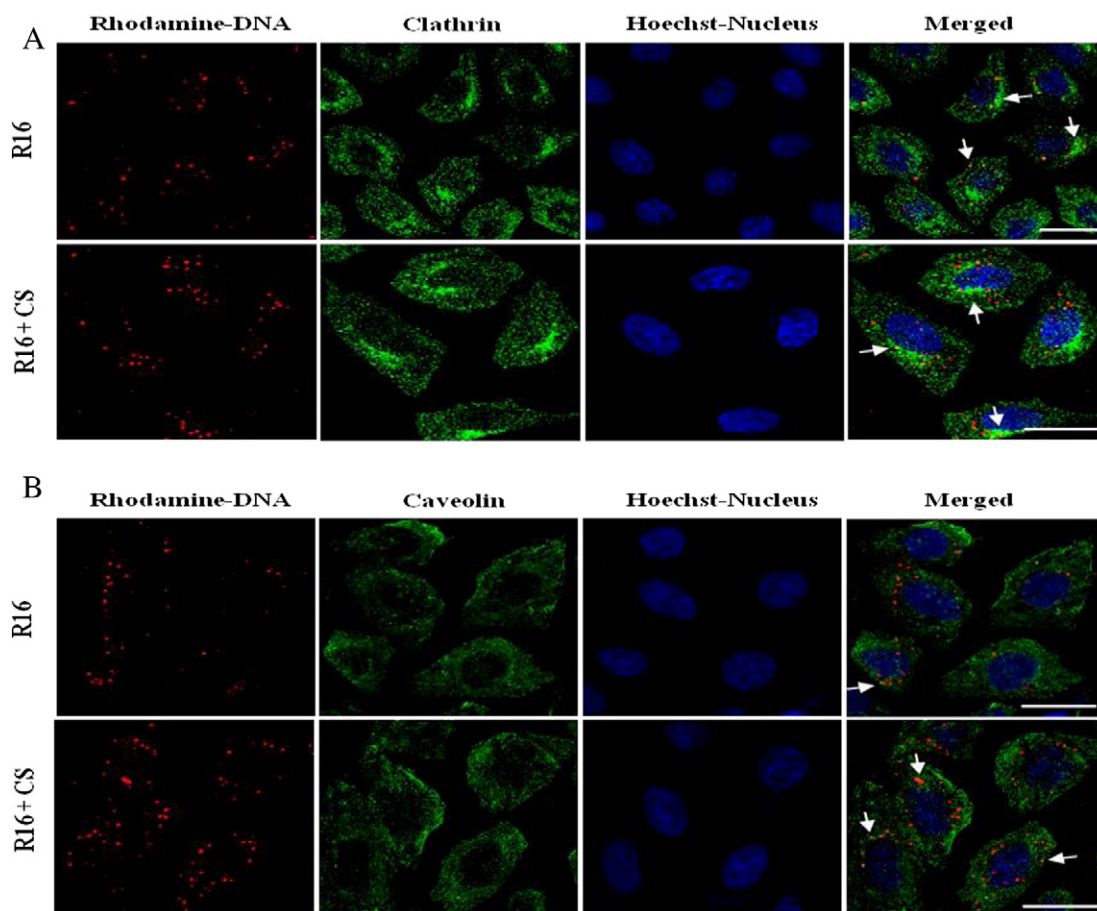


Fig. 4. Colocalization of R_{16} polyplexes with clathrin and caveolin-1 in the presence and absence of exogenous chondroitin sulfate in CHO-K1 cells. A and B, CHO-K1 cells were treated with R_{16} polyplexes (with rhodamine-labeled DNA at Z(+/–) 10.0) with or without CS (at GAG:peptide w/w of 0.5:1) (red) for 1 h at 37 °C, then fixed, permeabilized and treated with primary antibodies against clathrin (A) and caveolin-1 (B) overnight at 4 °C. Cells were then incubated with Alexa Fluor 488-labeled secondary antibody for 2 h, washed and imaged with a confocal microscope. Scale bar = 20 μ m.

genistein reduced the transfection efficiency of both the coated and native polyplexes considerably in the CHO cell lines, similar to what had been observed in the uptake studies shown in Fig. 3A, indicating the caveolar pathway as the major endocytosis route for gene delivery and expression. Presence of dimethylamiloride showed a considerable reduction in transfection efficiency of native polyplexes in pgsD-677 cells and a mild reduction in the other cell lines. However, the transfection efficiency of the coated polyplexes was also mildly reduced in all the CHO cells under similar conditions. Treatment with cytochalasin D resulted in a decrease in transfection efficiency of both the native and coated polyplexes by up to 10 times in the CHO cell lines. These trends are different from that observed in the uptake studies where both dimethylamiloride and cytochalasin treatment caused an increase in the uptake of the coated polyplexes and an inhibition in the case of the native polyplexes. It is possible that the actin cytoskeleton may play a role in the intracellular events following the uptake process in the case of both the native and CS coated polyplexes. On the other hand, nocodazole did not show a major inhibition and in most cases increased the transfection efficiency of the polyplexes. We have deliberated on the possible reasons for this difference in the Discussion section.

It is thus clear that in these cell lines, multiple pathways are involved in the uptake of the native and GAG coated polyplexes but the eventual gene expression is also determined by the intracellular events following the uptake process. In order to study the intracellular fate of the arginine polyplexes with and without exogenous CS after their endocytic uptake, we assessed whether these polyplexes go to the lysosomes by colocalization with a lysosomal marker, LysoTracker Green DND-26. After just 2 h of incubation, the native and coated R_{16} polyplexes

showed co-localization with LysoTracker to a considerable extent (Fig. 7A) (for R_{16} polyplexes, Mander's coefficient for fraction of polyplex overlapping with marker was 0.487 and 0.637 in CHO-K1 and pgsA-745 respectively and for polyplexes with exogenous CS, Mander's coefficient for fraction of polyplex overlapping with marker was 0.626 and 0.458 in the same cell lines respectively). Therefore, we next explored how the polyplexes with exogenous CS exhibit increased transfection efficiency in spite of their obvious localization within the lysosomes. For this, we estimated the transfection efficiency of the R_{16} polyplexes, with and without CS, in the presence of chloroquine and bafilomycin.

