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NEURONAL DELIVERY OF SMALL INTERFERING (SIRNA) BY CATIONIC LIPOSOME COMPLEX (CLC) *IN VITRO* AND *IN VIVO*.

Microbiology

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ABSTRACT

Objective: Small interfering RNA (siRNA), effectively used for therapy against several diseases. Transfection of macromolecule in neuronal cells is very hard. We used a N2A cells as neuronal model to optimize the siRNA transfection. The study optimized the synthesis of cationic liposome used for the transfection of siRNA followed by determination of silencing efficiency.

Method: In the study we used Neuro 2A (N2A) as neuronal model and Hep2 as non-neuronal model. RVG and RV-MAT peptide were commercially synthesized and chemically tagged with FITC. N2A and Hep2 were cultured in DMEM respectively. BALB/c mice have been used as *in vivo* model.

Results: Liposome were prepared by cationic lipid (DOTMA) and neutral lipid (DOPE), 1:1 molar ratio was found optimal for maximum positive charge on particle (*r = 0.9977, P< 0.001, 95% CI*). Particles achieved particle size below 100 nm in diameter (*r = -0.9925, p = 0.0075, 95% CI*). Cationic liposomal complex (CLC) specifically deliver siRNA in N2A cells and 100 pmole of *GAPDH* specific siRNA significantly silence the expression of mRNA. CLC having *GAPDH* specific siRNA specifically reduced the expression in brain tissue only as compared to other organ *in vivo*.

KEYWORDS

Introduction:

The nucleic acid based therapeutic approaches like anti-sense therapy; gene therapy and RNA interference are attracting the attention of researchers. These nucleic acid based approaches are highly specific and the chances of non-specific targeting are rare. RNA interference (RNAi), a sequence-specific posttranscriptional gene silencing process initiated by double stranded RNA (dsRNA) was originally described as a natural antiviral mechanism in plants.

Efficient gene silencing by RNAi depends upon the activity of siRNA and on the efficient delivery of siRNA in to cells. Hence high transfection efficiency at low amounts of siRNA is desirable for efficient gene silencing especially for *in- vitro* systems. In cell culture studies, delivery agents such as cationic lipids or polymers are required in order to attain significant antisense or siRNA effects. The cationic lipids in the liposomal formulation form complexes with DNA or RNA, leading to their enhanced cellular association.

Cationic liposome is one of most used non-viral vectors for pDNA, and siRNA *in vitro* and *in vivo* (Dass and Choong, 2006; Garcia-Chaumont et al., 2000; Zhang et al., 2007). Cationic liposomes are constituted of cationic lipids and helper lipids, such as DOPE and cholesterol, and have a unilamellar structure with a positive surface charge at neutral pH (pH 7.4). The cationic liposomes offer the attractive ability of complexing with nucleic acid molecules such as pDNA and siRNA. Cationic liposomes–nucleic acids complexes, termed lipoplexes, are basically formed through the spontaneous electrostatic interaction between the positively charged liposome with the negatively charged phosphate backbone of the nucleic acid (Lasic and Templeton, 1996) to transfer them into cells (Keller, 2005; Mahato et al., 1998). The resulting lipoplex can prevent nucleic acid molecules from degradation by metabolic enzymes, such as DNase or RNase, and overcome the electrostatic repulsion of the cell membrane, resulting in enhanced uptake by the cell (de Lima et al., 2001; Felgner et al., 1994; Lasic and Templeton, 1996). Cationic liposome are usually employed as a gene delivery system because of their low toxicity, low immunogenicity, ease of preparation (Koltover et al., 1999), size-independent delivery of nucleic acids, quality control and capacity for mass production at

reasonable cost (El-Aneed, 2004; Faraji and Wipf, 2009; Labat-Moleur et al., 1996).

Material and Methods

Cell culture

Mouse neuroblastoma; Neuro2a (N2A) and human epidermoid cancer cells (Hep-2) was obtained from National Centre for Cell Sciences (NCCS), INDIA. Stable porcine kidney cells (PS) cells were a kind gift from Dr. SudhanshuVrati from THSTI, India. Hep-2 and PS were cultured in Eagle's minimum essential media (EMEM; Sigma USA) supplemented with 10% fetal calf serum (FCS) (Gibco; Invitrogen, USA), penicillin/streptomycin (10000 U/ml penicillin and 10 mg/ml streptomycin) and L-glutamine (2 mM) (Sigma USA) at a constant temperature of 37^{0}C with 5% CO_2(Sanyo, Japan). N2A were grown in DMEM with similar conditions including additional supplement of 1X non-essential amino acid (NEAA) and 100 mM sodium pyruvate (Gibco USA).

