Journal of Medical Microbiology and Infectious Diseases

JoMMID
ISSN: 2345-5349
eISSN: 2345-5330

In vitro Delivery of HIV-1 Nef Antigen by Histidine-rich nona-arginine and Latarcin 1 peptide

Fatemeh Namazi[1], Azam Bolhassani[2]*, Seyed Mehdi Sadat[2], Shiva Irani[1]

[1]Department of Biology, School of Basic Science, Science and Research Branch, Islamic Azad University, Tehran, Iran;
[2]Department of Hepatitis and AIDS, Pasteur Institute of Iran, Tehran, Iran

ARTICLE INFO

Original Article

Keywords: Therapeutic vaccine, HIV-1 Nef, Cell-penetrating peptides, Transfection

Received: Jul. 27, 2019
Received in revised form: Oct. 13, 2019
Accepted: Oct. 14, 2019
DOI: 10.29252/JoMMID.7.4.107

***Correspondence**
Email: A_bolhasani@pasteur.ac.ir
Tel: +98 21 64112240
Fax: +98 21 66465132

ABSTRACT

Introduction: The Nef accessory protein is an attractive antigenic candidate in the development of HIV-1 DNA- or protein-based vaccines. The most crucial disadvantage of DNA and protein-based vaccines is their low immunogenicity, which can be improved by cell-penetrating peptides (CPPs) as effective carrier molecules. **Methods:** In this study, the HIV-1 Nef protein was generated in the *Escherichia coli* expression system for *in vitro* delivery using a novel CPP, Latarcin 1 peptide, in a non-covalent manner. Also, the Histidine-rich nona-arginine peptide was utilized to transfer the HIV-1 Nef gene. The size, morphology, and zeta potential of the complexes were evaluated by scanning electron microscopy (SEM) and Zetasizer. The efficiency of cell transfection was studied using a fluorescence microscopy and flow cytometry for the DNA/CPP complexes and western blot analysis for the protein/CPP complexes. **Results:** The Nef protein generated in the BL21 strain migrated as a dominant band of ~30 kDa in SDS-PAGE. The SEM data confirmed the formation of stable complexes with a size below 200 nm. MTT assay demonstrated that the complexes did not represent any considerable cytotoxic effect compared to untreated HEK-293T cells. The results of fluorescence microscopy, flow cytometry, and western blotting revealed that the Nef DNA and protein constructs could be significantly transfected into HEK-293T cell line using these CPPs. **Conclusion:** These data suggest that the Histidine-rich nona-arginine peptide and Latarcin 1 peptide as CPPs can be considered as a promising approach in the development of the HIV-1 vaccine for gene or protein delivery.

INTRODUCTION

Human immunodeficiency virus (HIV) infection is one of the leading causes of mortality in the world, and by 2017, 36.9 million people were estimated to live with this infection [1]. According to genetic features and antigen variations, HIV comprises two types, *i.e.*, HIV-1 and HIV-2 [2]. The HIV genome consists of two single-stranded RNA molecules enclosed within the core of the virus and includes about nine genes encoding structural, regulatory, and accessory proteins involved in the viral life cycle [2-4]. Among the accessory proteins encoded by the HIV genome, Nef protein is an attractive vaccine target; it is involved in viral pathogenesis, is expressed early in the viral life cycle, and harbors several T and B cell epitopes [5]. Nef is a 27-32 kDa protein, which is associated with high viral load and progression to AIDS [6]. Depending on its intracellular localization, Nef protein interferes with signal transduction pathways or modulates cell surface expression of many membrane-associated proteins in infected cells [7]. Nef is essential for virus survival because it reduces the detection of infected cells by CTLs through down-regulation of MHC-I molecules and allows the evasion of the immune system and inhibits the apoptosis of infected cells [8, 9]. This protein prevents the induction of antiviral immune responses by inhibiting MHC class II [8].

Previous trials have shown that humoral or cellular responses alone are not sufficient to control HIV infection, and an effective vaccine should stimulate both arms of the immune system [10]. DNA and protein-based vaccines are a successful approach for the generation of antigen-specific immune responses, but due to poor immunogenicity of both strategies, it is necessary to use efficient methods to increase their potency. One of these methods is the combination of antigens with active carrier molecules [11]. Recently, cell-penetrating peptides (CPPs) received particular attention as efficient cellular delivery vectors. The CPPs are short cationic or amphipathic peptides that can enter the cells and mediate uptake a wide range of macromolecular cargo such as plasmid DNA, proteins, peptides, nanoparticles and drugs [12, 13]. Therefore, it is essential to find novel and non-toxic CPPs capable of transporting various cargoes [14].