Chloroquine accumulates in the endosomes due to their low pH and causes swelling and disruption of these vesicles by osmotic effects [37]. Hence entrapped polyplexes, if any, are released into the cytosol. Bafilomycin A1 is a specific inhibitor of the vacuolar type H^+ -ATPase, and thus inhibits acidification of the endosomes preventing their maturation and fusion into lysosomes [38]. Any endosomal escape mechanism of polyplexes that is dependent on endosomal acidification is therefore inhibited by bafilomycin treatment. Addition of chloroquine during incubation of the polyplexes with the cells resulted in an increment, by an order of magnitude, in the transfection efficiency of the native R_{16} polyplex in the CHO cell lines (Fig. 7B). However, for the coated polyplex, the increase was less than 1.5 times under similar conditions. This implied that the coated polyplexes have improved ability to escape from endosomes and are not strongly dependent on endosomal rupture caused by chloroquine, while the native polyplexes get entrapped in the endosomes and require chloroquine to enhance the endosomal release and subsequent increase in transfection.

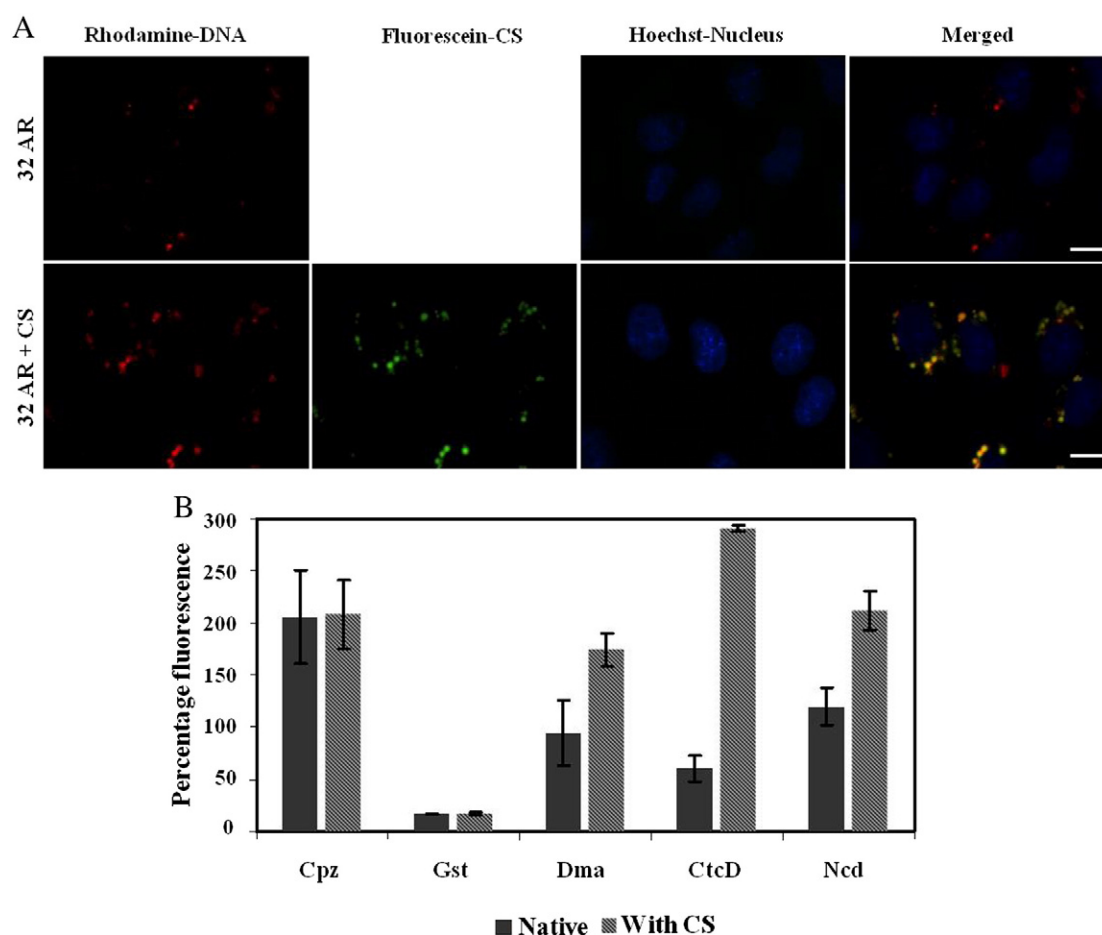


Fig. 5. Localization of 32 AR polyplexes with and without exogenous chondroitin sulfate and their uptake in the presence of endocytic inhibitors. **A**, 32 AR polyplexes at Z (+/–) 10.0 containing rhodamine-labeled DNA, with and without fluorescein-labeled chondroitin sulfate at GAG:peptide ratio of 0.3:1, were incubated with CHO-K1 cells for 4 h at 37 °C. The cell nucleus was stained with Hoechst 33342. Cells were then washed with PBS containing heparin and imaged by fluorescence microscopy. Scale bar = 20 μ m. **B**, Cells were treated with the endocytic inhibitors for 1 h prior to the addition of 32 AR polyplexes (with fluorescein labeled DNA at Z (+/–) 10.0) with or without CS (at GAG:peptide w/w of 0.3:1). After subsequent incubation at 37 °C for 4 h, cells were washed and analyzed by flow cytometry. Results are plotted as a percentage of the uptake in untreated cells. Values are given as mean \pm S.D. of three independent experiments. Cpz, chlorpromazine; Gst, genistein; Dma, dimethylamiloride; CtcD, cytochalasin D; Ncd, nocodazole.

