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Novel Rath peptide for intracellular delivery of protein and nucleic acids

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ABSTRACT

In the present study, a novel cell penetrating peptide (CPP) named as Rath, has been identified from the avian infectious bursal disease virus. It has the potential to penetrate and translocate cargo molecules into cells independent of temperature. Additionally, it can deliver oligonucleotide in 30 min and antibodies within an hour intracellular to chicken embryonic fibroblast primary cells. As an ideal delivery vehicle, it has the ability to protect the cargo molecules in the presence of serum, nucleases and has minimal or no cytotoxicity at even higher peptide concentrations studied. The biophysical characterizations showed that Rath has a dominant β structure with a small α helix and has remarkable binding ability with protein and DNA. Thus, the characterization of unique Rath peptide to deliver protein or nucleic acid into the cells with non-covalent interaction could be used as an effective delivery method for various cell based assays.

An improved method for the delivery of cargo molecules into cell is essential for various gene and protein therapies. Although viral vectors remain the most efficient gene transfer system, safety concerns regarding their use in humans have increased the importance of non-viral delivery systems. Furthermore, non-viral delivery has several advantages over viral delivery system as they are simple to use, easy to produce, do not induce specific immune responses, and exhibit lesser cytotoxicity [1].

Cell penetrating peptides (CPPs) or protein transduction domains promote the cellular uptake of various molecules in vitro and in vivo [2].The major drawback of currently available CPP based delivery is the need to covalently conjugate CPP to cargo molecules for each experiment. Hence, CPP which could deliver with non-covalent interaction has attracted the attention for peptide based delivery system. Inspite of discovery of large group of CPP after first discovery of Penetratin 13 years ago [3], very few CPPs has all the attributes needed for the ideal delivery peptide which also lack one or other properties.

In the present study, we have identified a novel CPP from infectious bursal disease virus (IBDV) for the first time which is a member of genus *Avibirnavirus* of the family Birinaviridae. The potential CPP was derived from the C-terminus of VP5 protein and named 'Rath' peptide. Unlike widely used peptides, the development of a single peptide for the delivery of protein as well as nucleic acid with non-covalent interaction has added to versatility of the peptide based delivery system. Interestingly, it has unique properties such as it has low cytotoxicity, dominant β-structure and interaction with wide range of molecules such as nucleic acid and protein. Thus, the attempts were made to prove all the characteristics needed to be the best candidate for peptide based delivery system.

Materials and methods

Peptide synthesis and analysis. A cell penetrating peptide (HHKRRDLPRKPE) and its amphipathic carrier Rath peptide (TPWWRLWTKWHHKRRDLPRKPE) were synthesized by standard solid phase methodology using F-moc chemistry [4]. The specific labeling at N-terminus of bead bound peptide was achieved by conjugating the FITC and analyzed by HPLC.

Transfection of peptides to cells. The overnight grown Vero cells in GMEM supplemented with 10% fetal bovine serum and antibiotics were transfected with 10 μM of FITC labeled 12-mer or 22-mer peptides for 30 min in serum free media. The cells were washed with calcium and magnesium free PBS (pH 7.0) and fixed with 4% paraformaldehyde and washed again with PBS.

For intracellular delivery of other cargo molecules, the preformed complex was made. Approximately, to 100 μl of PBS (pH 7.2) the peptide was added first with subsequent addition of respective oligonucleotide/antibody or plasmid and incubated for 20–30 min at 37 °C. These preformed complexes were overlaid on Vero cell or CEF cells for 30 min to 3 h in serum free media. The cells were finally washed extensively several times with PBS, fixed with 4% paraformaldehyde solution, washed again 3–4 times and visualized under UV light by fluorescence microscope. The experiments were repeated at 4 °C also in which cells were pre-chilled on ice for 2 h and then transfection was carried out as mentioned earlier.

For the plasmid transfection, the Rath–plasmid complex were replaced by GMEM with 5% fetal calf serum at 2 h post transfection, and cells were again incubated for 48–72 h. In positive control, cells were transfected using standard calcium-phosphate method. After 48 and 72 h, cells were washed, fixed and subjected to fluorescence microscopy.