[DOI: 10.29252/JoMMID.7.4.107]

Histidine-rich nona-arginine (HR9), a cationic peptide containing 21 amino acids, enter the cells through direct translocation. Guanidinium-head groups of arginines are thought to initiate HR9's contact with the cell membrane. Hydrophobic cysteines and histidines help the formation of a pore, and the peptides translocate by diffusing on the surface of the pore [15]. Latarcin 1 peptide (LDP) is an amphipathic peptide derived from Lachesana tarabaevi spider toxin that influences the permeability of biological membranes [16].

In this study, we evaluated the potency of novel cell-penetrating peptides for the delivery of Nef gene and protein in HEK-293T mammalian cell line. We utilized HIV-1 Nef accessory protein as the antigenic model for the development of HIV-1 therapeutic vaccine in the future.

MATERIAL AND METHODS

Construction of the recombinant eukaryotic and prokaryotic expression vectors. The pEGFP-N1-*nef* eukaryotic expression vector and pET23a-*nef* prokaryotic expression vector were previously provided in our laboratory. The full-length *nef* gene [17] was subcloned from the pUC19-*nef* vector into the *Nhe*I/*Pst*I cloning sites of the pEGFP-N1 and the *Eco*RI/*Sal*I sites of the pET-23a vector.

Generation of HIV-1 Nef protein. In order to express the Nef protein, BL2 (DE3) *Escherichia coli* strain was transformed with the recombinant pET23a-*nef*. Then, the single clone was cultured in LB-broth at 37˚C. The precultured cells were added to Ty2x medium and induced with 1 mM IPTG (Sinaclon, Iran) when the culture reached an OD_{600} of 0.7-0.8. The expression of the Nef protein was optimized at different incubation times (*i.e.*, 2, 3, 4, and 16 h) after induction at 37˚C. The cell pellets were harvested and analyzed by 12.5% SDS-PAGE. The Nef protein was purified by affinity chromatography using a Ni-NTA agarose column under native conditions (*i.e.*, 300 mM imidazole buffer, pH=8) by increasing the concentration of imidazole according to the manufacturer's instructions (Machery-Nagel, Germany). Further purification of Nef protein was carried out using the reverse staining method [18]. Then, the pure protein was dialyzed against PBS1X, and its concentration was evaluated by Bradford protein assay kit (Thermo Fisher Scientific, USA) and NanoDrop spectrophotometer (Thermo Scientific, USA), and stored at -70˚C until used. Finally, endotoxin contamination was assessed by Limulus Amebocyte Lysate (LAL) QCL-1000 (Lonza, USA).

Preparation and characterization of the Nef DNA/HR9 complexes. The HR9 cationic peptide (CHHHHRRRRRRRRRRHHHHHC) was synthesized by Biomatik Corporation (Canada). For preparation of the non-covalent pEGFP-N1-*nef*/HR9 complexes, the HR9 solution was added to 2 µg of pEGFP-N1-*nef* at different N/P ratios (*i.e.*, molar ratios of basic amino acid residues in the HR9 peptide to DNA phosphates) of 0.5, 1, 2, and 5 in apyrogenic water, and incubated for 45 min at room temperature. The formation of the pEGFP-N1-*nef*/HR9 complexes was confirmed by gel retardation assay. The size and morphology of the complexes at N/P ratio of 5 were investigated using a scanning electron microscope (FEI Quanta 200 ESEM, Philips, Netherlands). In addition, the zeta potential of the complexes was evaluated by the Zetasizer Nano ZS instrument (Malvern Instruments, UK).

Preparation and characterization of the Nef protein/LDP complexes. The LDP-NLS amphipathic peptide (KWRRKLKKLRPKKKRKV) [16] was synthesized by Biomatik Corporation (Canada). The formation of non-covalent Nef/LDP-NLS complexes with molar ratios of 1:2, 1:5, 1:10, 1:15, 1:20, and 1:30 (1 µg Nef protein) was performed in PBS and incubated for 60 min at room temperature. The presence of the complexes was confirmed by 12.5% SDS-PAGE. The size and morphology of the complexes were determined at a molar ratio of 1:10 (protein: CPP) using a scanning electron microscope (FEI Quanta 200 ESEM) (Philips, Netherlands). Also, the zeta potential of the complexes was assessed by the Zetasizer Nano ZS instrument (Malvern Instruments, UK).

