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Contrasting effects of cysteine modification on the transfection efficiency of amphipathic peptides

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ABSTRACT

Delivery of DNA to cells remains a key challenge towards development of gene therapy. A better understanding of the properties involved in stability and transfection efficiency of the vector could critically contribute to the improvement of delivery vehicles. In the present work we have chosen two peptides differing only in amphipathicity and explored how presence of cysteine affects DNA uptake and transfection efficiency. We report an unusual observation that addition of cysteine selectively increases transfection efficiency of secondary amphipathic peptide (Mgpe-9) and causes a drop in the primary amphipathic peptide (Mgpe-10). Our results point the effect of cysteine is dictated by the importance of physicochemical properties of the carrier peptide. We also report a DNA delivery agent Mgpe-9 exhibiting high transfection efficiency in multiple cell lines (including hard-to-transfect cell lines) with minimal cytotoxicity which can be further explored for *in vivo* applications.

1. Introduction

Gene therapy relies on efficient delivery of nucleic acids into cells. A safe, stable and efficient vector is a prerequisite for successful gene therapy. Non-viral vectors which range from liposomes to nanoparticles are being developed for this purpose [1–4]. Cationic lipids and polymers are the most extensively studied non-viral vectors which can deliver nucleic acids by formation of nanocomplexes [5,6]. Although some of them have been taken ahead in clinical trials, most of these systems suffer from poor *in vivo* stability and low transfection efficiency of the nanocomplexes [7–10]. Cell penetrating peptides constitute another promising class of non-viral delivery vector system which is relatively safe, biocompatible and easy to synthesize [11–14]. However, similar problem of efficiency exists with these systems as well. Designing of efficient vectors for nucleic acid delivery thus remains a challenge.

One of the strategies to improve the gene delivery efficiency of polymers/peptides is to improve the stability of the nanocomplexes that they form with DNA. It has been shown that high molecular weight (HMW) polymers/peptides are more potent to deliver DNA as compared to their low molecular weight (LMW) counterparts due to higher stability of the polyplex formed between these polymers and DNA [15–17]. However HMW carriers can be toxic, immunogenic or can activate the complement system [18–21]. The LMW counterparts are safer to use, more homogeneous and easy to modify but because of lower stability, mainly *in vivo*, suffer from lower transfection efficiency [22–24]. Inter-peptide cross-linking of LMW polymers has often been used to improve the transfection efficiency of LMW polyplexes *e.g.* glutaraldehyde cross-linking and disulfide cross-linking [25–27]. Slower reversal of Schiff bases in case of glutaraldehyde cross-linking and oxidative environment outside the cell and reducing environment inside the cell makes disulfide cross-linking more preferred strategy for cross linking of LMW polymers for cargo delivery [25,26,28,29]. Presence of cysteines can also help in membrane anchoring of the polyplexes and in addition, amenable to further modification to molecules like targeting ligands.

When cysteine residues are present in the LMW peptide/polymer, such disulfide bonds can be formed either by oxidative polymerization (*i.e.* by oxidizing the peptide using an oxidative agent such as DMSO) or by template polymerization (*i.e* DNA provides a template to the peptide for disulfide bond formation and polymerization) [27,30–32]. The resultant reducible polycations (RPCs) are likely to have potential for efficient DNA delivery as shown by several groups in the literature [30,33–38] For example, addition of sulfhydryl groups in peptides and polymers has been successfully used to improve stability with less toxicity and higher transfection

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efficiency *e.g.* in $(Lys)_{16}$ and (Cys-(D-R_9)-Cys) peptides [38,39]. Incorporation of different number of cysteines in CWK_{18} and endosomolytic TAT peptide (Tat-10H) showed that peptides having two cysteines at the terminals were most efficient instead of more than two cysteines per peptide or both the cysteines at a single terminal [37,40]. Similarly, presence of terminal cysteines in a series of arginine-rich peptides developed in our laboratory showed very high transfection efficiency in multiple cell lines [41]. Influence of polymerization strategies (*i.e.* whether template polymerization or oxidative polymerization is used) on transfection efficiency has been found to be peptide dependent. Using modified TAT peptide with terminal cysteine residues, it was shown that template polymerization and oxidative polymerization have comparable effect on transfection efficiency but in selected cases, template polymerization showed about 150-fold higher transfection when compared to oxidative polymerization [35]. RPCs formed with Cys-Lys_{10}-Cys peptide by oxidative polymerization showed higher transfection in multiple cell lines [42].