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Flow cytometry. The pGFP-CMV transfected Vero cells were trypsinized with 0.25% trypsin-versene and collected in PBS. The cells were then fixed with 0.2% paraformaldehyde solution for 2 h, washed with PBS and finally resuspended in PBS and analyzed by flow cytometry. GFP positive cells were counted on the basis of 10,000 events from 4 different groups of samples.

Cytotoxicity assay. The cytotoxicity assay was performed using cytoTox 96 (TM) kit as per the manufacturer's recommendations (Promega Inc., USA) after the complex of peptide and DNA transfected to Vero cells. The cell viability was quantitated from the pooled samples from three independent experiments based on lactate dehydrogenase released as a marker of cell damage.

Gel retardation assay. For studying binding Rath peptide binding with plasmid DNA, 100 ng of pGFP-CMV was incubated with different concentrations of Rath peptide (1–5 μg) and complex were incubated at 37 °C for 20 min and checked on 1% agarose gel after staining with ethidium bromide.

Serum protection assay. pGFP-CMV construct was incubated with different concentrations of peptide for 20 min at 37 °C. Then 200 μl of GMEM containing 10% serum was added and incubated again for 6 h at 37 °C. After 6 h, proteinase K (200 mg/ml) was added to each sample and incubated at 37 °C for 1 h to degrade serum proteins. The plasmid was extracted with phenol:chloroform and checked in agarose gel.

Nuclease protection assay. Approximately 200 ng of plasmid DNA pGFP-CMV was incubated with peptide at 37 °C for 20 min and then treated with DNAse I (2.8 U/μl) for 90 min at 37 °C and validated as mentioned above.

Fluorescence spectroscopy. Emission fluorescence spectra of peptide, labeled nucleotide, and antibodies were recorded on a Hitachi F-4500 spectrofluorometer at 20 °C using a slit width of 5/5 nm (exi/emi) with a slow scan of 15 nm/min. A 10 μM phosphate buffered saline; pH 7.0 was used as solvent. The emission spectra were recorded between 310 and 380 nm. A fixed concentration of peptide was titrated by increasing the concentration of plasmid DNA using tryptophan probe in Rath peptide to obtain fluorescence spectra. Further, fluorescence measurements were also performed by titrating a fixed concentration of FITC labeled antibody with different concentration of Rath peptide and the emission spectra were monitored within range of 510–550 nm, keeping excitation at 490 nm. Averaged fluorescence spectra were obtained by taking the average of at last three emission spectra. Titration curves were obtained by plotting relative fluorescence against perturbation or addition.

Circular dichroism (CD) spectroscopy. The CD spectra of peptide as well as its complex with nucleic acid were recorded in 10 μM phosphate buffered saline, pH 7.0 at 20 °C from 200 to 320 nm using a CD-spectropolarimeter (J-810 model, JASCO) with 0.2 cm path length cuvettes. The routine calibration of CD machine was done with d-10-camphosulphonic acid (60 mg/100 ml water). Data analysis and acquisition were performed with attached computer to spectropolarimeter. On an average, six scans were taken with scanning rate 20 nm/min. For structural analysis of peptide, CD measurements were recorded at different concentrations of trifluoroethanol. The spectra of plasmid alone as well as its interaction with peptide were recorded. In order to know the nature of conformation of peptide, CD spectra were obtained in water and with increasing concentrations of trifluoroethanol (TFE), a helicogenic solvent.

Transmission electron microscopy (TEM). The peptide alone and peptide/DNA complex were mixed with equal quantity of 2% PTA. Then all samples were loaded on Farmvar coated grids separately and visualized at different magnification for obtaining the high resolution transmitted image of the peptide and its complex under Transmission Electron Microscopy (A Philips model 100EX).

Results

Identification of novel cell penetrating peptide 'Rath' and its functional validation for in vitro delivery of small oligonucleotides, antibody, and plasmid

A cell penetrating peptide (CPP) and its amphipathic carrier named as 'Rath' peptide identified from VP5 protein of infectious bursal disease virus, were found to localize in the nucleus of cells at both 37 and 4 °C, at 10 μM concentration. Rath peptides delivered 18-mer oligonucleotide molecules equally within the cytoplasm as well as the nucleus of Vero cells within 30 min at 4 °C. Further, incubation of complex for 2 h showed complete internalization into the nucleus (Fig. 1).