Cell viability assay. HEK-293T cells (Human Embryonic Kidney 293T, Pasteur Institute of Iran) were cultured in RPMI 1640 medium (Kowsar Biotech co, Iran), supplemented with 5% FBS (Fetal Bovine Serum) (Biosera, France), 1% Gentamicin (Sigma, USA) at 37˚C and 5% CO_2 and after several passages, the cells (10^4 cells/well) were seeded into 96-well plates in RPMI 1640 medium supplemented with 5% FBS and cultured for 16 h at 37˚C in 5% CO_2. Then, the culture medium was replaced with fresh RPMI 1640. In order to evaluate the cytotoxicity of compounds, the pEGFP-N1-*nef* (2 µg), Nef protein (1 µg), HR9 (9.3 µg), LDP-NLS (10 µg), and pEGFP-N1-*nef*/HR9 complexes with an N/P ratio of 5, as well as, Nef/LDP-NLS complexes with a molar ratio of 1:10 [15, 16] were added to the cells and incubated for 48 h at 37˚C and 5% CO_2 without exchanging the media. After 48 h, the culture medium was removed and the cells were incubated with the MTT (5 mg/ml in PBS) (Sigma-Aldrich, Germany). After 3 h of incubation, the media was removed and the formazan crystals were dissolved in DMSO (Dimethyl Sulfoxide, Sigma-Aldrich, Germany). The absorbance was measured at 570 nm by a microplate reader. The untreated cells were used as a negative control (100% cell viability).

Transfection of HEK-293T cells using the DNA/CPP complexes. HEK-293T cells (5×10^4 cells/well) were seeded into 24-well plates (Greiner, Germany) in RPMI 1640 medium supplemented with 5% FBS at 37˚C in 5% CO_2 and the medium was replaced by the serum-free medium when the confluency of the cells reached approximately 80%. The pEGFP-N1/HR9 and pEGFP-N1-*nef*/HR9 complexes at N/P ratio of 5 were added to the cells and incubated for 1 h at 37˚C. Then, the HEK-293T cells were supplemented with fresh RPMI 1640, 10% FBS in a total volume of 250 µL medium without removal of the complexes overlay, and repeatedly incubated at 37˚C [15]. Moreover, TurboFect transfection reagent (Thermo Scientific, USA) was used as a DNA transfection reagent and positive control according to the manufacturer's instructions. Finally, the transfection efficiency of the complexes was monitored by fluorescence microscopy (Envert Fluorescent Ceti, Korea) and quantified by a FACSCalibur flow cytometer (Sysmex Partec, Germany) at 48 h post-transfection.

[DOI: 10.29252/JoMMID.7.4.107]

Transfection of HEK-293T cells using the protein/CPP complexes. In order to evaluate the transfection of the Nef protein/LDP-NLS complexes, HEK-293T cells (5×10^4 cells/well) were seeded into 24-well plates (Greiner, Germany) in RPMI 1640 medium supplemented with 5% FBS at 37˚C in 5% CO_2 atmosphere and the medium was replaced by the serum-free medium when the confluency of the cells reached approximately 80%. Then, 100 µl of Nef/LDP-NLS complexes (at a molar ratio of 1:10) was applied to the well and incubated for 2 h at 37˚C in an atmosphere containing 5% CO_2. Moreover, TurboFect (Pro-Ject ™ Reagent) was applied as a protein transfer reagent and positive control according to the manufacturer's instructions (Fermentas, Germany). Finally, the cells were harvested, and the delivery of the Nef protein was confirmed by western blot analysis using an anti-His antibody (Abcam, UK).

Statistical analysis. Statistical analysis (Student's t-test) was performed by Prism 5.0 software (GraphPad, San Diego, California, USA) to analyze the viability and cell transfection. The value of $p<0.05$ was considered statistically significant. Similar results were obtained in two independent experiments.

RESULTS

Generation of the HIV-1 Nef protein. The Nef protein was expressed in BL21 strain (DE3) *E. coli* at 16 h after induction, and successfully purified under native conditions. The Nef protein migrated as a bright band of ~30 kDa in SDS-PAGE (Fig. 1). The concentration of the Nef protein was 0.5 mg/mL.

Evaluation of the Nef DNA/HR9 complexes. The negatively charged pEGFP-N1-*nef* plasmid interacted with the HR9 cationic peptide for the generation of nanoparticles. The pEGFP-N1-*nef*/HR9 complexes did not migrate into the agarose gel at N/P ratio of 5, indicating the formation of complexes (Fig. 2). Moreover, the size and morphology of nanoparticles were analyzed by SEM, as shown in Fig. 3. The size of ~150-250 nm and non-spherical (cubic) shape were observed for HR9. SEM analysis of pEGFP-N1-*nef* plasmid showed a spherical shape with a size of ~150-250 nm. The average size of ~150-200 nm was observed for pEGFP-N1-*nef*/HR9 nanoparticles with a spherical shape. On the other hand, zeta potential analysis of pEGFPN1-*nef*/HR9 complexes was approximately 24.5 mV. Moreover, the pEGFPN1-*nef* plasmid had a surface charge of -9.27 mV with dynamic light scattering. This result indicated that the addition of HR9 to pEGFP-N1-*nef* reduced the size and increased the positive charge of the complexes.