Peptides and siRNA

Peptides RVG (test peptide; YTIWMPENPRPGTPCDIFTNSRG-KRASNG), which interacts specifically with the nicotinic acetylcholine receptor, RV-MAT (control peptide; MNLLRKIVKN-RRDEDTQKSSPASAPLDDG), RVG-9R (YTIWMPENPRPGTPCDIFTNSRGKRASNGGGGRRRRRRRRR) and RV-MAT-9R (MNLLRKIVKNRRDEDTQKSSPASA-PLDGGGGRRRRRRRRR) were commercially synthesized and purified by high-performance liquid chromatography (GL Biochem, Shanghai). RVG-9R and RV-MAT-9R peptides were also fluorescently labelled by FITC at amino terminus. In RVG-9R and RV-MAT-9R peptides, the C-terminal nine arginine residues were D-arginine.

Cationic Liposome Complex (CLC)

Liposome was prepared in various molar concentrations of the cationic lipid **DOTMA** [1,2-di-O-octadecenyl-3-trimethylammonium propane] and **DOPE** [1,2-dioleoyl-sn-glycero-3-phosphoethanolamine] (Avanti Polar Lipids, Alabaster, AL) in 1ml chloroform in a round-bottom flask. The solution was dried overnight in a rotatory evaporator. Liposomes (dried thin film of lipid) were rehydrated in 10% sucrose in water at 50 °C for 50 min, followed by incubation for 2 hours at room temperature and sonication. The complex was formed by self-assembly. Briefly, 100 pmole of siRNA (*GAPDH* specific) was diluted in OptiMEM (Invitrogen, Paisley, UK), incubated with 100 pmole of cationic liposome at room temperature followed by 1000 pmole of RVG-9R and RV-MAT-9R (control). The mixture was incubated at room temperature for 30 min. to allow complex formation.

Cellular uptake of peptide

In-vitro

Cellular uptake and distribution of RVG-9R-FITC and RV-MAT-9R-FITC peptide was qualitatively studied by fluorescence microscopy in N2A and Hep2 cells at different conc. Briefly, N2A and Hep2 cells were seeded on cover slip at a conc. of 3×10^5 cells/ml, incubated at 37° C for overnight. Next day the cells were switched in antibiotic and serum free media for 2 hours. The peptides (100 pmole, 1000 pmole and 2000 pmole) were diluted in 200 µl of serum free media containing 100 pmole of cationic liposome followed by addition on cells. Cells were incubated for 4 hours, replenished by growth media. After 24 hour cells were fixed and mounted in ProLong Gold antifade™ (Invitrogen, USA) fluorescent mounting medium and dried at room temperature overnight.

In-vivo

Four nanomole of cationic liposome were incubated with 40 nanomole (4-5 mg/kg body weight) of RVG-9R-FITC or control (RV-MAT-9R-FITC) in 1X sterile PBS for 15 minutes at room temperature. A total 100 µl of liposome- peptide complex were injected systemically by tail vein of BALB/c mice (n= 9). Mice were euthanized 12 hour later, dissected and brain harvested for cryostat sectioning thin smear preparation. Brain was mounted in OCT, 5µm thick sectioned at – 20 °C and mounted in glycerol. Smear was fixed and mounted in ProLong Gold antifade™ (Invitrogen, USA) fluorescent mounting medium and dried at room temperature overnight.

Optimization of siRNA transfection and silencing

In-vitro

GAPDH specific and unrelated Cy-3 labeled siRNA (100 pmol; Ambion, USA) was mixed with cationic liposome (100 pmole) and RVG-9R or RV-MAT-9R with different concentrations in serum-free DMEM for 15 min at room temperature. The complex was then added to N2A cell (plated at 3×105 cells/ml). After incubation for 4 h at 37 °C cells were replenished with 2 ml of fresh medium supplemented with 10% fetal bovine serum (Gibco, USA) and incubated culture for further 12 h before being examined by fluorescent microscopy. For quantitative estimation cells were trypsinized, washed with PBS followed by fixation with freshly prepared 0.5 % PFA. Cell suspension was immediately subjected to analysis by flowcytometry. Lipofectamine ® 2000 was used as positive control, performed in accordance with the manufacturer's instructions.