A caveolae-mediated mode of endocytosis has been suggested to traffic polymer–DNA complexes to the Golgi network [39,40]. In order to check whether the R_{16} polyplexes localize to the Golgi, we performed immunostaining of giantin, a Golgi marker, after incubation of CHO-K1 cells with R_{16} polyplexes for 1 h. As shown in Fig. 7C, the native R_{16} polyplexes exhibited very little co-localization with giantin, whereas the CS-coated R_{16} polyplexes co-localized to a larger extent with giantin, indicating their presence in the Golgi (Mander's coefficient for native R_{16} polyplexes and polyplexes with CS for fraction of polyplex overlapping with marker was 0.258 and 0.502 respectively). Therefore, it is possible that the coated polyplexes taken up by caveolar endocytosis are trafficked to the Golgi network before entry into the nucleus.

4. Discussion

Peptide-based gene carriers possess numerous advantages over other chemical vectors such as polymers and liposomes with respect to safety, sequence diversity and functionalization. Yet, peptide carriers have not found widespread clinical application mainly due to their low efficiency in achieving therapeutic levels of transgene expression. Therefore, their extensive use as gene delivery agents will benefit from designing of novel strategies to improve the efficiencies of current peptide vectors. There are few reports in the literature where attachment of polysaccharides, specifically sulfated GAGs like chondroitin sulfate, to polymers such as polyethylenimine [41] and chitosan [42] has been observed to improve their gene delivery and targeting efficiency

[43]. However, the effect of exogenous GAG addition on the gene delivery efficiency of peptide carriers has not been studied in detail.

Our group had first reported that small amounts of exogenous GAGs added to homoarginine (R_{16}) polyplexes increased their transfection efficiency considerably in both wild-type and GAG-deficient CHO cell lines [12]. The GAGs enhanced the stability of the polyplexes and protected the compacted DNA from nucleases. The polyplexes also showed more efficient cellular entry in the presence of GAGs than the native polyplexes. Atomic force microscopy studies had revealed an increase in size of the polyplexes on GAG addition. This was further confirmed using dynamic light scattering. The hydrodynamic diameters of the native R_{16} polyplexes (67.14 ± 0.86 nm) increase by about 20 nm on addition of GAGs at GAG:peptide w/w ratio of 0.5:1 (88.47 ± 0.14 nm). These results implied that the GAG possibly formed a 'GAG coat' by electrostatic interactions at the polyplex surface. A similar phenomenon was also observed with arginine-rich peptides containing different distributions of the positive residues, for example, 32 AR peptide, containing a GAG-binding motif with clustered arginine residues separated by neutral amino acids [23].

These observations pointed towards a hitherto unexplored role of GAGs in gene delivery mediated by peptides. Several intriguing questions stemmed from this unusual observation, the most pertinent being whether the presence of GAGs affects the mechanisms of cellular uptake, intracellular processing and localization and if so, whether such events affect the overall transfection efficiency. In the present study, we initially confirmed that the addition of exogenous CS improved the

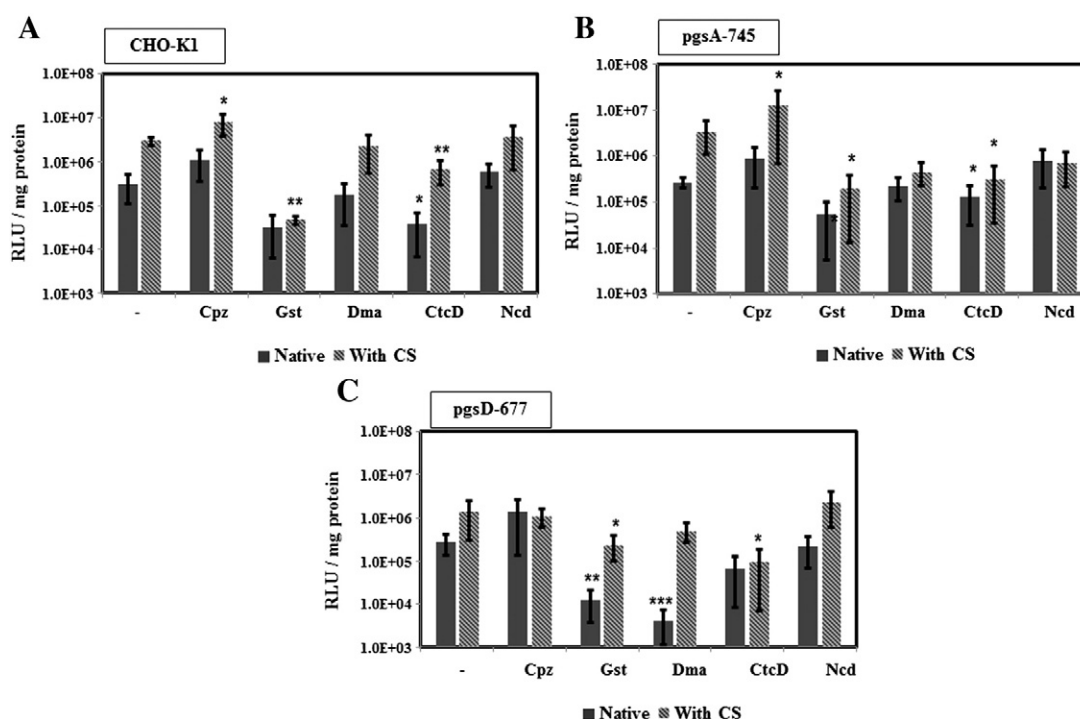


Fig. 6. Effect of different endocytic inhibitors on gene delivery efficiency of R_{16} polyplexes with and without exogenous chondroitin sulfate. A–D, Cells were pretreated with the respective inhibitors for 1 h before addition of R_{16} polyplexes at Z(+/–) 10.0 with or without exogenous CS at GAG:peptide w/w ratio of 0.5:1. Incubation was carried out for 5 h at 37 °C, and transfection efficiency was measured from luciferase activity after 24 h. Values are given as mean \pm S.D. of three independent experiments, each in duplicate (*** p < 0.005, ** p < 0.01, * p < 0.05). Cpz, chlorpromazine; Gst, genistein; Dma, dimethylamiloride; CtcD, cytochalasin D; Ncd, nocodazole.