Evaluation of the Nef protein/LDP complexes. The formation of Nef/LDP-NLS complexes was confirmed by SDS-PAGE analysis. As observed in Fig. 4, a chemical dissociation was detected as a band of ~30 kDa related to HIV-1 Nef protein, along with the LDP-NLS peptide band in SDS-PAGE indicating the formation of Nef/LDP-NLS complexes over a range of molar ratios of 1:2, 1:5, 1:10, 1:15, 1:20 and 1:30. Indeed, the formation of Nef/LDP-NLS complexes was detected using SDS-PAGE as two individual bands, indicating non-covalent interaction. Moreover, the size and morphology of nanoparticles were analyzed by SEM (Fig. 5). The size of ~400-500 nm and non-spherical (cubic) shape were observed for LDP-NLS peptide. SEM analysis of Nef protein showed a spherical shape with a size of ~300-450 nm. The average size of ~100-200 nm was observed for Nef/LDP-NLS nanoparticles with a non-spherical (pyramidical) shape. On the other hand, zeta potential analysis of Nef/LDP-NLS complexes was approximately 5.5 mV. Moreover, the Nef protein had a surface charge of -24.9 mV with dynamic light scattering. This result indicated that the addition of LDP-NLS to Nef protein reduced the size and increased the positive charge of the complexes.

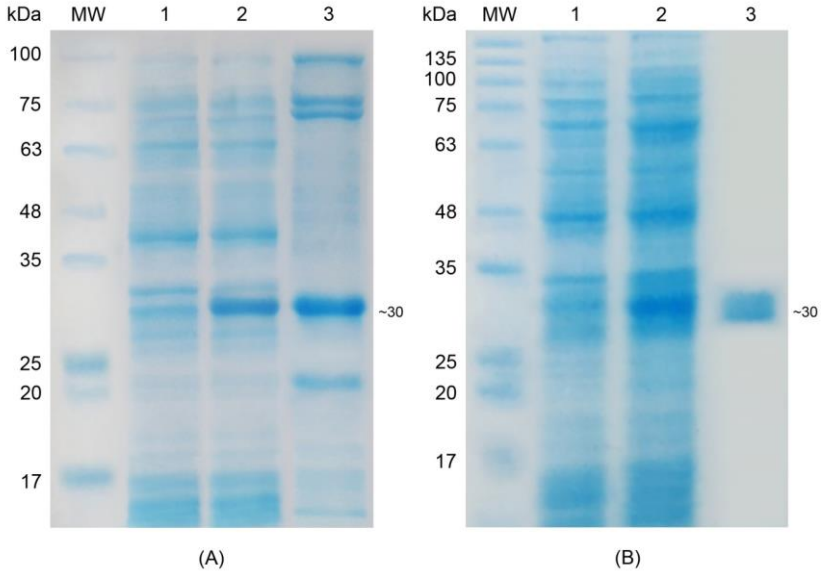

Fig. 1. Purification of HIV-1 Nef protein in *E. coli* expression system as shown in SDS-PAGE (A) and further purification using reverse staining method (B). (A) Lane 1, before induction; lane 2, 16 h after induction; lane 3, purified protein using affinity chromatography; (B) Lane 1, before induction; lane 2, 16 h after induction; lane 3, purified protein using reverse staining; MW, molecular weight marker (10-250 kDa, Cinaclon).

[DOI: 10.29252/JoMMID.7.4.107]

***In vitro* cytotoxicity of the pEGFP-N1-*nef*/HR9 and Nef/LDP-NLS nanoparticles.** According to the cell viability results, pEGFP-N1-*nef*/HR9 complexes at an N/P ratio of 5, as well as, the combination of LDP-NLS peptides with Nef protein at a molar ratio of 1:10 did not represent any considerable cytotoxic effect compared to untreated cells over a period of 48 h (p>0.05). Also, HR9 (9.3 μg), LDP-NLS (10 μg), pEGFP-N1-*nef* (2 μg), and Nef (1 μg) had no significant toxicity on the HEK-293T cells (Fig. 6).

Transfection of DNA and protein into HEK-293T cells by different CPPs. The efficiency of *nef* gene expression was assessed in a HEK-293T cell line using HR9 and TurboFect by fluorescence microscopy and flow cytometry at 48 h post-transfection (Fig. 7). The transfected cells by the pEGFP-N1/HR9, pEGFP-N1/TurboFect, pEGFP-N1-*nef*/HR9, and pEGFP-N1-*nef*/TurboFect showed spreading green regions as represented by fluorescent microscopy. The flow cytometry analysis showed that HR9- and TurboFect- based nanoparticles facilitated the uptake of pEGFPN1-*nef* into the cells**.** Our data indicated that the cellular uptakes of the pEGFP-N1-*nef*/HR9 and pEGFP-N1-*nef*/TurboFect complexes into the cells were 29.89% and 73.71%, respectively. The percentage of GFP expressing cells was 46.33% and 75.70% for pEGFP-N1 delivered by HR9 and TurboFect, respectively. The cells transfected with TurboFect and untransfected cells were used as positive and negative controls, respectively. On the other hand, the delivery of Nef protein was investigated in the HEK-293T cells transfected by the Nef/LDP-NLS nanoparticles at a molar ratio of 1:10 as compared to the cells transfected by Nef/TurboFect using western blotting. The dominant band of ~30 kDa was detected in the transfected cells with Nef/LDP-NLS and Nef/TurboFect nanoparticles using the anti-His antibody at 2 and 6 h post-transfection, respectively. This band was not observed in the untransfected cells (Fig. 8). Moreover, the transfected cells with Nef protein alone did not detect any related bands indicating that LDP-NLS peptide could transfer Nef protein into the cells.