In-vivo

CLCs (molar ratio of liposome:peptide:siRNA is 1:10:1) having *GAPDH* specific siRNA, was systemically injected 12 hour apart in BALB/c mice (n = 3 each group). Mice were euthanized 48 hour of inoculation, brain, liver, kidney and spleen was dissected out.

RNA extraction

The plates were harvested at defined time intervals; in brief the medium was poured off and washed with sterile PBS followed by trypsinization by 0.05 % Trypsin-EDTA solution. Cells were washed by PBS and centrifuged at 100 g followed by RNA extraction by RNAeasy mini kit (Qiagen) as per manufacturer protocol. The extracted RNA was eluted in 50ul of RNase, DNase and Protease free water, aliquoted into two nuclease free tubes and stored in -70 °C. RNA was treated by DNase to remove any DNA from the RNA preparation. In case of *in-vivo* condition Organs were homogenized in liquid nitrogen and RNA was extracted by Trizol. RNA was estimated by Nanodrop 2000 (Thermo Fisher).

cDNA synthesis

cDNA was prepared by Superscript™III (Invitrogen, USA) as per manufacturers protocol. Briefly, 10 µl of RNA was mixed with 100 nmol of random primers followed by RNA denaturation at 75C for 15 minutes. Tubes were immediately transferred to ice. A mixture containing dNTP, DTT and Superscript III Reverse transcriptase (Invitrogen, CA) was added and reactions was incubated at 25 C for 10 min followed by 42 C for 50 minutes and 75 C for 20 minutes.

Real Time PCR for *GAPDH* knockdown

Relative expression of *GAPDH*m-RNA was estimated by TaqMan Real time PCR. Primers/probe mix was purchased from commercial supplier (ABI, USA). Reaction was setup in 20 µl having 10 µl of TaqMan Universal PCR master mix (2X) (Invitrogen), 1 µl of 20X TaqMan primer and probe and 9 µl cDNA dilutet in RNase-free water. The Gene Knockdown was determined by $2^{-\Delta\Delta}$ Ct method. Data was normalized by TBP (TATA box binding protein for endogenous control).

Results

Liposomes were prepared by mixing differet molar concentration of cationic lipid **DOTMA** [1,2-di-O-octadecenyl-3-trimethylammonium propane] and neutral lipid **DOPE** [1,2-dioleoyl-sn-glycero-3-phosphoethanolamine]. Zeta sizer results suggest that increase in molar ratio of DOTMA relatively increased the zeta potential towards the positivity (*r = 0.9977, P< 0.001, 95% CI*) **(Fig. 1a)**. Results demonstrated that at 1:1 molar ratio of DOTMA and DOPE in liposomal particles achieved maximum zeta potential (59.3 ±3.5mV) as compared to other molar combinations (9.8 ± 1.9v) (*P< 0.001*). Results also indicate that no significant differences in particles zeta potential have been found even after increasing the molar conc. of DOTMA. (*P= 0.359*) **(Fig. 1a).**

The prepared cationic liposome was subjected to sonicate and results suggests that repeated pulse (4 times) of 30 seconds each was optimal to achieve particle size below 100 nm in diameter (*r = -0.9925, p = 0.0075, 95% CI*) **(Fig. 1b).** Sonicated liposome estimated for their size and found majority of them is below 100 nm diameter **(Fig. 1c).**

CLCs (liposome-peptide-siRNA) particles has been analysed by TEM **(Fig. 1d)**, results indicate most of the CLCs was spherical in shape and in range of up to 100 nm diameter. So, 1:1 molar ratio and 4 pulse of sonication were optimized for CLCs having higher particle charge ratio with optimal size, was used for entire set of experimentation.

