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Structural rearrangements and chemical modifications in known cell penetrating peptide strongly enhance DNA delivery efficiency

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ABSTRACT

Amphipathic peptides with unusual cellular translocation properties have been used as carriers of different biomolecules. However, the parameters which control the delivery efficiency of a particular cargo by a peptide and the selectivity of cargo delivery are not very well understood. In this work, we have used the known cell penetrating peptide pVEC (derived from VE-cadherin) and systematically changed its amphipathicity (from primary to secondary) as well as the total charge and studied whether these changes influence the plasmid DNA condensation ability, cellular uptake of the peptide–DNA complexes and in turn the efficiency of DNA delivery of the peptide. Our results show that although the efficiency of DNA delivery of pVEC is poor, modification of the same peptide to create a combination of nine arginines along with secondary amphipathicity improves its plasmid DNA delivery efficiency, particularly in presence of an endosomotropic agent like chloroquine. In addition, presence of histidines along with 9 arginines and secondary amphipathicity shows efficient DNA delivery with low toxicity even in absence of chloroquine in multiple cell lines. We attribute these enhancements in transfection efficiency to the differences in the mechanism of complex formation by the different variants of the parent peptide which in turn are related to the chemical nature of the peptide itself. These results exhibit the importance of understanding the physicochemical parameters of the carrier and complex in modulating gene delivery efficiency. Such studies can be helpful in improving peptide design for delivery of different cargo molecules.

1. Introduction

Development of methods to introduce therapeutic molecules like proteins, nucleic acids and drugs into cells has been a key area of biomedical research since the last two decades [1,2]. The cellular membrane is one of the major obstacles for the efficient delivery of such molecules since it allows only selective entry by passive or active transport mechanisms. Different kinds of physical (e.g. electroporation) and chemical methods (e.g. using agents like cationic liposomes, polymers and peptides) have been developed to facilitate the entry of these foreign molecules across the plasma membrane [3-5]. Such methods are preferred over viral routes of biomolecule transport due to the safety concern associated with the latter [6,7]. Peptides constitute a promising class of non-viral vectors as they are easy to synthesize and amenable to modifications for the attachment of different functional moieties [8,9].

Peptides used for nucleic acid delivery can be categorized into two classes: cationic peptides and amphipathic peptides. Cationic peptides are rich in basic amino acids like lysine and arginine and can be used for nucleic acid delivery *in vitro* and *in vivo*[10-14]. Amphipathic peptides are made of both hydrophobic and hydrophilic amino acids. These peptides are classified as primary or secondary depending upon the mutual arrangement of the hydrophobic and hydrophilic residues. In primary amphipathic peptides, one terminus has more hydrophobic amino acids and the other consists of hydrophilic amino acids (e.g., MPG, pep-1, pVEC) [15-18]. However, secondary amphipathic peptides adopt alpha helical structure in presence of the hydrophobic membrane where all the hydrophilic amino acids are arranged on one face of the helix and the hydrophobic amino acids constitute the opposite face (e.g., MAP, KALA, CADY) [19-21]. Most amphipathic peptides possess the ability to translocate across the cell membrane in a temperature and energy independent manner although more recent evidences also point towards endocytotic entry routes [22-24]. Efficient cellular uptake and endosomal escape properties of these peptides are the main reasons for their development as carriers of therapeutic cargo molecules [25–30]. For example, MPG, a primary amphipathic peptide and CADY, a secondary amphipathic peptide have been used as efficient agents for siRNA delivery [31,32]. Similarly the pep series of primary amphipathic peptides has been used for delivery of protein, peptides and PNA molecules using non-covalent association between the peptide and the cargo [33,34]. MAP and KALA have been used for PNA and DNA delivery and pVEC has been shown to deliver PNA and even a 67 kDa protein [18,20,35]. However, the selectivity and efficiency of

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cargo delivery vary considerably among different peptides. For example, CADY can efficiently deliver siRNA but is poor in delivering peptide or DNA; PPTG1 peptide, which shows strong delivery of plasmid DNA into both cultured cells and *in vivo*, is inefficient for the delivery of small nucleic acids [32,36]. Multiple parameters like chemical nature of the peptides, nature of the cargo, strategies for conjugation of the cargo with peptides, ability of the peptide and the peptide–cargo complex to interact with membrane components and mechanism of cellular uptake of the peptide–cargo complexes are involved in the process. It is thus difficult to establish a common set of conditions which are applicable for efficient delivery of all cargoes by all amphipathic peptides. Structure–activity studies are very important in this context. There are few examples in the literature where alterations in the chemical/structural parameters of the peptide have been found to be crucial for improving its cargo delivery efficiency. For example, in case of MPG, one amino acid change in its nuclear localization signal changes its cellular localization from nucleus to cytoplasm and makes it amenable to be used for siRNA delivery [31]. A novel derivative of the peptide CADY termed CADY2, can be used for protein and peptide delivery whereas CADY has been demonstrated to deliver short nucleic acids, in particular siRNA, with high efficiency [36]. Similar examples are also seen in case of fusogenic peptides like GALA in which introduction of several positive charges improves the DNA condensation ability [37]. Other peptides like ppTG1 and ppTG20 [38] which show high gene transfer *in vitro* and *in vivo* have been derived through chemical modification of the membrane destabilizing acidic peptide JTS-1 [39].