Rath peptide was found to deliver IgG antibody into Vero cells at 37 °C as well as 4 °C within an hour. The optimum concentration of 'Rath' peptide was found to be 8–16 μM but no delivery was observed below 4 μM and above 32 μM indicating optimum ratio was essential for the effective delivery of large proteins up to 180 kDa. Whereas, the efficient molar ratio of peptide to protein cargo (IgG antibody) was 285:1–570:1 indicating that more peptide molecules are required to carry one molecule of antibody.

Chicken embryonic fibroblast (CEF) primary cells were also transfected which showed Rath mediated internalization of oligonucleotides and antibody within 30 and 60 min as Vero cells. The optimum Rath peptide concentration for delivery in CEF cells was found to be 4–16 μM for oligonucleotides and 8–16 μM for antibody showing the fluorescence in most of the cells.

To evaluate potential to use as a general transfection reagent, the preformed complex with reporter plasmid was found to be delivered within 2 h with high level of expression evident by intense fluorescence of GFP in cell cytoplasm. The optimum concentration of peptide was found out to be 16–48 μM. The quantitation by flow cytometric analysis showed expression in most of the cells (70% cells).

Evaluation of effect on gel retardation with plasmid DNA, safety of cargo and cytotoxicity

To evaluate the interaction of plasmid and Rath peptide, the complexes were analyzed in agarose gel and showed a strong interaction in retarding plasmid in gel. The plasmid was completely protected with the degradation in the presence of Rath peptide at peptide to DNA ratio after 30:1 due to interaction with DNA in presence of serum protease and DNAse (Fig. 2).

The cytotoxicity was less than 4% at optimum effective concentration of 50 μM of Rath peptide which was the maximum peptide concentration generally used for transfection of DNA and protein into the cell. Even at very high concentration (75 μM), more than 92–95% cells were viable indicating Rath system to be highly safe to use for cell based assay.

Biophysical chacterization of Rath peptide and its interaction with the cargo molecule

Different secondary structures quantitation of Rath peptide showed β type (64.4%) confirmation in water. At 20% TFE concentration, 9.7% of alpha content was introduced whereas with further increase in 30%, TFE concentration up to β content dropped to 60.7% (Fig. 3). The CD spectroscopy also showed that it has titratable interaction with small oligonucleotides and plasmid.

Saturation in quenching on the emission fluorescence of Rath peptide on titration with plasmid DNA was observed at *P*/*N* ratio from 525:1 to 58:1, whereas for antibody at molar ratio of 10:1–15:1 confirming Rath peptide has strong binding with DNA and protein.

The peptide molecules self aggregated to form particles in the nanometer range of 10–60 nm size. As observed in CD spectroscopy, aggregate formation in plasmid DNA/Rath peptide interaction was started at the *P*/*N* ratio of 60:1. These aggregates were analyzed by TEM, which were within the nanometer range of 50 nm, 100 nm and 140 nm in size forming regular size of structures.

Discussion

The cell penetrating peptides (CPP) are the short stretches of amino acid with property to penetrate itself and translocate the cargo molecule after conjugation or complex formation. Various CPP identified are penetratin [3], *tat* derived [5], pVEC [6], model peptides [7,8], and transportan [9]. However, there are differences in the mechanism of internalization and delivery of cargo. Some have the potential to deliver protein other could deliver nucleic acid. In the present study, we have identified the novel CPP from VP5 protein domain of infectious bursal disease virus of poultry and exploited as a carrier for delivery of small oligo-