Cellular uptake of peptide and optimization of siRNA delivery (*In-vitro*)

RVG-9R- FITC has been tested qualitatively for their efficiency to transfect N2A and Hep2 cells. Qualitatively increased uptake of RVG-9R-FITC was observed in dose dependent manner from 100 pmole to 1000 pmole (**Fig 2A and B** respectively). But no significant variation was evident qualitatively in case of further increase of concentration up to 2000 pmole **(Fig. 2C)**. RVG-9R-FITC was also tested for their specificity towards cells of non-neuronal origin. The cellular uptake of RVG-9R-FITC was tested by targeting the cells with 1000 pmole **(Fig. 2D)** and 2000 pmole **(Fig. 2E)**, but failed to enter proven its specificity to neuronal cells only.

Delivery efficiency of CLCs containing 100 pmole of labeled with Cy3 siRNA and 100 pmole of cationic liposome with variable concentration of RVG-9R- FITC peptide was qualitatively assessed **(Fig 3).** Qualitatively no significant differences have been observed in delivery efficiency of CLCs having 1000 pmole **(Fig. 3B)** and 2000 pmole **(Fig. 3C)** of RVG-9R- FITC peptide, but control peptide (RV-MAT-9R-FITC) was found inefficient to deliver Cy3 labeled siRNA in N2A cells **(Fig. 3D, E).** Lipofectamine 2000 was used as positive control for transfection **(Fig. 3A).**

CLC mediated Cy3-siRNA transfection was also assessed quantitatively **(Fig. 4)**. RVG-9R mediated siRNA transfection was found equivalent to L2K **(Fig. 4A).** The optimal transfection was observed at Liposome peptide siRNA in 1:10:1 molar ratio **(Fig. 4C)**

Delivery of peptide *in vivo*

Liposome-peptide complex (molar ratio 1:10) having RVG-9R-FITC or control peptide, was systemically administered in tail vein of BALB/c mice. Mice was sacrificed after 12 hours, brain was cryostat sectioned. The thin smear of brain tissue treated with RVG-9R-FITC **(Fig. 5A)** showed intense fluorescence as compared to control (RV-MAT-9R-FITC) **(Fig. 5B).** The same were also evident in case of cryostat sections **(Fig. 5 C, D),** showed RVG have specificity to cross the BBB.

Optimization of siRNA transfection and silencing

To assess whether CLC carrying siRNA would be able to specifically deliver their cargo in-vitro, we used N2A and Hep2 cells. No statistical significant differences have been observed in mRNA expression level by 100 pmole of siRNA delivered by CLC having RVG-9R when tested in Hep2 (**Fig. 6 A**). siRNA delivered by CLC containing RVG-9R significantly reduced the expression of mRNA at the lower conc. ($P < 0.001$). Silencing by 100 pmole of siRNA was not statistically different as compared to 200 pmole (**Fig. 6B**; $P = 0.21$). The medium containing α-bungarotoxin prevented the inhibition of GAPDH mRNA expression (**Fig. 6B**; $P = 0.80$) proven receptor mediated entry of CLC complex. In case of *in vivo*(**Fig. 6C)**, significant reduction in GAPDH expression was only observed in BALB/c mice brain ($P < 0.001$) compared to other organs (kidney, liver and spleen). The control peptide was tested for N2A cells **(Fig. 6D)** was failed to reduce expression of *GAPDH* mRNA proven specificity of RVG-9R peptide towards the cells of neuronal origin. The results were same even after increase the amount of siRNA (200 pmole; $P = 0.72$).

Discussion:

We chemically synthesized RVG peptide reported from study by kumar et al that a 29 amino acid long Rabies virus glycoprotein (RVG) was able to cross the BBB and carry a siRNA if peptide was modified with poly arginine tailing (Kumar et al., 2007).

In this study we prepared cationic liposome by cationic lipid DOTMA [1,2-di-O- octadecenyl-3-trimethylammonium propane], and neutral DOPE [1,2-dioleoyl- sn-glycero-3-phosphoethanolamine], mixed in different concentration in chloroform. The prepared liposome was subjected to sonicate to achieve unilamilar and small size. After multiple pulses of sonic wave, it was observed most of the particles were below 100 nM in diameter and positive charge, evident by zeta sizer and TEM. The formulation and delivery of siRNA with synthetic nanoparticle formulations to cells faces many of the same challenges as those faced for pDNA delivery (Kwok and Hart, 2011). The size of the nanocomplexes should be less than 200 nm for efficient internalization by endocytic processes (Aoki et al., 2004; Gao et al., 2005). They should be stable and protect the siRNA from nucleases in the extracellular environment, but once inside the cell they should be able to escape endosomal/lysosomal compartments before possible degradation, then dissociate to release the siRNA in the cytoplasm (Jeong et al., 2011).