transfection efficiency of arginine-rich peptides including R_{16} and 32 AR, in multiple cell types, with different cell surface GAG compositions including a HS-deficient CHO mutant cell line, pgsD-677 which has CS as the major cell surface GAG species (Fig. 1). We also observed that even in the presence of serum the transfection efficiency of the CS-coated R_{16} polyplexes was higher than that observed with uncoated polyplexes in CHO-K1 cells (Fig. S5 in Supplemental information). This illustrated that the addition of exogenous GAGs to polyplexes of arginine-rich peptides can be used as a potential method to improve the gene delivery efficiency in different cell types and can also be extended for in vivo applications including dermal and ocular gene delivery. It also confirmed our previous observations that arginine-rich polyplexes, either with contiguous or spaced residues, enter cells independent of the cell surface GAGs.

We also observed that there is an increase in cellular uptake in the presence of small amounts of exogenous CS. We attempted to decipher whether this could be because of differences in the uptake routes of the native and CS-coated polyplexes. Elucidation of the uptake route was also important because although CS coating causes enhanced cellular uptake in all the cell lines, the extent of increase in transfection efficiency was different in different cell lines. Using chemical inhibitors for the commonly observed endocytic uptake routes, we found that both the wild-type and GAG-deficient CHO cells took up the native R_{16} polyplexes by multiple pathways including caveolae-mediated endocytosis and macropinocytosis, whereas the CS-coated polyplexes were predominantly taken up by the caveolar pathway (Fig. 3; large format views given in Supplemental Fig. S9). Caveolae-mediated endocytosis has also been reported to be functional in the uptake of other chemical carriers including lipoplex and chitosan in CHO-K1 cells [44,45]. Also, macropinocytic entry of arginine-rich peptides is well-documented [13,46,47].

Using positive markers for the pathways of clathrin- and caveolin-mediated endocytosis, we could however observe their co-localization

with both the native or CS-coated polyplexes (Fig. 3B–C). This was also confirmed by immunostaining using antibodies against clathrin and caveolin (Fig. 4). Therefore, looking at the effects of both chemical inhibitors and the positive markers for the endocytic pathways in total, it seemed most likely that both the R_{16} and 32 AR polyplexes were taken up by multiple pathways in CHO cells, regardless of the presence or absence of exogenous CS. The presence of multiple uptake pathways might also be responsible for the discrepancy observed between the magnitude of increase in the cellular uptake and transfection of R_{16} polyplexes in the presence of CS amongst different cell lines (Fig. 1), where all the pathways may not equally contribute to the ultimate level of gene expression. A complete investigation of the kinetics of entry of the polyplexes will therefore help in elucidating the exact time course of intracellular events and the role of a particular pathway in contributing to successful gene delivery ultimately.

The caveolae-mediated uptake of the R_{16} polyplexes was also found to largely contribute to the ultimate levels of gene expression in the CHO cell lines, since treatment with genistein decreased the transfection efficiency of both the native and CS coated polyplexes considerably (Fig. 6). Treatment with chlorpromazine caused stimulation in the transfection efficiency of R_{16} polyplexes in CHO cell lines, which was also observed in the cellular uptake studies. This phenomenon has been seen earlier also [48–50] and it is believed that inhibition of clathrin dependent endocytosis by chlorpromazine may enhance uptake by other pathways.

However, treatment with the inhibitors of the cytoskeletal network like cytochalasin D and nocodazole showed conflicting results in the pattern of uptake and corresponding transfection studies in the CHO cell lines. Presence of these inhibitors reduced the uptake of the native polyplexes whereas the uptake of the polyplexes with CS was either not affected or increased. Hence, our results indicate that the cytoskeletal network constituted by actin filaments and microtubules plays a major role in the uptake of the native arginine polyplexes, whereas its