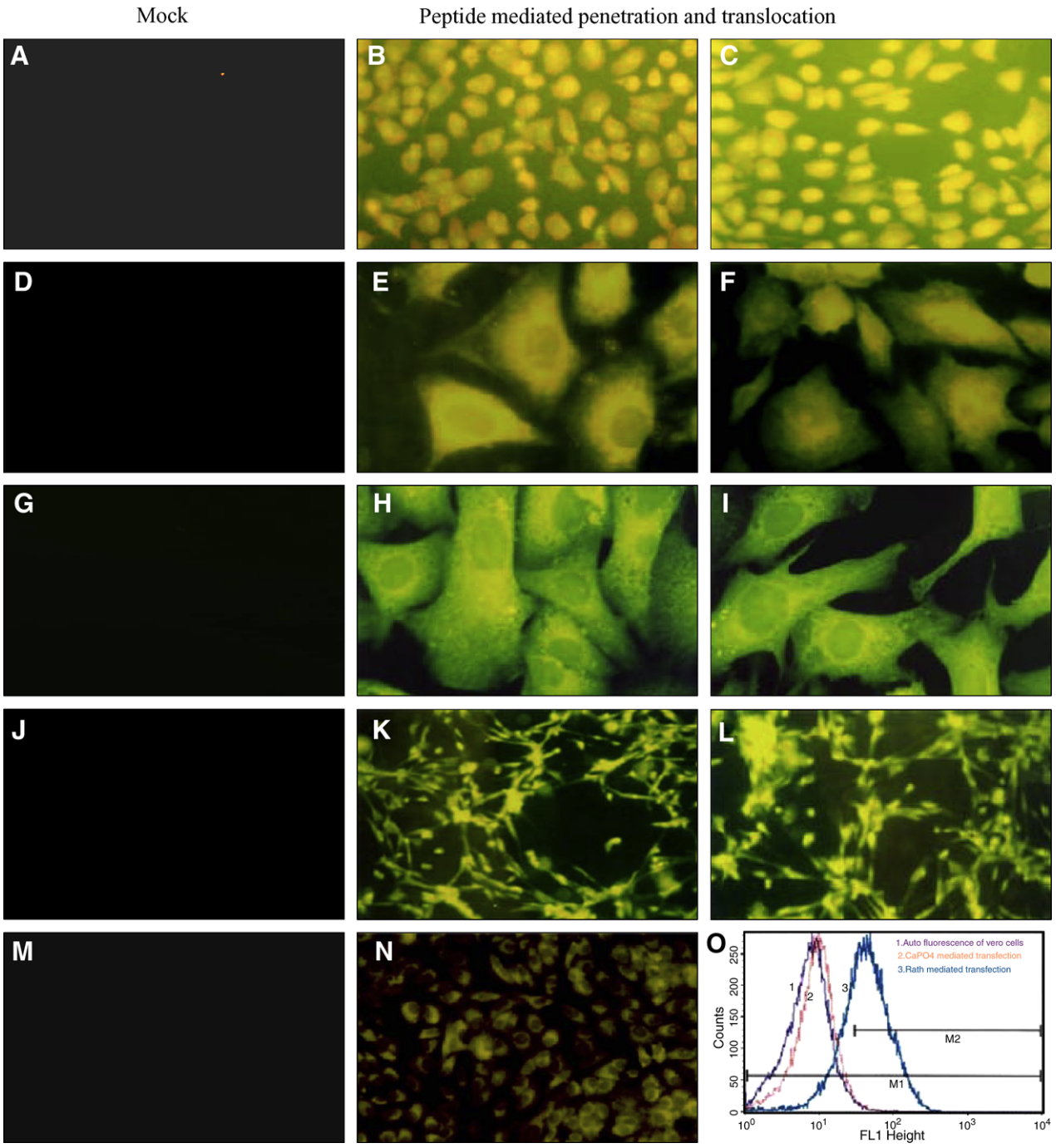

Fig. 1. Penetration and translocation of Cell penetrating peptide and its derivative Rath peptide. Vero cells without peptide as a control (A), incubated with FITC labeled 12 mer cell penetrating peptide (B) and its 22 mer Rath peptide (C) showed the internalization at 4 °C at 10 μM concentration within 30 min. Intracellular delivery of FITC conjugated 18 mer oligonucleotide at 4 °C after 30 min without Rath peptide (D), with shows localization in the cytoplasm and nucleus (E) whereas, after incubation for 2 h most of the oligonucleotides were visible in the nucleus (F). (G–I) Rath peptide mediated transduction of FITC-labeled complete antibody (IgG) to Vero cells at 4 and 37 °C to Vero cells within an hour showed that there is no significant difference in fluorescence at two different temperatures. (J–L) Delivery of 18 mer oligonucleotide and antibody to chicken embryonic fibroblast, primary cells, within an hour at 4 °C. (M–O) Transfection of pGFP-CMV constructs to Vero cells with 16 μM concentration of Rath peptide as for the plasmid DNA and quantitation of expression by green fluorescent protein expression after 48–72 h. Graph 1 represents auto fluorescence of Vero cells, Graph 2 shows percentage of cells showing green fluorescent protein expression using calcium-phosphate method and Graph 3 represents 70% percent of cells expressing green fluorescent protein by 16 μM final concentration of Rath peptide.

nucleotides, plasmid and large protein such as antibody. Further, its initial characterization revealed its unique properties to delivery to cells by non-covalent interaction with cargo molecules, minimal cytotoxicity and an efficient, rapid and safe delivery vehicle for cell based assays. The biophysical studies also confirmed the binding with cargo molecules with unique structure.

