Published in final edited form as:

Biomacromolecules. 2009 August 10; 10(8): 2189–2193. doi:10.1021/bm900370p.

Virus-Inspired Approach to Nonviral Gene Delivery Vehicles

D Roy Raghunath[†], Joseph Jerry[‡], and S. Thayumanavan^{*,†}

Departments of Chemistry and Veterinary and Animal Sciences, University of Massachusetts, Amherst, Massachusetts 01003

Abstract

The basic TAT peptide, responsible for translocation of the HIV-TAT protein, has been conjugated to a variety of artificial nanoscopic materials to transport them across the cellular membrane. However, attempts to translocate genes using the TAT-peptide had met with limited success. We hypothesized that the cationic nature of the peptide does not allow for displaying these peptides on the surface of the polyplex. To circumvent this potential issue, we have developed a new molecular design strategy where the TAT-peptide can be effectively displayed on the surface of the polyplex, thus enhancing gene expression.

Introduction

Correcting a genetic disorder by artificially incorporating a therapeutic gene into the cellular machinery is one of the attractive options in gene therapy.^{1–3} The promise of successful gene therapy relies on the development of a safe delivery material that can protect exogenous DNA from enzymatic degradation, efficiently translocate it into the nucleus and then allow the gene to be expressed.^{4,5} Gene delivery vehicles can be broadly classified into viral and nonviral ones.^{4,6,7} Viral vectors⁸ efficiently deliver exogenous therapeutic DNA into nucleus with a high possibility of long-term gene expression. While viral vectors exhibit excellent transfection, recent clinical trials raise significant safety concerns, including immunogenic and oncogenic effects.^{9,10} Recombinant viral vectors are also limited in the size of exogenous DNA that it can carry and its safe large scale production. On the other hand, nonviral vehicles have relatively limited safety concerns and could potentially be used for a wide range of sizes of DNA. However, the nonviral vectors generally suffer from poorer transfection efficiencies.¹¹ Therefore, it is necessary that strategies be developed for improving the safety issues of viral vectors or transfection efficiency of nonviral ones. This paper concerns the latter, more specifically on cationic polymers. Among several delivery materials, cationic polymers have gained attention because of their lower cost, robustness, and possibility to improve their biocompatibility.^{12–20} We report here on an approach that is inspired by a viral protein to improve the efficacy of a nonviral cationic polymer vector.

Human immunodeficiency virus (type 1, HIV-1) is a retro-virus that encodes an 86-amino acid protein called transactivator of transcription (TAT).^{21,22} It has been identified that a

*To whom correspondence should be addressed. thai@chem.umass.edu.

[†]Department of Chemistry.

[‡]Department of Veterinary and Animal Sciences.

basic sequence of 10 amino acids, known as the TAT-peptide (GRKKRRQRRR), within this protein is primarily responsible for translocation through the plasma membrane and for reaching the nucleus.^{23–25} Following this finding, it has been shown that conjugation of this peptide can enhance the cellular uptake of nanometer-sized structures such as magnetic and superpara-magnetic nanoparticles,^{26–28} liposomes,^{29–31} and heterologous proteins.³² Considering this well-recognized ability of TAT-peptide to take a variety of cargo across the cell membrane, it is easy to imagine that the incorporation of this peptide onto a cationic polymer chain is attractive for improving gene transfection efficiency. In fact, there are a few reports on utilizing this peptide for gene delivery.^{33–35} While these attempts have resulted in some enhancements in transfection efficiency, we recognize that the full potential of the TAT-peptide based transfection is not yet realized. We hypothesized that this is mainly because the TAT-peptide, being very cationic (primarily Arg- and Lys- units), may be engaged in condensing the negatively charged DNA. Therefore, the peptide is likely to be unavailable for cell surface recognition, a necessary feature for the peptide-mediated translocation. This issue does not exist in the TAT protein itself or other nano-objects that were translocated using this peptide. Here, we present a molecular design that circumvents this issue and thus enhance the gene delivery efficacy of cationic polymers.

Experimental Section

The β -gal reporter plasmid pCMV- β -gal was inserted in XL1 blue bacteria and grown in LB broth. The plasmid was purified using a plasmid purification kit (Qiagen maxi kit) and further purified by ethanol precipitation. Branched polyethyleneimine (PEI; M_w 25 KDa) and the short heterobifunctional linker containing maleimide and *N*-hydroxysuccinamide ester (MAL-propyl-NHS) were purchased from Aldrich. The linear PEI (M_w 25 KDa) was purchased from Polysciences, Inc. and was used as received. The long hydrophilic heterobifunctional linker poly(ethylene oxide) with maleimide and *N*-hydroxysuccinamide ester (MAL-PEO-NHS or MAL-PEG-NHS) of molecular weight 2 KDa were purchased from Creative PEGWorks and used without further purification.