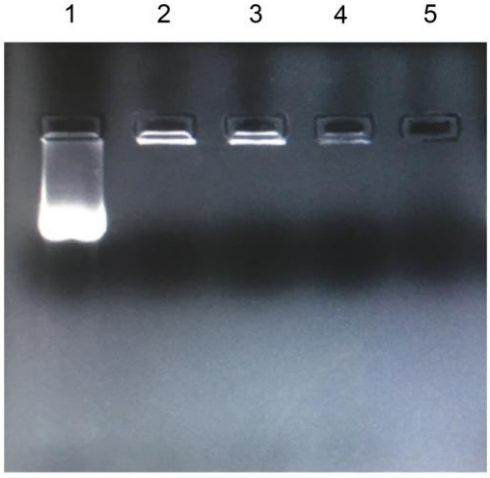

Fig. 2. Gel retardation assay of HR9 peptide complexed with pEGFP-N1-*nef* at different N/P ratios. Lane 1, naked plasmid DNA as a control (pEGFP-N1-*nef*); lane 2, N/P = 0.5; lane 3: N/P = 1; lane 4, N/P = 2; and lane 5, N/P = 5. The formation of the DNA/HR9 complexes was observed at an N/P ratio of 5.

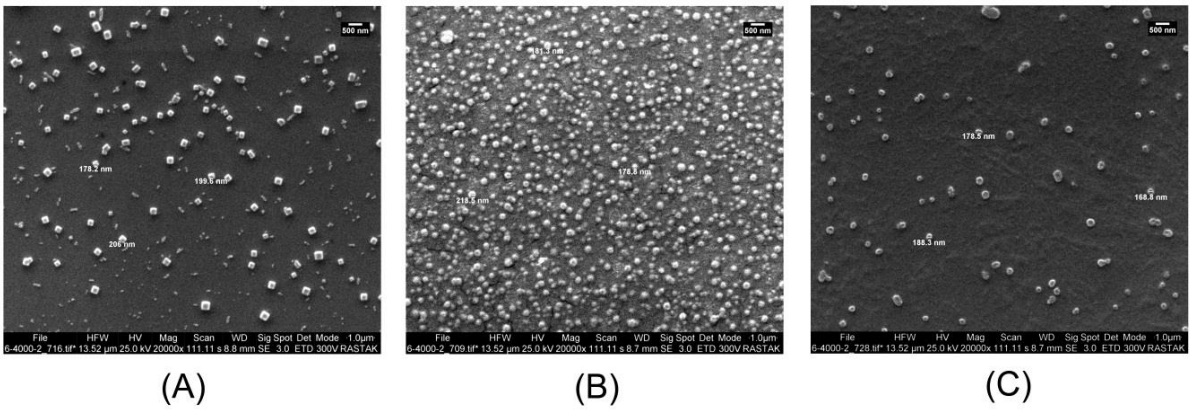

Fig. 3. The SEM micrograph of the pEGFP-N1-*nef*/HR9 nanoparticles. (A) HR9 peptide, (B) pEGFP-N1-*nef*, (C) pEGFP-N1-*nef*/HR9 complexes; The size of ~150-200 nm was observed for pEGFP-N1-*nef*/HR9 nanoparticles

[DOI: 10.29252/JoMMID.7.4.107]

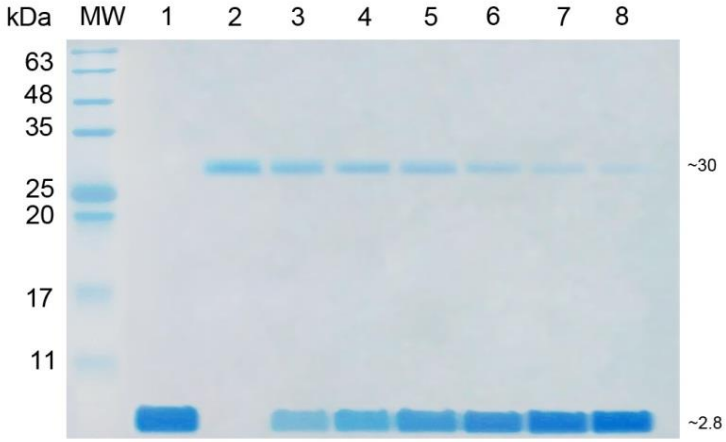

Fig. 4. Analysis of the Nef protein/LDP-NLS complexes at different molar ratios in SDS-PAGE. Lane 1, LDP-NLS peptide; lane 2, the purified Nef protein as a control; lanes 3-8, the Nef/LDP-NLS complexes at molar ratios of 1:2, 1:5, 1:10, 1:15, 1:20 and 1:30; MW, molecular weight marker (10-250 kDa, Sinaclon).