The literature presented above indicates that cysteine modification of LMW polymer/peptide systems is an efficient strategy to improve stability and transfection efficiency of the corresponding polyplexes. However, to the best of our knowledge, there are no studies on whether other physicochemical parameters of the peptide and the polyplex also dictate the effect of cysteine modification. In general, it has been shown that physicochemical properties of the peptide/polymer can influence interaction with cargo, polyplex size and morphology, polyplex uptake and finally transfection efficiency [43–46]. Peptides having similar amino acid composition but different amphipathicity have been shown to exhibit differences in DNA delivery efficiency [45,46].

In this study we attempted to explore whether cysteine modification for improvement of DNA delivery efficiency is controlled by the physicochemical properties of amphipathic peptides. We generated two cysteine modified amphipathic peptides (Mgpe-9 and Mgpe-10) which have the same chemical composition and only differ in their amphipathicity. We studied the effect of cysteine modification on DNA complexation, kinetics of polyplex formation, polyplex size, DNA uptake and transfection efficiency. Further we checked the effect of amphipathicity on polymer formation ability of the peptides and influence of polymerization strategy (oxidative polymerization and template polymerization) on transfection efficiency. We also checked the role of cell surfaces thiols and cellular glutathione in transfection efficiency. The transfection efficiencies of the peptides were also compared with that of commercially available agent Lipofectamine in multiple cell lines including hard to transfect cell lines.

2. Materials and methods

2.1. Materials

Peptides used in the study were custom synthesized (>95% purity) from G L Biochem (Shanghai) Ltd. Peptides were dissolved in de-ionized water at a concentration of 10 mg/ml and stored in small aliquots at −80 °C to avoid repeated freeze − thaw. Plasmid DNA was labeled using Label IT® Tracker Fluorescein Kit purchased from Mirus Bio Corporation. Luciferase assay kit was purchased from Promega. 2,2,2-Trifluoroethanol (TFE) was purchased from Sisco Research Laboratories Pvt Ltd. L-Buthionine-sulfoximine (BSO), 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and Dimethyl sulfoxide (DMSO) were purchased from Sigma. The plasmids pEGFP-C1, 4.7 Kb and pMIR-REPORT™ Luciferase, 6.47 Kb were purchased from Clonetech and Ambion respectively. GenElute HP Endotoxin-Free Plasmid MaxiPrep Kit from Sigma was used to obtain endotoxin free plasmids which were amplified in *Escherichia coli* DH5-α. The purity and concentration of pDNA was determined by the 260/280 UV absorbance ratio. Integrity of plasmid DNA was determined by agarose gel electrophoresis and restriction enzyme digestion. pDNA was stored at −20 °C after dissolving it in sterile nuclease free water. All other chemicals and cell culture media were purchased from Sigma unless stated otherwise.

2.2. Oxidative polymerization

To study the oxidative polymerization, 200 μg of peptide was dissolved in 20 μl of water to which 10 μl of DMSO was added as an oxidizing agent and incubated for 96 h at room temperature.

2.3. Preparation of peptide-DNA complex (polyplex)

Peptide-DNA complexes (polyplexes) were prepared at different charge ratios expressed as peptide nitrogen per nucleic acid phosphate (N/P), or Z (±) using fresh peptides or oxidized peptides.

$$\text{Charge ratio} = \text{Total positive charge of peptide}/\text{Total negative charge of DNA}$$

Briefly, the plasmid DNA stock was diluted to a concentration of 20–40 ng/μl as described in specific experimental details in de-ionized water and added drop-wise to an equal volume of the appropriate peptide dilution while vortexing. The polyplexes were incubated for 30 min or 1 h at room temperature before performing any experiment.