Synthesis of Conjugated Polymers

The branched PEI (2 mg) was dissolved in dry dichloromethane and added to a dichloromethane solution of either NHS-PEO-MAL (460 μ g) or the short bifunctional linker (MAL-propyl-NHS; 61 μ g). The mixture was allowed to stir for 2 h to allow the amine of the PEI to react with the NHS functionality. After the reaction, the solvent was removed and the product was dried in vacuum. The dried product was dissolved in water and was purified by dialysing against distilled water for 48 h. The polymer was lyophilized and then dissolved in HEPES buffer (10 mM, pH 7.2) and used for transfection experiment. The linear PEI was conjugated to the linker by reacting it with NHS-PEO-MAL in 10 mM sodium carbonate buffer of pH 9.0 for 24 h. The extended time was utilized to allow for the secondary amines of the linear PEI to react with the NHS-ester of the PEO linker. The product was purified by dialysing against distilled water for 48 h and then lyophilized.

Preparation of Polyplex

A total of 10 μL of pCMV- β -gal plasmid (0.2 μg) was mixed with 10 μL of polymer solution of appropriate concentration in HEPES buffer (10 mM, pH 7.2) to get different N/P ratios. N and P represent the number of nitrogens in the polymer and number of phosphate groups in the plasmid, respectively, for a particular solution (43 mg of PEI contains 1 mmol of “N”; 1 μg of DNA has 3.3 nmole of “P”; “N” of the TAT peptide is not considered in the N/P calculation for any of the complexes). The mixture was incubated at room temperature for 20–30 min for complete complexation. After the complexation, an appropriate amount of TAT-Cys-SH peptide (1 mg/mL solution in water; peptide and maleimide functionality of equimolar ratio) was added to the previous solution and again allowed 3–4 h for the peptide conjugation to the maleimide functionality of the other end of either short or long linker attached to PEI. These transfecting mixtures are used without further purification. In the case of lipofectamine 2000, 0.5 μL of 1 mg/mL solution was mixed with the plasmid. The complex was then diluted to 100 μL with the serum-free media and used as the transfecting mixture. To conjugate the TAT peptide first to the polymer, the polymer solution (10 μL) was first mixed with TAT-Cys-SH peptide and allowed to react for 3–4 h then 10 μL of plasmid was added to the polymer–peptide conjugate solution.

Cell Culture and Transfection

Human kidney cells (293T) and human breast cancer cells (MCF7) were cultured in Dulbecco's modified Eagle media with F12 nutrient (DMEM/F12) with 10% FBS (Atlanta Biologicals) supplement along with gentamicin and antibiotic antimycotic (Invitrogen Corp.) solutions at 37 °C in 5% CO₂ incubator. For transfection studies, cells were seeded in a 96-well plate at 10000 cells/well/100 μL of media for 24 h. The growth media was then immediately replaced with 25 μL of media without serum and antibiotics. The polyplex solution of 100 μL was added to each well and allowed for transfection for 2 h. The media was replaced with fresh media, and 48 h later, the transfection was measured.

Reporter Gene Transfection Efficiency Assay

The reporter β -gal expression assay was performed following the manufacturer protocol (Promega). In short, after the transfection, the cells were washed two times with 1 $\times$ PBS and then lysed the cells with the lysing buffer. The β -gal protein was then collected and allowed to react with its substrate until (30 min to 5 h) it produced a faint yellow color. It is important to note that a variable time of above reaction allowed us to better compare the relative transfection of different polyplexes. The enzymatic reaction was stopped by addition of 1 M sodium carbonate solution, and the absorbance was measured at 420 nm. An aliquot of 20–30 μL of the cell lysate was used to measure the total amount of the protein for each well. This data was then used to normalize the transfection efficiencies. The total protein content in the lysate was measured by BCA assay kit (Pierce) where the provided albumin protein was used as the control to obtain the calibration curve.

X-gal Staining

The microscopic images of the β -gal transfection were obtained by treating the β -gal expressed cells with β -gal staining solution (X-gal kit, Invitrogen) following the

manufacturer protocol. The transfection protocol used in this experiment is the same as the above reporter gene transfection efficiency assay, except the cells were seeded in a six-well plate at 100000 cells/well. pCMV- β -gal (4 μ g) of plasmid was complexed with PEI, PEI-MAL, and PEI-PEO-MAL, respectively, at a N/P ratio of 50. After allowing the complexation, TAT-Cys-SH was added only to PEI-MAL and PEI-PEO-MAL and incubated at room temperature for the reaction with the maleimide functionality. Following the transfection, the transfected cells were stained with X-gal solution and pictures were taken at 20 $\times$ magnification through light microscopy.