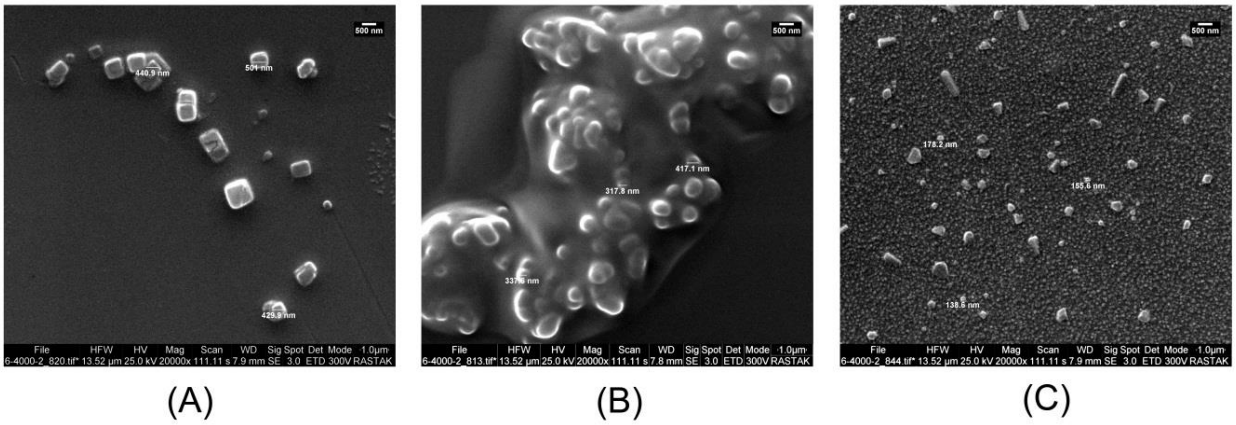

Fig. 5. The SEM micrograph of the Nef protein/LDP-NLS nanoparticles formed at a molar ratio of 1:10 with 20,000 × magnification. (A) LDP-NLS peptide, (B) Nef protein, (C) the Nef/LDP-NLS complexes; the average size of ~100-200 nm was observed for the Nef/LDP-NLS nanoparticles.

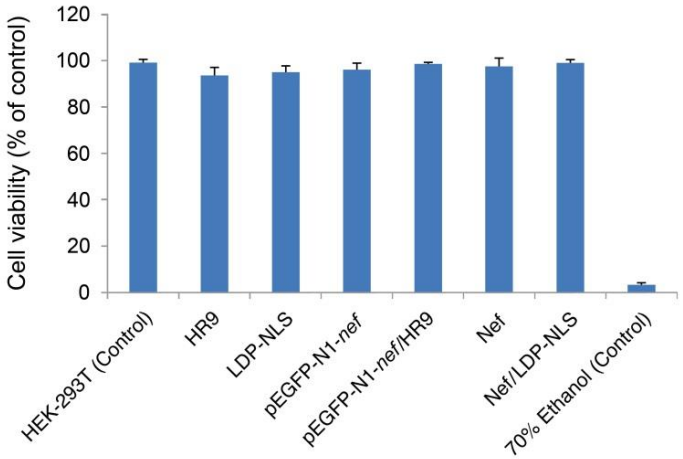

Fig. 6. Cell viability assay: HEK-293T cells were treated with HR9 (9.3 µg), LDP-NLS (10 µg), pEGFP-N1-*nef* (2 µg), pEGFP-N1-*nef*/HR9 (N/P: 5), Nef (1 µg), Nef/LDP-NLS (molar ratio of 1:10).