In the present work, we have used the peptide pVEC (derived from VE-cadherin) as a model peptide system and systematically changed its amphipathicity as well as the total charge and studied their influence on the ability of the peptide to condense plasmid DNA, the cellular uptake of the peptide–DNA complexes and in turn the efficiency of plasmid DNA delivery. Primary amphipathic peptides were changed to their secondary counterparts by rearranging the amino acids while keeping the composition identical. In addition, variants were created where the number of charges was increased by replacing the three neutral or hydrophobic amino acids with arginine in both primary and secondary amphipathic peptides. Our results interestingly identify a combination of nine arginines along with secondary amphipathicity as a prerequisite for improving the plasmid DNA delivery efficiency of pVEC, particularly in presence of an endosomotropic agent like chloroquine. We show that this variant creates small and monodisperse nanoparticles which exhibit higher cellular uptake as compared to the large, aggregated and primarily hydrophobic complexes formed by pVEC with DNA. Introduction of histidines further increases the DNA delivery efficiency even in absence of chloroquine.

2. Materials and methods

2.1. Materials

Peptides (>95% purity) were custom synthesized by G.L. Biochem (Shanghai) Ltd. The plasmids pEGFP-C1, 4.7 kb (Clonetech) and pMIR-REPORT™ Luciferase, 6.47 kb (Ambion) were amplified in *E. coli* DH5-α and purified using GenElute HP Endotoxin-Free Plasmid MaxiPrep Kit (Sigma). Luciferase assay kit was purchased from Promega. Label IT® Tracker Fluorescein Kit for labeling plasmid DNA was purchased from Mirus Bio Corporation. All other chemicals and cell culture media were from Sigma unless stated otherwise.

2.2. Preparation of peptide–DNA complex (polyplex)

Polyplexes were prepared at different charge ratios expressed as peptide nitrogen per nucleic acid phosphate (N/P), i.e. $Z(+/-)$. Briefly, the plasmid DNA stock was diluted to a concentration of 20–40 ng/μl and added drop-wise to an equal volume of the appropriate peptide dilution while vortexing. The polyplexes were incubated for 30 min at room temperature before performing any experiment.

2.3. DNA condensation assays

The electrophoretic mobility of the polyplexes at different charge ratios was studied using agarose gel electrophoresis. 20 μl of the polyplex having 200 ng total DNA was loaded in each case onto 1% agarose gel containing ethidium bromide. Electrophoresis was carried out at 100 V in 1× TAE buffer (pH 7.4) for 30 min. DNA condensation was also measured from the inhibition of DNA-intercalated EtBr fluorescence signal in the presence of peptides. The assay was carried out in black 96-well format plates (Nunc) where 4.22 μl EtBr (10 ng/μl) and 20 μl DNA (20 ng/μl) i.e., one EtBr molecule per 6 base pairs of DNA, was dispensed in each well and incubated in dark for 5 min at room temperature. 20 μl of peptide solution at increasing charge ratio was then added and the plate incubated for another 10 min in dark. Fluorescence was measured in DTX 880 Multimode detector (Beckman Coulter) using 535 SL EXP 1 excitation and 595 SL EMP 1 emission filters. The fluorescence of DNA with EtBr was taken as the maximum, i.e. 100% and the relative percentage decrease in fluorescence signal was calculated at increasing charge ratio and plotted as percentage of maximum (% of Max).

2.4. Assays for analyzing stability of polyplexes against anionic challenge

Polyplexes were prepared at $Z(+/-)$ of 10 and incubated for 30 min at room temperature. These were treated with increasing amount of anionic agent heparin (H3149-100KU) in wt/wt (heparin/peptide) ratios ranging from 0.1:1 to 3:1, incubated for a further 30 min and run on 1% agarose gel. The amount of the DNA released from the polyplexes was compared with the bare DNA band. The stability of the polyplexes was also checked with EtBr assay. Heparin at increasing amounts, was added to black 96 well plates (Nunc), followed by addition of 20 μl of polyplex and 10 μl EtBr (4.22 ng/μl) and incubated for 15 min at room temperature in the dark. The fluorescence of DNA with EtBr was taken as 100% and the relative percentage increase in fluorescence signal was calculated at increasing concentration of heparin.

2.5. Determination of particle size, zeta potential and morphology of the polyplexes

The mean hydrodynamic diameter and zeta potential of polyplexes prepared at charge 10.0 at 10 ng/μl and 25 ng/μl of DNA concentration respectively in deionized water were measured by Zeta sizer Nano ZS (Malvern Instruments, UK) at a fixed angle of 173° at 25 °C. Minimum of 3 readings were recorded for each sample with replicates. The morphologies of the different polyplexes were analyzed using Atomic Force Microscopy (AFM). The polyplexes were prepared at two different charge ratios (3.0 and 10.0) and incubated for 30 min. The polyplex (4 μl) was loaded between two freshly cleaved pieces of mica to ensure even spreading of solution on mica surface, kept for 2 min and dried in air. Imaging was carried out with 5500 Scanning Probe Microscope (Agilent Technologies, Inc., AZ) using AAC mode in air with 225 μm long silicon cantilevers (Agilent Technologies, Inc.) with resonance frequency of around 75 kHz and force constant of 2.8 N/m. Scan speed used was 1 line/s. Image analysis was performed using PicoImage software.

2.6. Transfection efficiency measurement using luciferase assay

Chinese hamster ovary cells (CHO-K1) were maintained in Ham's F-12K media and MCF-7 cells in low glucose DMEM supplemented with 10% (v/v) Fetal bovine serum (Life Technologies, USA) and