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ORIGINAL RESEARCH

Conformational analysis of Infectious bursal disease virus (IBDV) derived cell penetrating peptide (CPP) analogs

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Abstract

Aim: This study was designed to develop peptide analogs of Infectious Bursal Disease (IBD) virus VP5 protein segment having cell penetrating ability to improve their interaction with cargo molecule (Nucleic acid) without affecting the backbone conformation.

Materials and Methods: IBDV VP5 protein segment designated as RATH peptide were synthesized using solid phase peptide synthesis and their solution conformation was elucidated using CD spectroscopy in polar (water) and apolar (TFE) solvents. Cell penetrating ability of RATH-$CONH_2$ was observed using FITC labeled peptide internalization in to HeLa cells under fluorescent microscopy. The efficacy of RATH analog interactions with nucleic acids was evaluated using FITC labeled oligonucleotides by fluorescence spectroscopy and plasmid constructs in gel retardation assay.

Results: CD spectra of RATH analogs in water and apolar trifluroethanol (TFE) helped to compare their secondary structures which were almost similar with dominant beta conformations suggesting successful induction of positive charge in the analogs without affecting back bone conformation of CPP designed. Cell penetrating ability of RATH $CONH_2$ in HeLa cell was more than 90%. The fluorescence spectroscopy and plasmid constructs in gel retardation assay demonstrated successful interaction of amide analogs with nucleic acid.

Conclusion: Intentional changes made in IBDV derived peptide RATH COOH to RATH $CONH_2$ did not showed major changes in backbone conformation and such modifications may help to improve the cationic charge in most CPPs to interact with nucleic acid.

Keywords: IBDV, conformationa analysis, gel retardation assay, RATH, VP5 protein

Introduction

Cellular delivery of various biological molecules like oligonucleotides, peptides, plasmids, siRNA etc is major thrust areas in newer drug developments. Various strategies have been used to achieve cellular delivery with the use of cationic lipids, polymers; viral vectors reviewed time to time [1-5]. While understanding the mechanisms of delivery using these materials it was observed that none of these system are full proof and suffers from inherent disadvantages like delivery efficiency, cytotoxicity, adverse immune reactions and bio-safety as in case of live viral vectors [6-7]. While addressing these issues new class of cell penetrating peptides (CPP) were discovered and presently gaining importance for cellular delivery of biomolecules such as protein and nucleic acid [8-10]. Delivery of nucleic acids in the form of complete gene, gene construct as plasmid, DNA vaccine and antisense oligonucleotides remains a challenge because these molecules cannot invade cell membrane barriers, otherwise we would have got genetic contamination [11-13]. Therefore to use these molecules for therapeutic purpose delivery vehicles are essentially required, to realize their potential in target cell. Identification of biologically compatible CPP for *in vivo* delivery is important and requires comprehensive biophysical studies which may help to improve their cell penetrating abilities along with its cargo carrying capacity [14-16]. Such studies are important to determine the structural conformation, molecular interaction and charge based interactions of CPP with cargo. Elucidating the conformation of CPP and its interaction with cargo using tools like CD spectroscopy and fluorescence supported by scattering and gel retardation assay may give incite in to the mechanism of cellular transduction. In-silico analysis using bioinformatics tools may allow pre assessment of likely CPP domains and it was successfully done by us to arrive at novel RATH peptides, CPP derived from IBDV [17]. The systematic replacement of the residues by alanine in CPP sequence has been used to know the role of individual amino acids in the CPP for transduction and cargo carrying capacity. Here analogs syntheses of CPP were made to understand the role of

charge on CPP and CPP-cargo complex and importance of positive charge in nucleic acid transduction was established.

Tat protein of HIV which is known to help internalize the whole protein into cell and have role in virus propagation, was reported to have a consensus cell penetrating domains which led to the construction of various CPP analogs used successfully in transduction of nucleic acids in to cells [18-20]. Since IBD is also referred as AIDS of poultry because of the immune-suppression caused by the virus, and role of non structural VP5 protein of IBDV in viral propagation and pathogenesis is well documented. When VP5 protein of IBDV and *Tat* of HIV were subjected to *in-silico* analysis at consensus sequences was found to be present in the C-terminal of VP5 protein of IBDV that helped to construct the RATH peptide as a delivery system to deliver both protein and nucleic acid in primary cell culture (CEF) as well established *Vero cell* line [17]. The analog synthesis of RATH peptide led to a novel peptides for delivering protein and nucleic acids with least cytotoxicity where in the basic NLS (nuclear localization signals) and membrane transduction domains were separated by a SQP peptide spacer [21] We further designed RATH analogs by replacing acidic residue by neutral amino acids with similar dimensions to improve the positive charge on the delivery peptides in turns improve the transfection efficiency of the RATH peptides.