Rath peptide was found to penetrate and translocate the cargo at low temperature as well as room temperature which is the feature of endocytosis independent pathway shown by some other peptide such as MPG [10] and third helix of *Antennapedia* [11]. It also shows delivery dependent on time and final concentration of peptide. In order to overcome the major limitations of CPP based delivery system that it needs covalent interaction, we

delivered antibody of around 180 kDa to different cells within an hour without covalent interaction with Rath peptide. This indicates the diversity and rapidity for protein delivery. Although transduction of protein cargo by covalent attachment of protein to CPP through generation of fusion protein product or chemical conjugation with Tat peptide has been demonstrated [12,13] but very few comparative studies are available [14]. Further, it maintains the cargo in a biologically active state and does not interfere with plasmid expression showed by efficient expression reporter plasmid in almost 70% of cells as one of the best general transfection reagents. Similar, applications of TAT peptide [15] and H1 based delivery system [16] has also been reported earlier.

Besides diversity and good transfection ability of carrier peptide, Rath peptide has essential attributes for the ideal delivery of molecules such as (a) the ability to protect the cargo molecule in intracellular environment by enzymatic degradation (b) have good biophysical interaction with the cargo molecule, and (c) most importantly have minimal or no cytotoxicity at effective concentration. The series of experiments attributed Rath peptide as an ideal carrier CPP for in vivo delivery of proteins and DNA. In the biological condition, depending on the results of nuclease and serum protection assays, there was no degradation of DNA at certain ratio of peptide: DNA, which could be due to formation of protective cage around cargo. The phenomenon is explained earlier for the Trp-cage formation [17]. The Rath peptide sequence contains four tryptophan residues creating a highly hydrophobic environment and may be responsible for cage formation which needs to be confirmed further by NMR spectroscopy. Some amphipathic peptides cause damage to the cell membrane [18] and recent study showed that toxicity of CPPs depends heavily on peptide concentration, cargo molecule and coupling strategy [19]. However, Rath was found to be safe to deliver cargo into the cells at the ratios studied in the present report. Even at very high concentration required to achieve delivery, there is negligible or very low cytotoxicity.

Biophysical characterization reconfirmed the unique properties of Rath peptide and specific interactions. It has a dominant β -structure devoid of any α-helical structure similar to peptide pep-1 [20]. It has self-assembling property as well has interaction with protein and nucleic acids cargo molecules forming regular size of particles with plasmid. Previously, the complexes of carrier peptide and plasmid were reported in range of 30–80 nm at plasmid concentration of 20 mg/ml [21]. However, we have not tried to study the detailed mechanism of action. Most cationic CPPs internalized by endocytosis by binding to cell-associated glycosaminoglycans but the detailed mechanisms involved remain controversial [22]. To provide insight into delivery of protein and nucleic acid, it would be interesting to know detailed mechanism of Rath peptide compared to other CPPs.

Thus, the identification and characterization of novel CPP from Infectious bursal disease virus, with the unique properties and tremendous and DNA with non-covalent interaction, might prove good candidate for various cell based assays.