[DOI: 10.29252/JoMMID.7.4.107]

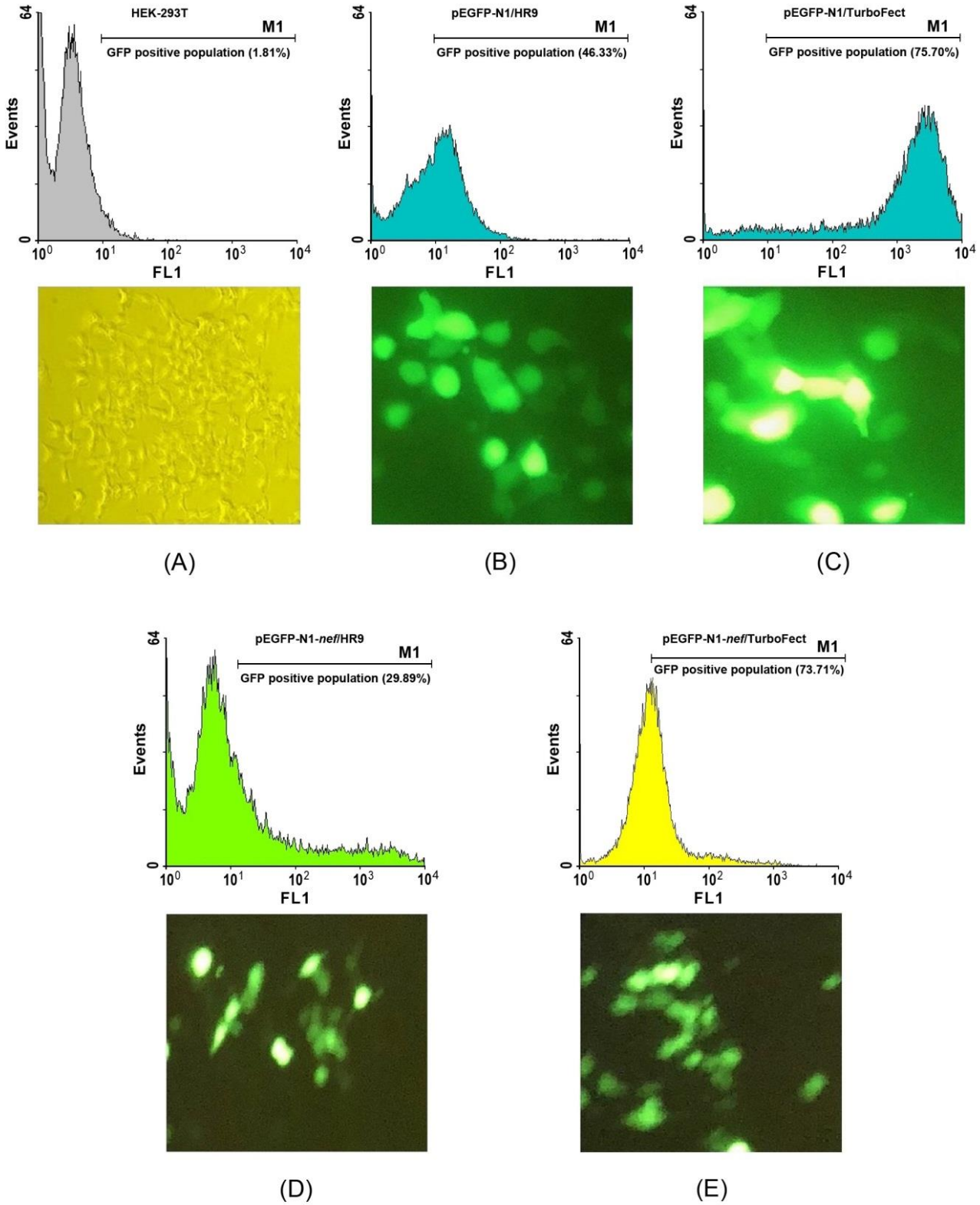

Fig. 7. *In vitro* transfection analysis using fluorescence microscopy and flow cytometry. (A) Untransfected HEK-293T cells (negative control), (B) cells transfected with pEGFP-N1/HR9, (C) cells transfected with pEGFP-N1/TurboFect (positive control), (D) cells transfected with pEGFP-N1-*nef*/HR9, (E) cells transfected with pEGFP-N1-*nef*/TurboFect

[DOI: 10.29252/JoMMID.7.4.107]