As the transfection efficiency depends on the interactions of CPP with cargo molecules and the conformations of CPP is detrimental to the cargo carrying ability, therefore conformation of designed RATH analogs was investigated *vis a vis* RATH peptide with SQP peptide spacer in solution using CD spectroscopy is being presented in this study.

Materials and Methods

All the chemicals, solvents and reagents used were of analytical grade. N'N' Dimethyl formamide, N' N' dimethyl acetamide, diethyl ether were from Merck (India), piperidine was from SDFCL (India). Fmoc-amino acid derivatives and coupling reagent O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) were from Nova-Biochem (Germany), Diisoprpylcarbodiimide (DIPIC), 4 N'N' dimethyl aminopyridine DMAP and diisopropylamine (DIEA) were purchased from Sigma(USA). The cleavage reagents such as TFA, Phenol, EDT and mercapto-ethanol were from Merck (India). Spectroscopic grade water and trifluoroethanol used in CD experiments were from SDFCL (India).

Synthesis of peptides: Synthesis of RATH peptide analog with SQP peptide spacer having sequence TPWWRLWTKWSQPHHKRRDLPRKPE (RATH-COOH) was carried out using Fmoc chemistry in solid phase peptide synthesis. Wang resin (Nova Biochem Germany, loading efficiency 0.9 mmol/g of resin) was used as solid supports to have free carboxyl group at C-terminal.. Whereas, synthesis of another analog with same spacer having sequence TPWWRLWTKWSQ PHHKRRNLPRKPQ (RATH-$CONH_2$) was performed on Rink amide resin (GL Bioscience, 0.42 mmole/g of resin), this will render charge neutral amide at C terminal, in order to have increased net positive charge on the peptide. In solid phase synthesis first amino acid loading on resin is crucial step and chemistry to be used for coupling is governed by nature of resin used, therefore in case of Wang resin first amino acids Fmoc-Glu (t-butyl) –OH was added using preformed symmetrical anhydrides made using two equivalent of Fmoc-amino acid and one equivalent DIPIC at 0 ºC for 30 min in DMF. While using Rink amide resin Fmoc-amino acid was added directly by making its in-situ active ester using HOBt-HBTU. Always for first amino acid coupling active Fmoc-amino acid was taken 5 times more equivalent to the loading efficiency of the Resin. Loading of first amino acid was determined by quantifying Fmoc group on the resin spectrophotometrically as follows. Loading efficiency or coupling efficiency was determined by estimating Fmoc group per amount of resin, which was removed by treating with 20% piperidine in DMF. The UV absorbance at 290 nm was used for estimating coupling efficiency.
Following steps were performed.

a. Small amount of resin was removed from the vessel, washed four times with DMF and dried by washing with dry methanol.
b. 1 mg of dried Fmoc-glycine resin was taken in micro centrifuge tube in triplicate.
c. 3 ml of freshly prepared 20% piperidine in DMF (v/v) was dispensed in each tube containing 1 mg of Fmoc-glycine resin. The resin beads were agitated for 2-3 min and centrifuged to settle down the resin.
d. The O.D of solution was read at 290 nm considering 20% piperidine solutions as blank.

Coupling efficiency was determined from the correlation Table-1 and mean of three samples was taken.After removal of Fmoc group from anchoring amino acid on the resin, made using treatment of 25% piperidine in DMF (v/v), next Fmoc-amino acids was coupled using HOBt –HBTU activation and coupling for 1hr. DIEA concentration of 6% (v/v) was maintained in coupling reactions to facilitate the amino acids coupling and elongation of peptide chain on the resin. For first amino acid coupling on to resin N'N' Dimethylacetamide was used as solvent instead of DMF used in subsequent couplings and synthesis steps. The schematic of the synthesis used is given in figure-1.

The peptides after deprotection and cleavage from the resins were precipitated using of TFA (cleavage reagents) using dry and chilled diethyl ether (10 fold volumes) and precipitated peptides were filter/ centrifuged and were desalted.