Results and Discussion

Our approach is based on a comb-polymer with the cationic polymer backbone to condense the DNA, while the TAT-peptide is displayed at the distal comb terminus. We hypothesized that a long neutral, hydrophilic linker between the positively charged polymer backbone and the peptide ligand will obviate the continuum of positive charges. Thus, it provides the prospect for the TAT-peptide to be displayed on the surface of the polyplex and thus be available for cell surface recognition. In such a case, the DNA will be selectively and effectively condensed by the polymer backbone. If the cationic peptide is intimately associated with the polymer backbone, the peptide will be less available due to the condensation with the DNA, as schematically shown in Figure 1.

Synthesis of the Polymer and the Polyplex

To test the above hypothesis, we synthesized a comb polymer, where branched PEI (M_w 25 KDa) is the main cationic polymer backbone and poly(ethylene oxide) (PEO or PEG, 2 KDa) is the long hydrophilic linker between the PEI backbone and the TAT peptide. The synthetic approach involved the reaction of the PEI with the bifunctional PEO, as shown in Scheme 1. The PEO contains a maleimide functionality and an activated *N*-hydroxysuccinimide (NHS) ester moiety to react with thiols and amines, respectively. Thus, the primary amine groups in PEI react with the NHS ester to form the covalent amide conjugation. The maleimide functionality on the other end of the PEO spacer is now available for reaction with a thiol. We conjugated the TAT-peptide to this polymer by incorporating cysteine at the C-terminus of the peptide (Scheme 1).

To further ensure that the TAT-peptide is indeed available for cellular recognition, we carried out the conjugation of the TAT-peptide through the maleimide reaction after the formation of the polyplex (polymer/DNA complex), that is, after complete condensation of the negatively charged plasmid. The PEI-PEO comb polymer is used at different ratios to optimize the complexation with the plasmid. Finally, the peptide is added to the complex solution to covalently attach peptide to the surface of the polyplex. In all cases, only 2% of the available amines in PEI were reacted with the bifunctional PEO (based on the feed ratio). Thus, the amount of the TAT-peptide conjugated to the polyplex is less than 2% of the primary amines in PEI. This is because (i) a small percentage of the peptide should be sufficient for enhanced transfection, if our design hypothesis works, and (ii) extensive conversion will reduce the amount of amine functionalities available for condensing the DNA. Also, we measured the size of the particles to be 98 ± 10 nm. The particles sizes were similar for both PEI-based and PEI-PEO-TAT polyplexes.

Transfection Studies

The polyplexes were tested for transfection using a human kidney cell line (293T) with pCMV- β -gal plasmid as the reporter. After 48 h of transfection, the amount of β -galactosidase expression was measured by reacting with the β -galactosidase substrate. The gene expression is then normalized with respect to the inherent amount of cellular protein. The normalization thus eliminates any differences in transfection due to possible cell death.

Results from β -gal expression experiments with PEI-PEO-TAT conjugates show that these polymers perform much better than PEI, which is considered to be among the better cationic polymers for gene delivery. Qualitative comparison of transfection efficiencies between the vehicles could be seen from the difference in the light microscopy pictures (Figure 2). After transfection with the pCMV- β -gal plasmid, the cells were treated with X-gal solution. The X-gal solution stains only the transfected cells to blue color. Although it is a qualitative representation, it does clearly show that the PEI-PEO-TAT polymer outperforms the transfection efficiency of PEI. For more quantitative comparison the relative luminescence from the substrate of β -gal was measured after lysing the transfected cells (Figure 3). These results support our design hypothesis that the TAT-peptide could indeed be used to enhance transfection efficiency. Interestingly, we also observed that that our cationic polymer has surpassed the efficiency of lipofectamine 2000, a commercially available standard for liposome-based transfection agents.

It is important to note that the relative transfections of polyplexes are expressed in RLU/mg of protein. To better compare the transfection between polyplexes, the β -gal reaction was stopped at different time intervals. Thus, the relative transfection number varies with the β -gal reaction time. For the transfection of a set of polyplexes that we have in each of the figures in this manuscript, the measurements were performed under identical conditions. However, it is difficult to compare two sets of data based on the absolute RLU numbers because of the variable time of β -gal reaction for each set of transfection experiments. To calibrate this, lipofectamine 2000 is used as a standard transfecting reagent that provides a reference in comparing different sets of data.

If our design hypotheses were correct, then the transfection efficiency should be lower when the TAT-peptide is conjugated to the polymer before mixing with the plasmid to obtain the polyplex. In fact, at the N/P ratio of 40 or less, such a polyplex afforded only 25% of the transfection efficiency (or lesser) compared to the one where the peptide was attached after the polyplex formation (Figure 4). We also envisioned that at a higher N/P ratio, the sequence of plasmid complexation and peptide attachment should not matter. Because at a high N/P ratio it is statistically possible for a reasonable number of peptide ligands to be displayed on the polyplex surface. We found this was indeed the case when the N/P ratio is 60.