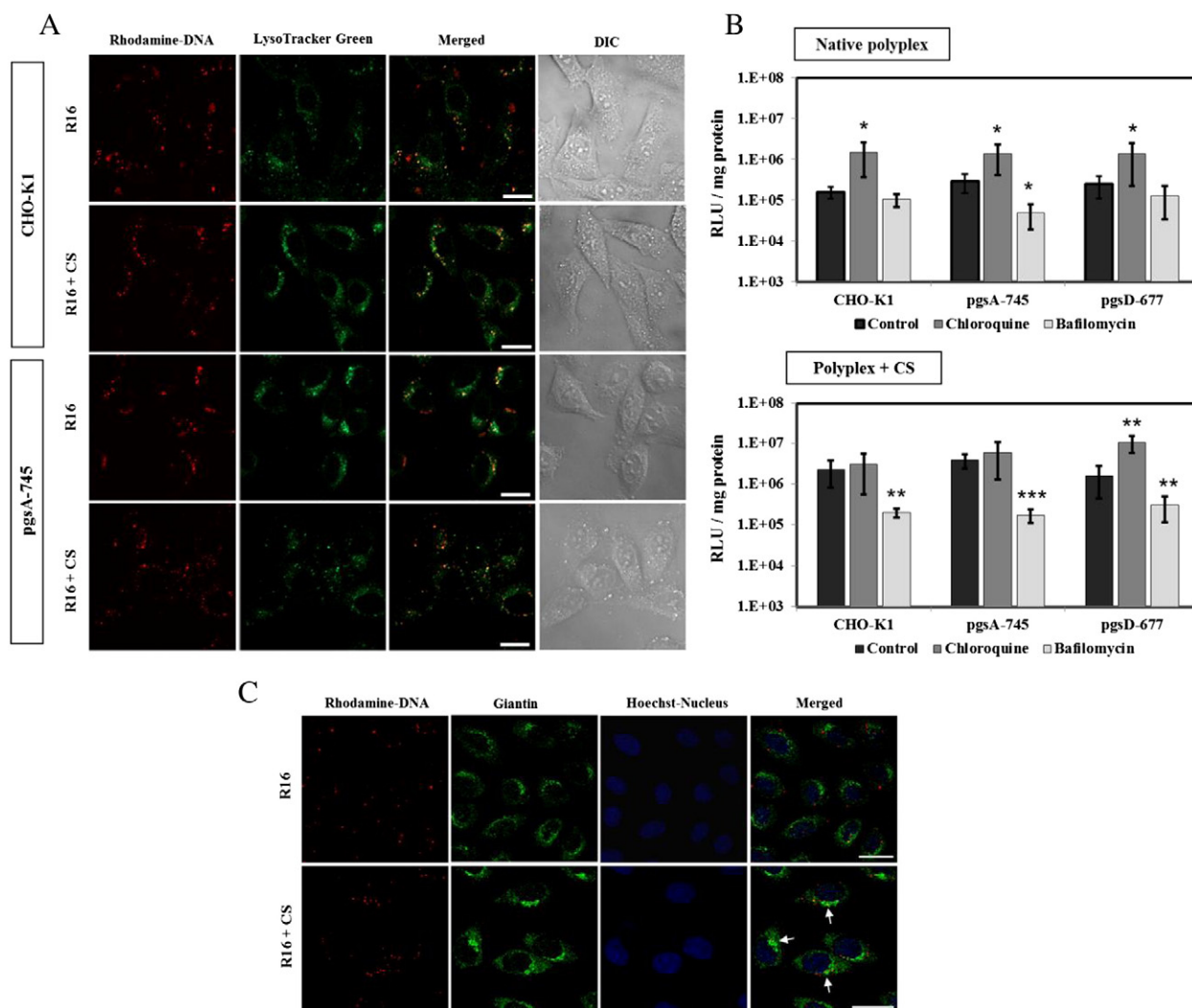


Fig. 7. Endosomal escape of R₁₆ polyplexes in the presence and absence of exogenous chondroitin sulfate. **A**, Cells were incubated with R₁₆ polyplexes (with rhodamine-labeled DNA at Z(+/-) 10.0) with or without CS (at GAG:peptide w/w of 0.5:1) and incubated for 2 h at 37 °C. LysoTracker Green DND-26 (150 nM) was added during final 5 min of incubation. Cells were washed and imaged with a laser scanning confocal microscope. Scale bar = 10 µm. **B**, Cells were incubated with R₁₆ polyplexes (at Z(+/-) 10.0), with and without CS (at GAG:peptide w/w 0.5:1) along with chloroquine (100 µM) and bafilomycin A1 (100 nM) for 5 h at 37 °C. Transfection efficiency was measured after 24 h from luciferase activity. Values are plotted as mean ± S.D. of three independent experiments, each in duplicate (**p < 0.005, *p < 0.01, *p < 0.05). **C**, CHO-K1 cells were treated with R₁₆ polyplexes (with rhodamine-labeled DNA at Z(+/-) 10.0) with or without CS (at GAG:peptide w/w of 0.5:1) for 1 h at 37 °C, then fixed, permeabilized and treated with primary antibody against giantin for 2 h at room temperature. Cells were then incubated with Alexa Fluor 488-labeled secondary antibody for 2 h, washed and imaged with a confocal microscope. Scale bar = 20 µm.

depolymerization seemed to be helpful for the uptake of the CS-coated polyplexes. Yet, co-localization of either polyplexes with the actin filaments could not be observed (Fig. S2). Since the actin filaments and microtubules are known to be active in the various endocytic entry mechanisms and are also required for vesicle transport intracellularly [51–53], the mechanism of entry of the CS-coated polyplexes independent of the cytoskeleton is rather intriguing, as also is its role in the uptake of the native R₁₆ polyplexes. But cytochalasin treatment reduced the transfection efficiency of both the native and the CS-coated polyplexes implying that actin polymerization may play a more important role in the subsequent intracellular processing of both the polyplexes. On the other hand, nocodazole treatment increased the transfection efficiency in most cases. Nocodazole is known to inhibit endosomal maturation by disrupting the microtubules, and therefore polyplexes might be able to escape the lysosomal degradation leading to better transfection efficiency in the presence of this drug [54,55].

We also analyzed whether the presence of the GAG coat which helped in stabilization of the polyplexes and caused changes in the intracellular routing was actually retained during the cellular entry and

intracellular events. We observed greater accumulation of the polyplex around the nuclear periphery in the presence of CS than in the case of the native polyplexes after 4 h of incubation for both the peptides studied (Figs. 2 and 5A); thereby confirming the increased cellular uptake seen earlier for the polyplexes with CS. We also observed co-localization of CS-coated R₁₆ polyplexes with a Golgi marker, giantin, indicating their trafficking to the Golgi network (Fig. 7C). Subsequently, to elucidate the localization of the polyplexes around the nucleus, we checked for the co-localization of the R₁₆ polyplexes with ER-Tracker Green which stains the endoplasmic reticulum and with FM 4-64 which stains vesicular structures (Figs. S6 and S7 respectively in Supplemental information). However, no co-localization was observed under these conditions; hence, the polyplexes were most probably in the cytosol from where they could gain nuclear access. Nevertheless, our results indicated that in all the cell lines used here, the CS coat remained associated to the polyplex. Thus, clearly the GAGs are carried along with the polyplexes and their presence might be conferring both extracellular stability and aid in intracellular accumulation. Sandgren et al. had earlier demonstrated that the protein transduction domain of HIV Tat could

target polyanions including exogenous heparan sulfate intracellularly to the nucleus [56]. Our observation not only corroborates this but also goes one step further to demonstrate that this happens in arginine-rich peptides in different cell types even when they are complexed with nucleic acids.