Purifications and characterizations of peptide: The peptides were purified from RP-HPLC using semi-

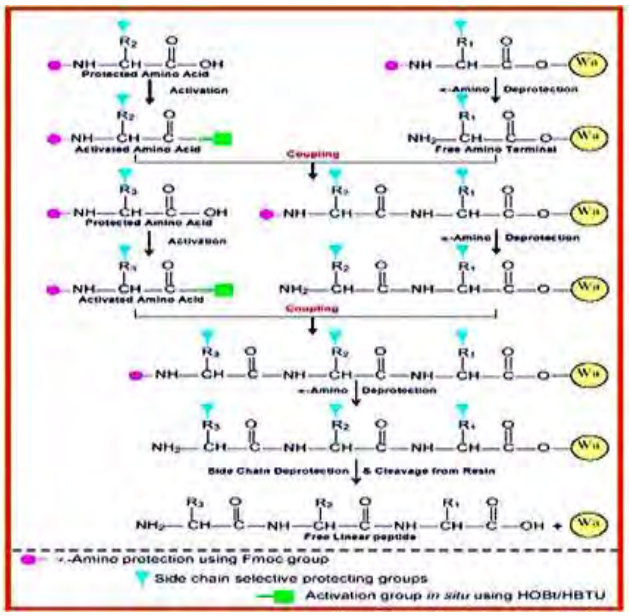

Figure-1. Schematics of solid peptide synthesis

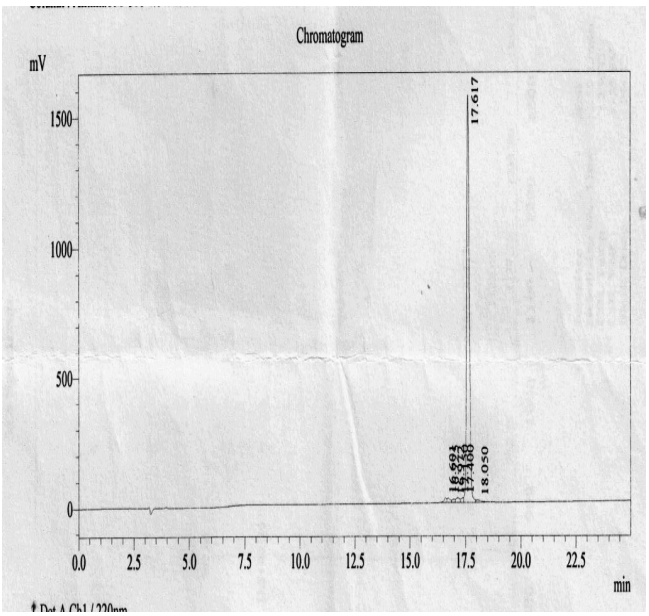

Figure-2. Analysis of CPP analog RATH-COOH by RP-HPLC using Acetonitrile:water gradient .

Table-1. Correlation table for estimation of coupling efficiency.

Fmoc (µ mole)	OD at 290 nm	Fmoc (µ mole)	OD at 290 nm
0.1	0.165	0.6	0.99
0.2	0.33	0.7	1.155
0.3	0.495	0.8	1.32
0.4	0.66	0.9	1.485
0.5	0.825	1	1.65

Table-2. Percent secondary structure of RATH analogues in polar and apolar solution

	Helix			Beta			Random coil			Turn		
	0%TFE	40%TFE	60%TFE	0%TFE	40%TFE	60%TFE	0%TFE	40%TFE	60%TFE	0%TFE	40%TFE	60%TFE
RATH-COOH	2.8	14.6	9	34.6	24	38.6	51.8	47.7	45.6	10.8	13.6	6.8
RATH-CONH2	1.0	10	6.4	42	35.6	42.5	55.8	45.7	49.2	1.2	8.6	1.9

preparative column (RP-C18; 7mm x 30 mm, with 10 micron particles size) and purified peptide was analyzed further on reversed phase analytical column (RP-C18; 4mm x 15mm, with 5 micron particles size) and characterized for molecular weight using Mass spectroscopy. The HPLC analysis of peptides are given in figure-2.

CD spectroscopy: CD machine (JASCO model –J810 spectropolarimeter, Japan) was calibrated using standard solution of D-(+)-10 camphorsulphonic acid in dioxane. A rectangular quartz cell of 0.1 cm path length was used to record the CD spectra of peptide solutions in 190-260 nm wavelength of far UV range. The peptide concentrations were uniformly maintained as 0.1mg/ml of solution in different solvent compositions. The CD spectra were expressed in molar ellipticity, [θ], as a function of wavelength calculated as below

$[\theta] = 100.x.\psi/c.l$ in degree.cm^2.decimole^{-1}

Where, ψ is observed elipticity in milli-degree, c is concentration in mol/lit and lis path length in cm. Quantification of different secondary structure from CD spectra of peptide was carried out using inbuilt Spectra Manager software (Table-2).