Our hypothesis also should follow that if the long PEO linker is replaced with a much shorter linker, then the peptide will be closely associated with the cationic backbone and thus might be less available for enhancing transfection efficiency (Figure 1). We tested this by conjugating the same cysteine-terminated TAT-peptide by the same procedure but with a propyl linker (labeled as PEI-TAT). Although the efficacies from these vehicles were

understandably better than PEI itself, these were consistently lower than that of the PEO-based ones (see Figure 3 for comparison).

We then asked ourselves whether the observed enhancement in the transfection is due to the display of the TAT peptide or the inherent transfection ability of just the PEI-PEO conjugated polymer. To distinguish between these two possibilities, we conjugated a nonpeptide molecule, ethanethiol, to the PEI-PEO polymer in the place of the TAT-peptide and tested its transfection ability. Transfection ability of the above conjugated PEI-PEO-Et polymer with respect to 293 T cell was found to be unchanged compared to the parent PEI polymer (Figure 5). Thus, this control experiment supports our hypothesis that the observed enhancement in the delivery efficiency is indeed due to the cellular recognition of the TAT peptide.

We also tested the effectiveness of the strategy with a different kind of polyethylenimine to ensure consistency. For this purpose, we attached TAT peptide to the commercially available linear PEI (M_w 25 KDa). The gene transfection efficiency of linear PEI covalently attached to TAT peptide through PEO spacer is compared to the unconjugated linear PEI (Figure 6). The linear PEI-PEO-TAT exhibits enhanced transfection compared to both linear PEI and lipofectamine 2000. Thus it clearly supports our molecular design scope for free display of a charged peptide for cellular recognition. While we are satisfied with the enhancement of transfection efficiencies of cationic polymeric vectors with respect to human kidney cell line 293T (Figure 3), we wanted to ensure that the TAT peptide display in cellular recognition is not special or limited to the above cell line. Thus, we tested transfection efficiency of the TAT conjugated branched PEI for a very different cell line, MCF 7 (human breast cancer cell line). The transfection efficiency is shown in Figure 7. Once again, we were gratified to find that there is a significant enhancement in transfection efficiency.

Conclusion

We have shown here that (i) virus-inspired attachment of the TAT-peptide on cationic polyplexes affords significant enhancement in transfection efficiency, (ii) attachment of the peptide ligand in a postpolyplex formation step affords a better display of the peptide ligand, and (iii) the length of the linker between the DNA-binding polymer backbone and the peptide ligand significantly influences its availability and thus transfection. The approach outlined here opens up new opportunities for not only enhancing the transfection efficiency, but also for effective incorporation of ligands for targeted delivery. Such approaches are part of the ongoing efforts in our laboratory.

Acknowledgments

This work was supported by NSF-MRSEC and the University of Massachusetts President's Science and Technology Initiative program. We thank Dr. Ellen Dickinson and Pardeep Thandi for their generous help.

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Figure 1.

Illustration of the strategy to effectively display cationic peptides on a polyplex surface for recognition. When the positively charged peptide is attached to the polymer backbone with a short linker, the peptide display is not effective for recognition as this is also likely engaged in the DNA complexation (top). The peptide display is enhanced by increasing the length of the neutral and hydrophilic linker (bottom).

Figure 2. Microscopic images comparing the transfection efficiency of PEI-PEO-TAT and PEI. The blue color stained cell represents transfected cell. Cells transfected with pCMV- β -gal plasmid and TAT peptide represent the control.

Figure 3.

Comparison of gene transfection efficiencies of branched PEI, TAT-conjugated to PEI with a short linker, and TAT-conjugated to PEI with a short linker. Lipofectamine 2000 is used as a standard for comparison of transfection efficiencies.

Figure 4. Effect of addition sequence upon transfection efficiency. PEI-PEO-plasmid-TAT represents the addition of TAT-peptide after polyplex formation. PEI-PEO-TAT-plasmid represents the conjugation of TAT-peptide to the polymer prior to polyplex formation.

Figure 5.
Comparison of gene transfection of our PEI-PEO-TAT-based delivery vehicles with PEI-PEO-Et in 293 T cells.

Figure 6. Transfection efficiency of linear PEI-PEO-TAT polymer in comparison to linear PEI and lipofectamine 2000 at different N/P ratios.

Figure 7.
Comparison of gene transfection of our TAT-based delivery vehicles with PEI and lipofectamine with respect to MCF 7 cells.

Scheme 1.
Synthesis of the Comb Polymers PEI-PEO-TAT and PEI-TAT