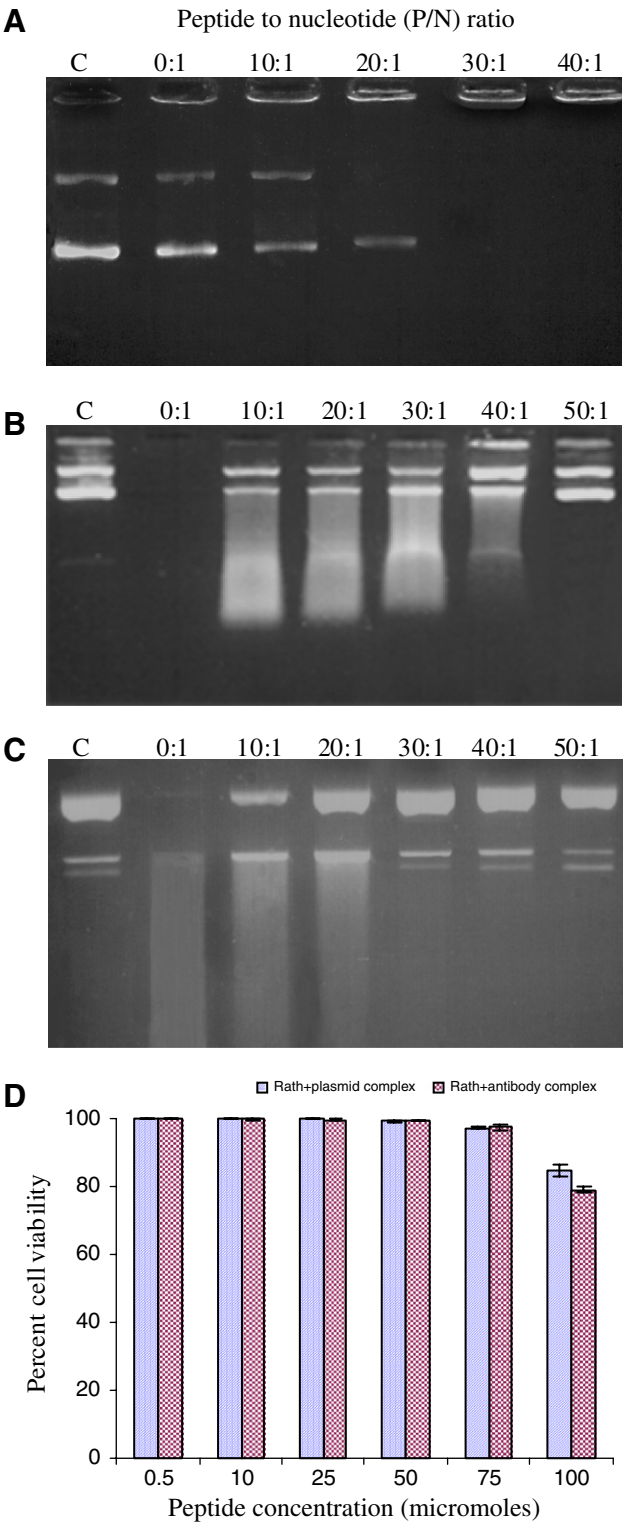

Fig. 2. Evaluation of gel retardation, protection and cytotoxicity of the Rath peptide. (A) DNA binding assay with plasmid DNA in 1% agarose gel where Lane C—denotes 200 ng plasmid as a control without peptide, whereas lanes 1–5 show Rath peptide with 200 ng plasmid at charge ratio 0:1, 10:1, 20:1, 30:1, and 40:1, respectively. The binding was observed at charge ratio 10:1 and 20:1 showing retardation clearly whereas, at 30:1 and 40:1 there was complete retardation in injection well, so no migrated DNA bands were visible in agarose gel in these lanes. (B) The stability of cargo DNA in presence of 'Rath' peptide was checked in serum and the partial protection was achieved at charge ratio 10:1–20:1 and complete protection at 30:1, 40:1, and 50:1 charge ratio. (C) Rath peptide could form cage around the DNA and protect it from DNAse1 degradation. At charge ratio of peptide to DNA 10:1, 20:1, and 30:1 partial protection was achieved whereas, at charge ratio 40:1 and 50:1, it was protected from degradation by nucleases. (D) Percent cell viability in presence of complexes of Rath peptide with the DNA/protein showed that it has very less cytotoxicity. Even at very high concentration (75 μM) needed to deliver plasmid and antibody, 92–95% cell viability was observed showing Rath as a safe delivery vehicle.