Cell culture: Hela cells were trypsinized and seeded in 24 well plate having $1x10^5$ cells per well to form uniform monolayer for 24 hr in DMEM with 10% FBS. Cells were washed twice with serum free media before transfection. The solution of FITC labeled peptides in autoclaved water, 0.1µM-1 µM concentrations filtered through 0.22 µm filters and were overlaid on cells in different wells in 100 µl of serum free media at neutral pH. Peptides were allowed to internalize for 30 min at 37 °C and then cells were washed twice and fixed with 4% paraformaldehyde in 1X PBS (w/v) and peptides internalization was observed under fluorescent microscope (Nikon Japan).

Fluorescence spectroscopy: RATH analogs were titrated with the FITC labeled oligonucleotides and their extrinsic fluorescence was recorded as function of wavelength. A 10 pmole of FITC labeled oligonucleotides were excited at 488 nm wavelengths and emission spectra were recorded from 400-600 nm. Gradual increments of 10 pmole RATH peptide were added to FITC labeled oligonucleotides and spectral changes if any as a result of interaction were recorded. Spectra graphs were plotted from average of six readings at individual wavelength. Spectral changes were observed

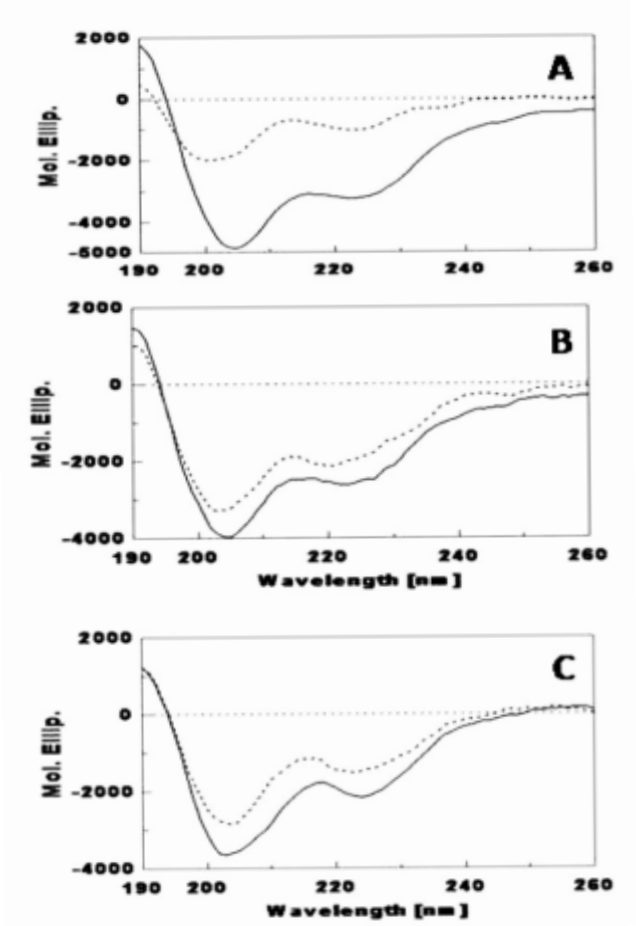

Figure-3. CD spectra of CPP analog namely RATH-COOH (solid line) and RATH-$CONH_2$ (dotted line) in different polar and aploar solutions: A- water; B -40% and TFE C-60%TFE

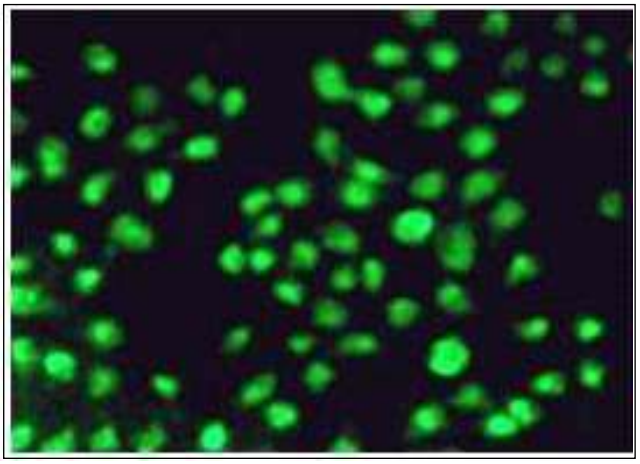

Figure-4. Cellular transduction of FITC labeled RATH-$CONH_2$ in HeLa cells under fluorescence microscope