2.4. EtBr exclusion assay

2.4.1. For DNA condensation

Intercalation of ethidium bromide (EtBr) into DNA increases the fluorescence quantum yield of the dye and gives 10 fold higher fluorescence. Binding of the peptide to DNA excludes EtBr from the DNA and that results in decrease in fluorescence intensity. This drop in fluorescence is used as a measure of DNA condensation. The assay was carried out in black 96-well plates (Nunc) where 4.22 μl EtBr (10 ng/μl) and 20 μl plasmid DNA (20 ng/μl) *i.e.*, one EtBr molecule per 6 base pairs of DNA, was dispensed in each well and incubated at room temperature for 5 min in dark. 20 μl of peptide solution at increasing charge ratio was added and further incubated for 10 min in dark. Fluorescence was measured in microplate reader (Infinite 200 Pro, Tecan Inc., Maennedorf, Switzerland) using 535 excitation and 595 emission filters. The fluorescence of DNA only with EtBr was taken as the maximum, *i.e.* 100% and the relative percentage of fluorescence signal was calculated at increasing charge ratios and was plotted as a percentage of maximum (% of Max). Each experiment was done in duplicates at least three times.

2.4.2. For DNA release

Anionic challenge destabilizes the complex and releases DNA. The relative amount of anionic agent required to destabilize the complex is a measure of its stability. The stability of the polyplexes was analyzed by EtBr assay. Increasing amounts of heparin was added to black 96 well plates (Nunc) (according to wt/wt ratios heparin/peptide), followed by the addition of 40 μl of polyplex and 4.22 μl EtBr (10 ng/μl) and incubated at room temperature for 15 min in the dark. The fluorescence of DNA only with EtBr was taken as 100% and the relative percentage of fluorescence signal was calculated at increasing concentrations of heparin.

2.5. Determination of particle size and zeta potential using dynamic light scattering (DLS)

The mean hydrodynamic diameter and zeta potential of polyplexes were measured at 25 °C using a dynamic light scattering device (Malvern, Zeta sizer Nano ZS90) at a fixed angle of 90°. Polyplexes were prepared at charge ratio 10.0 using 50 ng/μl concentration of DNA in deionized water then incubated at room temperature for 1 h and after that the mean hydrodynamic diameter and zeta potential were measured. Minimum of 3 readings were recorded for each sample with replicates.

2.6. SYBR gold exclusion assay

SYBR gold dye shows 1000 folds higher fluorescence (100 times higher than EtBr) when it binds to DNA. If the peptide binds more strongly to DNA and forms more linkages, it should exclude more dye from the DNA and thus more decrease in fluorescence would be observed. The assay was carried out in black 96-well plates (Nunc) where 20 μl DNA (20 ng/μl) and 1× SYBR Gold was dispensed in each well and incubated at room temperature for 20 min in dark. 20 μl of peptide solution according to charge ratio 5.0 was then added and the fluorescence intensity was monitored for 0–120 min (at an interval of 5 min) at an excitation wavelength of 495 nm and emission wavelength of 537 nm using a microplate reader (Infinite 200 Pro, Tecan Inc., Maennedorf, Switzerland). The fluorescence of DNA only with SYBR-Gold was taken as the maximum, *i.e.* 100% and the relative percentage of fluorescence signal was calculated at increasing time points and was plotted as a percentage of maximum (% of Max).

2.7. Ellman's assay

Estimation of free thiols in fresh peptides as well as DMSO treated (96 h) peptides was carried out by incubating 20 μl of peptide solutions at different concentrations with 140 μl of 360 μM of DTNB (or Ellman's reagent) in 1× Phosphate Buffer Saline (PBS). Absorbance measurements were performed after 5 min in microplate reader (Infinite 200 Pro, Tecan) at 405 nm wavelength. Absorbance of known

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