Since most of the endocytic uptake routes involve endosome maturation and fusion with lysosome, we also studied the intracellular trafficking of the polyplexes after their cellular entry. We observed that both the native and coated R_{16} polyplexes co-localized with the lysosomes to some extent. This was checked both at 2 h incubation and 4 h incubation, the latter being the time point at which the transfection efficiency was measured (Fig. 7A and Fig. S8 (Supplemental information)). At the longer time point, the amount of co-localization of the coated R_{16} polyplex with the lysosomal marker was reduced (as seen from lower Mander's coefficient of co-localization in Fig. S8), while the co-localization of the native polyplex remained same. In view of the fact that lysosomal localization resulted in degradation of the polyplexes, it was important to determine how the polyplexes coated with GAGs could accumulate in large quantities at the nuclear periphery and also lead to enhanced transfection efficiency. For this, we examined the transfection efficiencies of the native and CS-coated R_{16} polyplexes in the presence of endosomal disruption agents such as chloroquine and bafilomycin. Chloroquine treatment increased the transfection efficiency of the native polyplexes by an order of magnitude while the effect on the CS-coated polyplexes was much smaller (Fig. 7B). In addition, bafilomycin treatment reduced the transfection efficiency of the CS-coated polyplexes to a larger extent than the native polyplexes. These results implied that although the polyplexes indeed got entrapped in the endosomes, the CS coat helped in efficient endosomal escape. Yet, the mechanism by which CS can aid endosomal escape is not clear and would be an interesting prospect to explore.

In summary, we demonstrated that addition of small amounts of exogenous GAGs to polyplexes of arginine-rich peptides improved their efficiency of overcoming the various hurdles faced during the process of gene delivery. We have shown that the exogenous GAGs remain associated with the polyplexes during their entire journey by forming a ternary complex with the polyplex, or possibly by forming a 'GAG coat' on their surface. This association enhanced the stability of the arginine-rich polyplexes and offered better protection against nucleases *in vitro* [12]. The GAG-coated polyplexes also showed better cellular association and cell entry than the native polyplexes. Irrespective of the multiple endocytic routes that could be adopted, the presence of GAGs helped the polyplexes to escape the endosomes more efficiently and accumulate at the nuclear periphery. Hence, we demonstrated the potential applicability of addition of exogenous GAGs to arginine-based peptide carriers as a paradigm to improve their gene delivery efficiency without the need for chemical conjugation.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

Acknowledgements

This work was supported by Council of Scientific and Industrial Research (CSIR), Govt. of India (Project CSC0302). RJN thanks the Department of Biotechnology (DBT), Govt. of India for fellowship. RS, DN and GP thank CSIR for fellowship. Help of Mr. Manish Kumar in some of the microscopy experiments is acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbmem.2015.01.012>.