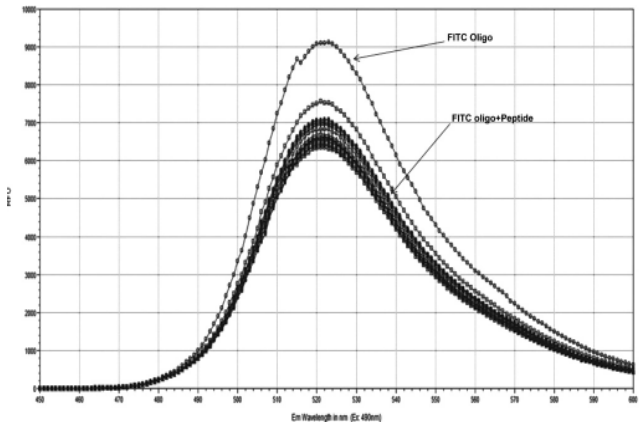

Figure-5. Fluorescent spectroscopy showing interaction of RATH-$CONH_2$ of FITC labeled oligonucleotide

from 400 to 600 nm using Spectra Max M5 Molecular Devices multimode reader in fluorescent mode.

Gel Retardation assay: A fixed amount of 200 ng known plasmid construct was treated with different concentrations of CPP analog RATH-$CONH_2$ at 2.5 to 40 P/N ratios in 10 mM Phosphate Buffer for 20 min in order to form a Peptide Nucleic acid complex. These complexes were run in 0.8% agarose gel electrophoresis and electrophoretic mobility of complexes were seen after staining with ethidium bromide and observed in a UVP gel documentation system.

Result and Discussion

Development and improvement in cellular transduction by developing efficient delivery vehicles is required for advance therapeutic applications of new generation drug molecules in post-genomic era. Use of CPP in drug delivery has proven its superiority over other delivery mechanisms due to reduced toxicity and adverse immunoreactions of their metabolites [17]. In the present study, synthesized CPP analogs as RATH-COOH and RATH -$CONH_2$ were purified by RP-HPLC using acetonitrile: water gradient and characterized by mass-spectroscopy (MS). The HPLC chromatogram of purified peptide RATH-COOH is given in figure- 2. Peptide RATH COOH is having homogeneity in analytical RP-HPLC and eluted at retention time of 17.49 min (figure-2) and showed mass of 3364.3Da which is comparable to the calculated mass of the peptide 33646.3 Da. The chromatograms and mass spectra of other analog RATH-$CONH_2$ obtained are not shown here. In this study we have synthesized a new RATH-$CONH_2$ analog by making some amino acid modifications with aim to increase the positive charge in the peptide to make it more cationic CPP analog to improve cargo carrying ability. Effect of such alteration on secondary structure and *in-silico* analysis was done. As we have replaced the negatively charged amino acids such as Glutamic acid and Aspartic acid, first and seventh amino acids from C terminal respectively that resulted in significant change in pK values of these two analogs of CPP. The pK values of RATH-COOH increase from 11.66 to 12.58 along with considerable increase in positive charge from 5.24 to 7.24 of RATH-$CONH_2$ peptide analog. This improvement in positive charge of peptide RATH-$CONH_2$ make it more cationic a property of CPP that can help in increasing the cargo carrying ability since nucleic acid CPP interaction are charge based phenomenon. The charge on the peptide backbone decides the conformation and conformational stability of peptides/ proteins in solution it was

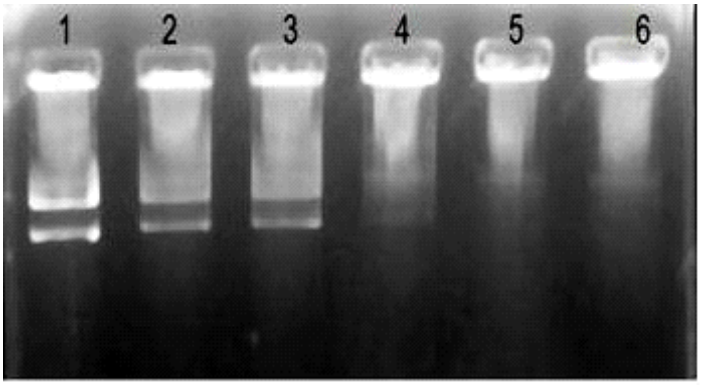

Figure-6. Gel- retardation assay of plasmid construct on interaction with RATH-$CONH_2$ analog at different P/N ratio agarose gel electrophoresis using 0.8% gel.
Lane 1. Plasmid construct
Lane 2. Plasmid-Peptide 2.5/1
Lane 3. Plasmid-Peptide 5.0/1
Lane 4. Plasmid-Peptide 10.0/1
Lane 5. Plasmid-Peptide 20.0/1
Lane 6 Plasmid-Peptide 40.0/1