In this study the liposome, peptide and siRNA in different molar ratio at room temperature, allowed to form a complex which is self-assembled molecular structure, which was optimized to package and deliver siRNA. Previous studies have shown the importance of the liposome component of CLCs for plasmid transfection efficiency and so this component was assessed for optimal siRNA transfection. Liposome, peptide and siRNA was mixed in different conc. and optimized for transfection qualitatively and quantitatively. Similar findings were conferred by pullford et al. in prion based study (Pulford et al., 2010). CLC formulations comprising modifications and variations of the lipid and peptide components have been developed for *in vitro* transfer to a range of cell types (Meng et al., 2004; Parkes et al., 2002; Uduehi et al., 2003; White et al., 2003; Writer et al., 2006) and *in vivo* gene transfer to lung (Tagalakis et al., 2008), vascular tissues and tumors.

It was previously reported that RVG29 can bind specifically to the AchR on neuronal cells and might be internalized into the cells via receptor-mediated endocytosis. Thus, N2A cells expressing the AchR were selected as an *in vitro* model to screen and optimize the CLC stoichiometry. Our results showed that the highest transfection efficiency was achieved with the CLC containing RVG-9R at molar ratio of 1:10:1 of liposome, peptide and siRNA respectively. The quantitative analysis based on the delivery of Cy3-siRNA revealed that liposome-RVG-9R could deliver siRNA into N2A cells but not into the receptor-negative Hep2 cells. More remarkably, the transfection efficiency of lipo-RVG-9R toward N2A cells was approximately 1-fold higher compared with that of Lipofectamine 2000. *In vitro* experiments showed the specificity of CLC (RVG-9R) for N2A but not for hep2 cell. Results are in agreement with pulford el al. (Pulford et al., 2010).

To assess whether CLC loaded with siRNAs would be able to specifically deliver their cargoes *in vitro*, we used N2A and Hep2 cells. Both cell lines were treated for 48 h with Cy5-labeled unrelated and GAPDH specific siRNA alone (siRNA), delivered by CLC and L2K. High delivery efficiency was confirmed by fluorescence microscopy, suggesting that CLC mediated delivery of siRNA can be as efficient as state-of-the-art transfection reagents. Most of the siRNA was found deposited in the cytoplasm. This contrasts with branched PEI (25 kDa), which is a less effective delivery reagent and deposits siRNA predominantly in the nucleus (Kwok and Hart, 2011). The CLC delivered siRNAs were detected intracellularly as early as 6 hour after theinitiation of transfection incubations, which is in line with the observations following L2K transfections (Griesenbach et al., 2006).

In our study we found dose dependent knockdown where we used previously validated GAPDH siRNA *in vitro* and *in vivo*. Further, specifically blocking the acetylcholine receptor (the cellular target of the RVG peptide) with α- bungarotoxin, substantially reduced the knockdown of GAPDH, showing that the RVG required the receptor for cellular uptake and targeting specificity. Repeated Injection of CLC having GAPDH specific siRNA (three dose of 50 ug each) produced a significant GAPDH silencing in brain only, inferred its neuronal specificity. Other organs (Liver, kidney and spleen) did not express any variation in GAPDH. The results are in agreement with Erviti et al, reported brain specific delivery of exosome (Alvarez-Erviti et al., 2011).

The similar combination of components of CLC could be used further for delivery of other macromolecule across the BBB.

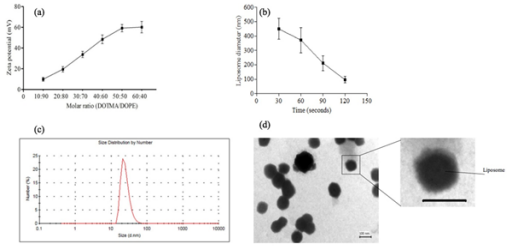

Figure 1 Liposome preparation and characterization. (a) Cationic liposome was prepared by using various molar ratios of DOTMA and DOPE and 1:1 was optimal for maximum zeta potential (n = 5; mean ± SD). **(b)** Liposome was sonicated by 4 pulse of 30 sec. each for particle distribution under 100 nm in diameter (n = 5; mean ± SD). **(c)**

Representative graph from zeta sizer for particle size. **(d)** Representative picture of Transmission Electron Microscopy (TEM) of cationic liposome.