References

- [1] I. Nakase, H. Akita, K. Kogure, A. Gräslund, U. Langel, H. Harashima, S. Futaki, Efficient intracellular delivery of nucleic acid pharmaceuticals using cell-penetrating peptides, *Acc. Chem. Res.* 45 (2012) 1132–1139.
- [2] J. Hoyer, I. Neundorff, Peptide vectors for the nonviral delivery of nucleic acids, *Acc. Chem. Res.* 45 (2012) 1048–1056.
- [3] V.P. Torchilin, Peptide vectors for the nonviral delivery of nucleic acids, *Adv. Drug Deliv. Rev.* 60 (2008) 548–558.
- [4] I.A. Ignatovich, E.B. Dizhe, A.V. Pavlitskaya, B.N. Akifiev, S.V. Burov, S.V. Orlov, A.P. Perevozchikov, Complexes of plasmid DNA with basic domain 47–57 of the HIV-1 Tat protein are transferred to mammalian cells by endocytosis-mediated pathways, *J. Biol. Chem.* 278 (2003) 42625–42636.
- [5] H.H. Kim, H.S. Choi, J.M. Yang, S. Shin, Characterization of gene delivery *in vitro* and *in vivo* by the arginine peptide system, *Int. J. Pharm.* 335 (2007) 70–78.
- [6] I.A. Khalil, S. Futaki, M. Niwa, Y. Baba, N. Kaji, H. Kamiya, H. Harashima, Mechanism of improved gene transfer by the N-terminal stearylization of octarginine: enhanced cellular association by hydrophobic core formation, *Gene Ther.* 11 (2004) 636–644.
- [7] I.A. Khalil, K. Kogure, S. Futaki, S. Hama, H. Akita, M. Ueno, H. Kishida, M. Kudoh, Y. Mishina, K. Kataoka, M. Yamada, H. Harashima, Octaarginine-modified multifunctional envelope-type nanoparticles for gene delivery, *Gene Ther.* 14 (2007) 682–689.
- [8] V.P. Torchilin, T.S. Levchenko, R. Rammohan, N. Volodina, B. Papahadjopoulos-Sternberg, G.G. D'Souza, Cell transfection *in vitro* and *in vivo* with nontoxic TAT peptide-liposome-DNA complexes, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 1972–1977.
- [9] M. Belting, Heparan sulfate proteoglycan as a plasma membrane carrier, *Trends Biochem. Sci.* 28 (2003) 145–151.
- [10] G.M. Poon, J. Gariépy, Cell surface proteoglycans as molecular portals for cationic peptide and polymer entry into cells, *Biochem. Soc. Trans.* 35 (2007) 788–793.
- [11] S.M. Fuchs, R.T. Raines, Pathway for polyarginine entry into mammalian cells, *Biochemistry* 43 (2004) 2438–2444.
- [12] R.J. Naik, P. Chandra, A. Mann, M. Ganguli, Exogenous and cell surface glycosaminoglycans alter DNA delivery efficiency of arginine and lysine homopeptides in distinctly different ways, *J. Biol. Chem.* 286 (2011) 18982–18993.
- [13] J.M. Gump, R.K. June, S.F. Dowdy, Revised role of glycosaminoglycans in TAT protein transduction domain-mediated cellular transduction, *J. Biol. Chem.* 285 (2010) 1500–1507.
- [14] H.L. Amand, H.A. Rydberg, L.H. Fornander, P. Lincoln, B. Norden, E.K. Esbjörner, Cell surface binding and uptake of arginine- and lysine-rich penetrating peptides in absence and presence of proteoglycans, *Biochim. Biophys. Acta Biomembr.* 1818 (2012) 2669–2678.
- [15] A. Subrizi, E. Tuominen, A. Bunker, T. Rog, M. Antopolsky, A. Urtti, Tat(48–60) peptide amino acid sequence is not unique in its cell penetrating properties and cell-surface glycosaminoglycans inhibit its cellular uptake, *J. Control. Release* 158 (2012) 277–285.
- [16] A. Ziegler, Thermodynamic studies and binding mechanisms of cell-penetrating peptides with lipids and glycosaminoglycans, *Adv. Drug Deliv. Rev.* 60 (2008) 580–597.
- [17] A. Walrant, I. Correia, C.Y. Jiao, O. Lequin, E.H. Bent, N. Goasdoué, C. Lacombe, G. Chassaing, S. Sagan, I.D. Alves, Different membrane behaviour and cellular uptake of three basic arginine-rich peptides, *Biochim. Biophys. Acta* 2011 (1808) 382–393.
- [18] M. Magzoub, K. Kilk, L.E. Eriksson, U. Langel, A. Gräslund, Interaction and structure induction of cell-penetrating peptides in the presence of phospholipid vesicles, *Biochim. Biophys. Acta* 1512 (2001) 77–89.
- [19] K.A. Mislick, J.D. Baldeschwieler, Evidence for the role of proteoglycans in cation-mediated gene transfer, *Proc. Natl. Acad. Sci. U. S. A.* 93 (1996) 12349–12354.
- [20] M. Ruponen, S. Ylä-Herttuala, A. Urtti, Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies, *Biochim. Biophys. Acta* 1415 (1999) 331–341.
- [21] M. Ruponen, S. Rönkkö, P. Honkakoski, J. Pelkonen, M. Tammi, A. Urtti, Extracellular glycosaminoglycans modify cellular trafficking of lipoplexes and polyplexes, *J. Biol. Chem.* 276 (2001) 33875–33880.
- [22] M. Tyagi, M. Rusnati, M. Presta, M. Giacca, Internalization of HIV-1 Tat requires cell surface heparan sulfate proteoglycans, *J. Biol. Chem.* 276 (2001) 3254–3261.
- [23] R.J. Naik, A. Chatterjee, M. Ganguli, Different roles of cell surface and exogenous glycosaminoglycans in controlling gene delivery by arginine-rich peptides with varied distribution of arginines, *Biochim. Biophys. Acta Biomembr.* 1828 (2013) 1484–1493.
- [24] D. Vercauteren, R.E. Vandenbroucke, A.T. Jones, J. Rejman, J. Demedter, S.C. Smedt, N.N. Sanders, K. Braeckmans, The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls, *Mol. Ther.* 18 (2010) 561–569.
- [25] W.S. Rasband, ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, 1997–2012. (<http://imagej.nih.gov/ij/>).
- [26] S. Bolte, F.P. Cordelières, A guided tour into subcellular colocalization analysis in light microscopy, *J. Microsc.* 224 (2006) 213–232.
- [27] L.H. Wang, K.G. Rothberg, R.G. Anderson, Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation, *J. Cell Biol.* 123 (1993) 1107–1117.
- [28] T. Akiyama, J. Ishida, S. Nakagawa, H. Ogawara, S. Watanabe, N. Itoh, M. Shibuya, Y. Fukami, Genistein, a specific inhibitor of tyrosine-specific protein-kinases, *J. Biol. Chem.* 262 (1987) 5592–5595.
- [29] L. Pelkmans, D. Puntener, A. Helenius, Local actin polymerization and dynamin recruitment in SV40-induced internalization of caveolae, *Science* 296 (2002) 535–539.
- [30] M.A. West, M.S. Bretscher, C. Watts, Distinct endocytic pathways in epidermal growth factor-stimulated human carcinoma A431 cells, *J. Cell Biol.* 109 (1989) 2731–2739.