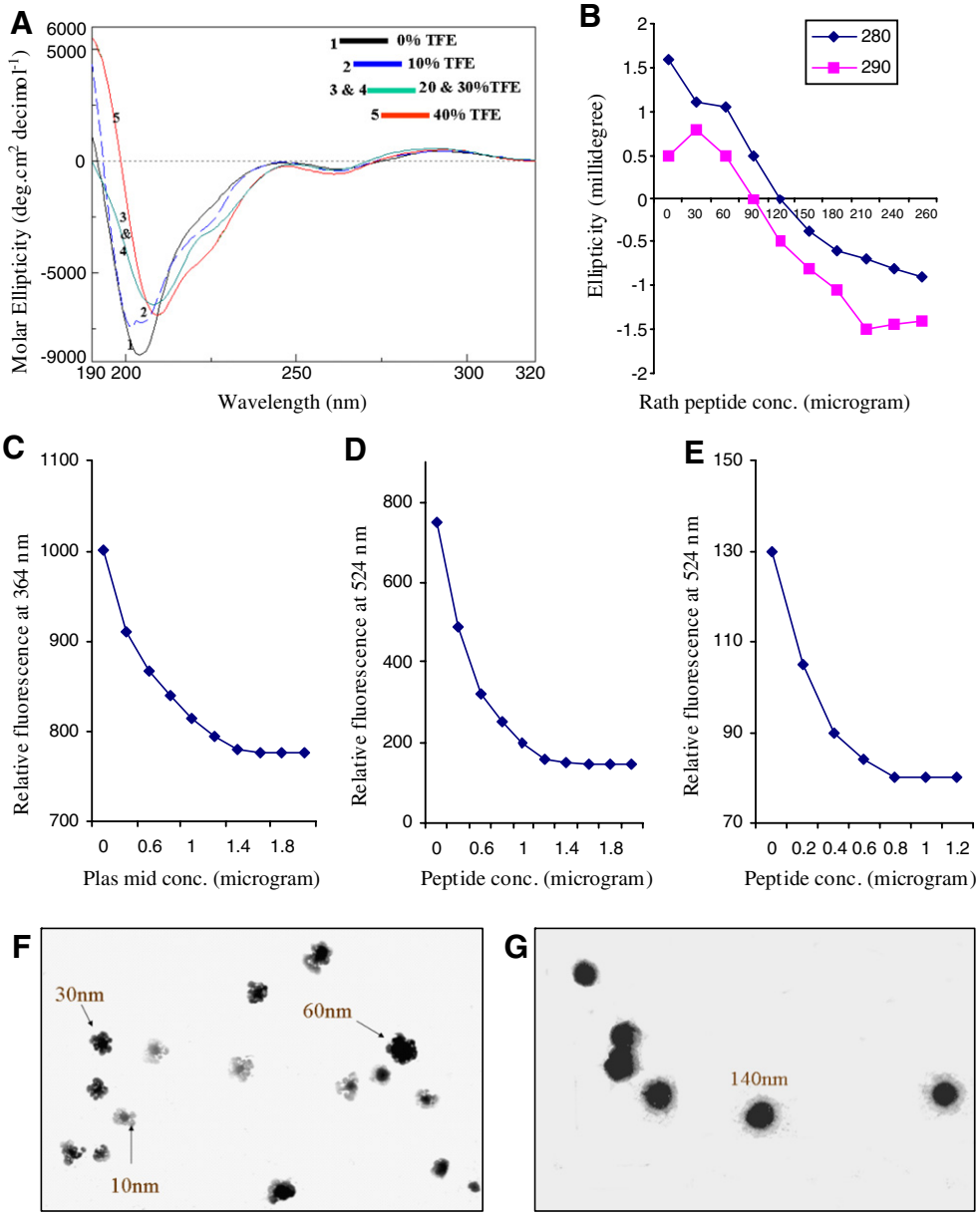

Fig. 3. Biophysical characterization of Rath peptide and its interactions with cargo molecules. (A) Structure determination of Rath peptide in which CD spectra of peptide in water, as a function of increasing concentration of trifluoroethanol from 10 to 40%. (B) CD spectra of plasmid DNA with increasing concentration of Rath peptide fron 0 to 260 μm of plasmid DNA, shows conformational changes in the plasmid DNA after interaction with Rath peptide at 280 and 290 nm. The aggregations were started at the ratio 60:1. (C) Quenching in the relative fluorescence at 364 nm with increasing concentration of plasmid from 0.2 to 1.8 μg of plasmid. (D) The titration of FITC labeled oligonucleotide with the increasing concentration of Rath peptide at 524 nm shows quenching after 10:1. (E) Changes in the relative fluorescence of FITC conjugated antibody after the titration with peptide showed saturation after 10:1 and 15:1 at 524 nm. (F) Self aggregates of Rath peptide were found in the range of 10–60 nm, whereas the complex of plasmid with peptide (G) were approximately 100–140 nm range at *P*/*N* ratio of 60:1.

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