worthwhile to see the change in conformation of these two peptide analogs of RATH molecules. For this, we attempted to change the charge residue by a neutral residue with same geometry expecting that backbone may not have much change in the secondary structure whereas essential positive charge will be added to the molecule. With this expectation structure of these two peptide analogs were determined by CD spectroscopy. The CD spectra of peptides in water and apolar solvent like TFE were recorded at a uniform concentration of 0.1mg/ml and are shown in figure- 3A, 3B and 3C. For comparison of CD spectra of both peptides in water are shown in Figure- 3A and effect of addition of apolar solvent like TFE is shown in figure- 3B and 3C at 40% and 60% TFE concentration in water (v/v) respectively. It was interesting to observe that both peptides have a similar feature of CD spectra showing double dichroic negative CD troughs in the solvent conditions used in present study, both polar and apolar environment except for a minor increase and decrease in the molar ellipticity values. No significant change was observed in the CD features and it is suggestive of more or less similar secondary structures populations of these peptide analogs in different environment. Therefore, our second analogs RATH-$CONH_2$ having same secondary structures as that of proven CPP peptide RATH-COOH [21] and may be a better CPP for nucleic acid transductions due to higher positive charge as compare to the later molecule.

To evaluate the internalization, RATH-$CONH_2$ peptide was FITC labeled and tested i*n vitro* cell culture in HeLa cells. Different concentrations of this peptide from 0.1µM to 1µM were tested for its cellular uptake and the results of internalization were observed by fluorescence microscopy and found to have approximately 90 % internalization at 1 µM peptide concentrations as shown in figure-4. This internalization study confirms a successful cellular delivery of RATH-$CONH_2$ analog.

In order to see the property as that of a CPP the analog RATH-$CONH_2$ was further studied for its interaction with nucleic acids molecule using extrinsic fluoresce of FITC labeled oligonucleotides in a spectrofluorimeter. The results obtained are depicted in figure- 5 where it is clearly seen that the extrinsic fluorescence maxima observed at 528 nm due to FITC fluorescence is perturbed with incremental addition of RATH-$CONH_2$ peptide. The quenching of 528 nm maximum can be attributed to the interactions of charged peptide (energy sink) with FITC oligo a property essential for a delivery systems to carry cargo molecule. Further interaction of this peptide with a whole plasmid, that can be a DNA vaccine or any other constructs requiring transduction in cell, was evaluated by charge neutralization of the DNA and cage formation around cargo molecule was seen by change in electrophoretic mobility of plasmid DNA as a result of peptide additions to plasmid molecule the results obtained are shown in figure-6. which clearly demonstrate the retardation of plasmid mobility as result of peptide interactions seen in lane no 4, 5 and 6 at P/N ration of 10:1 and above. These results are suggestive of possible use of RATH-$CONH_2$ as delivery vehicle for nucleic acids transduction and improvement in the positive charge with out affecting the backbone conformation as that of proven CPP peptide RATH-COOH [21] may lead to improvement in cargo carrying ability of this newer analogs derived basically from IBDV VP5 segment.

Conclusions

Development of CPP requires a comprehensive biophysical characterization of these novel peptides by analogs synthesis. Here we have demonstrated using CD spectroscopy the retention of similar backbone conformation as that of proven CPP peptide RATH-COOH with improvement in cationic charge on RATH-$CONH_2$ which may improve the cargo carrying ability of CPP further. Successful internalization of RATH-$CONH_2$ in HeLa cells and its interaction with the nucleic acids and plasmids seen by fluorescence spectroscopy and gel retardation assay, speaks about the property of this peptide analog as a possible delivery systems and nucleic acid transduction in to cells.

Authors' Contribution

VGJ, and SK designed the study. VGJ, AKS, KC and MVB performed the experiments. VGJ, AKT and SK analyzed the data. VGJ and SK drafted and revised the manuscript. All authors read and approved the final manuscript.

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Competing interests

Authors declare that they have no competing interest.

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