- [31] J.A. Cooper, Effects of cytochalasin and phalloidin on actin, *J. Cell Biol.* 105 (1987) 1473–1478.
- [32] J.R. Peterson, T.J. Mitchison, Small molecules, big impact: a history of chemical inhibitors and the cytoskeleton, *Chem. Biol.* 9 (2002) 1275–1285.
- [33] D. Vercauteren, J. Rejman, T.F. Martens, J. Demeester, S.C. De Smedt, K. Braeckmans, On the cellular processing of non-viral nanomedicines for nucleic acid delivery: mechanisms and methods, *J. Control. Release* 161 (2012) 566–581.
- [34] I.A. Khalil, K. Kogure, H. Akita, H. Harashima, Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery, *Pharmacol. Rev.* 58 (2006) 32–45.
- [35] V. Puri, R. Watanabe, R.D. Singh, M. Dominguez, J.C. Brown, C.L. Wheatley, D.L. Marks, R.E. Pagano, Clathrin-dependent and -independent internalization of plasma membrane sphingolipids initiates two Golgi targeting pathways, *J. Cell Biol.* 154 (2001) 535–547.
- [36] J. Rejman, V. Oberle, I.S. Zuhorn, D. Hoekstra, Size-dependent internalization of particles via the pathways of clathrin and caveolae-mediated endocytosis, *Biochem. J.* 377 (2004) 159–169.
- [37] A. Gonzaleznoriega, J.H. Grubb, V. Talkad, W.S. Sly, Chloroquine inhibits lysosomal-enzyme pinocytosis and enhances lysosomal enzyme-secretion by impairing receptor recycling, *J. Cell Biol.* 85 (1980) 839–852.
- [38] E.J. Bowman, A. Siebers, K. Altendorf, Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells, *Proc. Natl. Acad. Sci. U. S. A.* 85 (1988) 7972–7976.
- [39] M.J. Reilly, J.D. Larsen, M.O. Sullivan, Polyplexes traffic through caveolae to the Golgi and endoplasmic reticulum en route to the nucleus, *Mol. Pharm.* 9 (2012) 1280–1290.
- [40] K.M. Fichter, N.P. Ingle, P.M. McLendon, T.M. Reineke, Polymeric nucleic acid vehicles exploit active interorganelle trafficking mechanisms, *ACS Nano* 7 (2013) 347–364.
- [41] A. Pathak, P. Kumar, K. Chuttani, S. Jain, A.K. Mishra, S.P. Vyas, K.C. Gupta, Gene expression, biodistribution and pharmacoscintigraphic evaluation of chondroitin sulfate-PEI nanoconstructs mediated tumor gene therapy, *ACS Nano* 3 (2009) 1493–1505.
- [42] K. Hagiwara, M. Nakata, Y. Koyama, T. Sato, The effects of coating pDNA/chitosan complexes with chondroitin sulfate on physicochemical characteristics and cell transfection, *Biomaterials* 33 (2012) 7251–7260.
- [43] Y. Lo, K. Sung, C. Chiu, L. Wang, Chemically conjugating polyethylenimine with chondroitin sulfate to promote CD44-mediated endocytosis for gene delivery, *Mol. Pharm.* 10 (2013) 664–676.
- [44] A.W. Wong, S.J. Scales, D.E. Reilly, DNA internalized via caveolae requires microtubule-dependent, Rab7-independent transport to the late endocytic pathway for delivery to the nucleus, *J. Biol. Chem.* 282 (2007) 22953–22963.
- [45] K.L. Douglas, C.A. Piccirillo, M. Tabrizian, Cell line-dependent internalization pathways and intracellular trafficking determine transfection efficiency of nanoparticle vectors, *Eur. J. Pharm. Biopharm.* 68 (2008) 676–687.
- [46] I. Nakase, M. Niwa, T. Takeuchi, K. Sonomura, N. Kawabata, Y. Koike, M. Takehashi, S. Tanaka, K. Ueda, J.C. Simpson, A.T. Jones, Y. Sugiyama, S. Futaki, Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement, *Mol. Ther.* 10 (2004) 1011–1022.
- [47] I.A. Khalil, K. Kogure, S. Futaki, H. Harashima, High density of octaarginine stimulates macropinocytosis leading to efficient intracellular trafficking for gene expression, *J. Biol. Chem.* 281 (2006) 3544–3551.
- [48] M.A.E.M. van der Aa, U.S. Huth, S.Y. Hafele, R. Schubert, R.S. Oosting, E. Mastrobattista, W.E. Hennink, R. Peschka-Suss, G.A. Koning, D.J.A. Crommelin, Cellular uptake of cationic polymer–DNA complexes via caveolae plays a pivotal role in gene transfection in COS-7 cells, *Pharm. Res.* 24 (2007) 1590–1598.
- [49] D. Vercauteren, M. Piets, L.J. van der Aa, M.A. Soraj, A.T. Jones, J.F.J. Engbersen, S.C. De Smedt, K. Braeckmans, Flotillin-dependent endocytosis and a phagocytosis-like mechanism for cellular internalization of disulfide-based poly(amido amine)/DNA polyplexes, *Biomaterials* 32 (2011) 3072–3084.
- [50] S. Peng, M.T. Tseng, Y. Ho, M. Wei, Z. Liao, H. Sung, Mechanisms of cellular uptake and intracellular trafficking with chitosan/DNA/poly(γ -glutamic acid) complexes as a gene delivery vector, *Biomaterials* 32 (2011) 239–248.
- [51] S.D. Conner, S.L. Schmid, Regulated portals of entry into the cell, *Nature* 422 (2003) 37–44.
- [52] G. Apodaca, Endocytic traffic in polarized epithelial cells: role of the actin and microtubule cytoskeleton, *Traffic* 2 (2001) 149–159.
- [53] E.E. Vaughn, D.A. Dean, Intracellular trafficking of plasmids during transfection is mediated by microtubules, *Mol. Ther.* 13 (2006) 422–428.
- [54] N. Bayer, D. Schober, E. Prchla, R.F. Murphy, D. Blas, R. Fuchs, Effect of bafilomycin A1 and nocodazole on endocytic transport in HeLa cells: implications for viral uncoating and infection, *J. Virol.* 72 (1998) 9645–9655.
- [55] S. Hasegawa, N. Hirashima, M. Nakanishi, Microtubule involvement in the intracellular dynamics for gene transfection mediated by cationic liposomes, *Gene Ther.* 8 (2001) 1669–1673.
- [56] S. Sandgren, F. Cheng, M. Belting, Nuclear targeting of macromolecular polyanions by an HIV-Tat derived peptide, *J. Biol. Chem.* 277 (2002) 38877–38883.