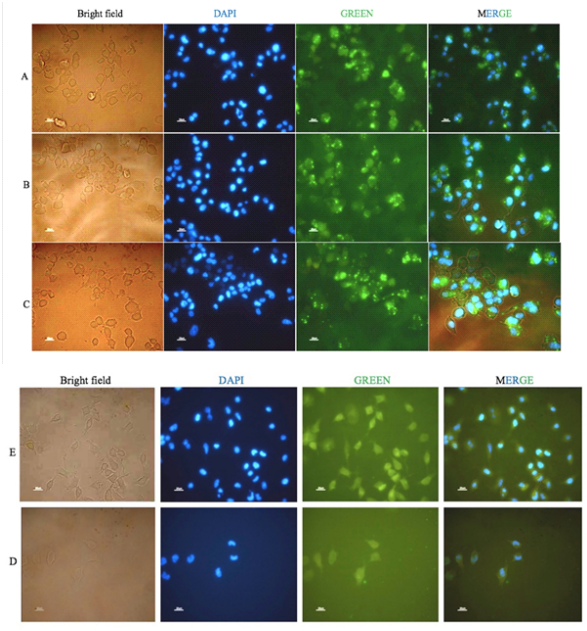

Figure: 2 Qualitative cellular uptake of RVG-9R peptide labelled with FITC. (a-c) N2A cells were treated with RVG-9R-FITC in different concentration; **(A)** 100 pmole, **(B)** 1000 pmole and **(C)** 2000 pmole.Hep2 (non-neuronal) cells treated with 1000 pmole**(D)** and 2000 pmole**(E)** of RVG-9R-FITC.

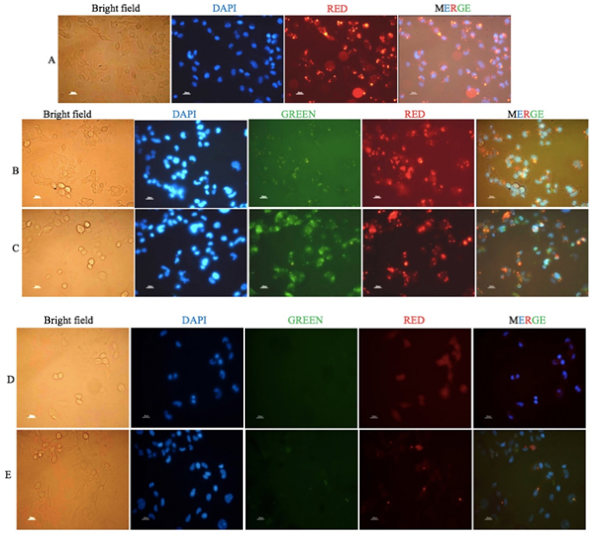

Figure 3 Qualitative delivery of Cy3 labeled siRNA (100 pmole) in N2A cells. N2A cell treated with CLCs containing 100 pmole of cationic liposome and **(B)** 1000 pmole and **(C)** 2000 pmole of RVG-9R-FITC. Delivery of Cy3 labeled siRNA (100 pmole) by CLCs having 1000 pmole**(D)**and 2000 pmole**(E)**of RV-MAT-9R-FITC. Lipofectamine 2000 used as positive control for transfection**(A)**.

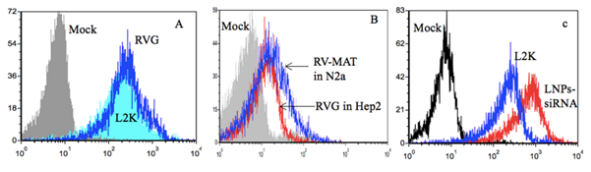

Figure 4 Delivery of Cy3 labeled siRNA in N2A cells estimated quantitatively by flow-cytometry. (A) Delivery of fluorescent labeled siRNA (100 pmole), complexed with RVG-9R (1000 pmole). **(B)** Hep2 and N2A cells were treated by RVG-9R-Cy3 complex. **(C)** Transfection of Cy3 labeled siRNA by CLCs (molar ratio of liposome:peptide:siRNA 1:10:1). Untreated cells act as mock and L2K (lipofectamine 2000) as positive control for transfection.

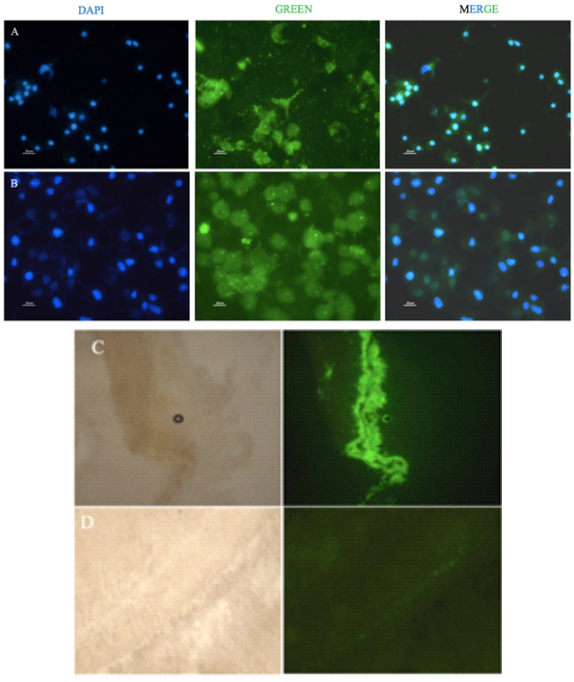

Figure 5 RVG-9R-FITC peptide delivery across the BBB. Thin smear of brain tissue, systemically administered by CLC (molar ratio 1:10) complex containing RVG-9R-FITC **(a)** and control (RV-MAT-9R-FITC) **(b)**. Representative cryostat section of BALB/c mice brain treated i.v. by RVG-9R- FITC **(c)** and control **(d)**.

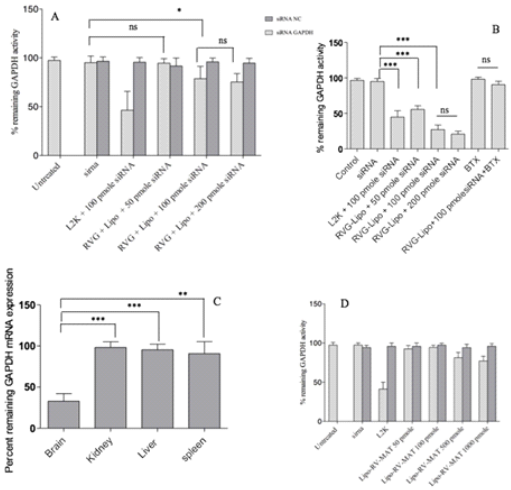

Figure 6 In-*vitro* and *in-vivo* inhibition of *GAPDH* gene (a) Inhibition of *GAPDH* in non-neuronal cells (Hep2 cell) by CLC containing RVG-9R (1000 pmole)-liposome (100 pmole) and different concentration of siRNA (50, 100, and 200 pmole). **(b)** RTN containing cationic liposome (100 pmole) and RVG-9R (1000 pmole) transfected different concentration of *GAPDH* specific siRNA (50, 100 and 200 pmole) in presence or absence of α bungarotoxin (500 nM). (c) *GAPDH* expression in different organs of BALB/c mice treated with RTN containing *GAPDH* specific siRNA (50 µg; 4 mg/kg of body wt.). (d) Delivery of *GAPDH* specific siRNA (100 pmole) by RTN containing cationic liposome (100 pmole) and different concentrations of control peptide (50, 100, 500 and 1000 pmole) in N2A cells.Relative expression of *GAPDH* was estimated by real time PCR ($2^{-\Delta\Delta ct}$), compared with control (untreated cells) and normalized with TBP. L2K with 100 pmole of siRNA act as a positive silencing control. *, ** and *** indicates $P < 0.05$, $P < 0.01$and $P < 0.001$ respectively. ns indicates non-significant.

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