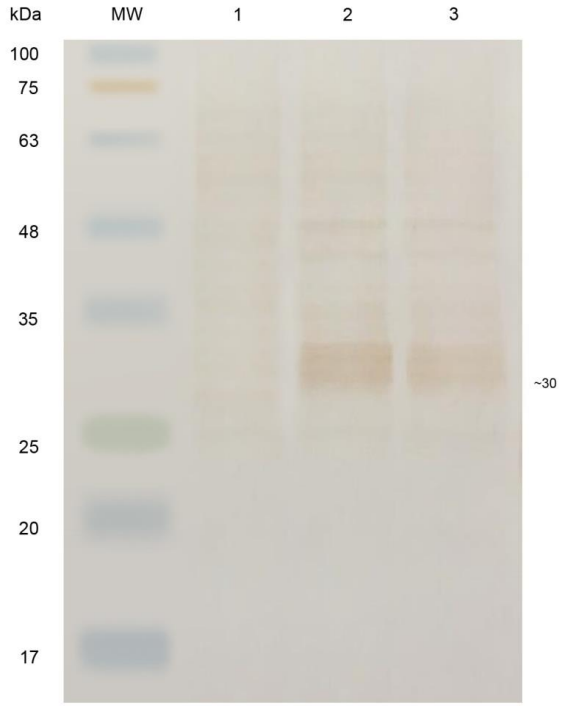

Fig. 8. Delivery efficiency of Nef protein using LDP-NLS and TurboFect in HEK-293T cell line. Lane 1, un-transfected HEK-293T cells (negative control); lane 2, cells transfected with Nef/LDP-NLS; lane 3, cells transfected with Nef/TurboFect (positive control); MW, molecular weight marker (10-250 kDa, Cinaclon).

[DOI: 10.29252/JoMMID.7.4.107]

DISCUSSION

Therapeutic vaccination is an extraordinary approach to stimulate HIV-specific immune response in infected patients [19]. The studies showed that DNA and protein-based vaccines are considered as a successful approach for the development of antigen-specific immune responses against viral diseases [11]. Some studies have focused on using Nef accessory protein, which is an essential target for the design of genetic vaccines due to its critical role in the viral life cycle and pathogenesis [20]. In one study, the HIV-1 Nef-expressing plasmid could induce Nef-specific cytotoxic T lymphocytes [21]. In another report, HIV-1 Nef DNA prime/protein boost vaccine increased the humoral and cellular immune responses against HIV-1 Nef [20]. Cosma *et al*. designed a vaccine based on MVA (Modified Vaccinia virus Ankara) vector that expressed the HIV-1 *nef* gene. The results showed that vaccination with MVA-*nef* was safe and could induce Nef-specific $CD4^+$ T-cell responses [19]. Moreover, vaccination with MVA-*nef* was associated with recognition of new HIV-1 T-cell epitopes and an increase in $CD4^+$ and $CD8^+$ T cells [5, 22].

The most crucial drawback of DNA and protein-based vaccines is their low immunogenicity [11]. New strategies have been developed to overcome this problem such as the use of carriers (*e.g.*, CPPs) [14]. CPPs showed the ability to penetrate cell membranes and were capable of transferring non-covalently or covalently biological cargoes with high efficiency and low toxicity into the cells [23, 24]. The formation of non-covalent complexes was often preferred because of easy handling, uptake efficiency, and auto-release of cargo into cells [25]. However, the optimal conditions for each cargo must be determined according to the CPP and cell type [26]. In the current study, the potency of HR9 and LDP-NLS cell-penetrating peptides was evaluated for *in vitro* delivery of pEGFP-N1-*nef* and Nef protein, respectively. HR9 is a peptide containing histidine, arginine, and cysteine residues. Probably, histidine and cysteine residues in HR9 facilitated complex passing through lipid bilayers by the direct membrane translocation [15]. In one study, HR9 peptide was used to deliver the plasmid DNA into insect Sf9 cells. The results of the MTT assay showed that this CPP was not cytotoxic and the transferred plasmid DNA was expressed in cells [27]. In another report**,** HR9 was used to transfer the plasmid DNA into human cells in a non-covalent fashion [28]. Other studies also indicated that HR9 was able to form stable complexes with nanomaterials including DNAs and quantum dots, and the complexes could be effectively internalized into cells in a short period.

The results elucidated that the zeta potential of complexes plays a major role in determining transfection efficiency, while particle size has a minor effect on cell permeability [15, 28-30]. In the current study, the HR9 cell-penetrating peptide formed stable nanoparticles with the pEGFP-N1-*nef* at an N/P ratio of 5, with a size below 200 nm. The spherical shapes observed for the pEGFP-N1-*nef*/HR9 complexes. The SEM results showed that the addition of HR9 peptide to pEGFP-N1-*nef* reduced the size of the complexes. On the other hand, Zetasizer analysis showed that the combination of negatively charged pEGFP-N1-*nef* with positively charged HR9 formed positively charged complexes that could pass through the membrane. These data suggested that the size and positive charges of

pEGFP-N1-*nef*/HR9 complexes can be considered as important factors for transport across the cell membrane of HEK-293T cells. MTT assay showed that the pEGFP-N1-*nef*/HR9 complexes at an N/P ratio of 5 did not represent significant toxicity for the cells, and thus can be used *in vivo*. Moreover, *in vitro* delivery of pEGFP-N1-*nef*/HR9 (29.89%) was confirmed by flow cytometry and fluorescence microscopy. Several studies indicated that transfection efficiency depends on the properties of both CPP and cargo, as well as the transfection conditions and the cell lines used [31].

Herein, the Nef protein was expressed in *E. coli* and purified by affinity chromatography using Ni-NTA agarose column and then reverse staining. The novel LDP-NLS cell-penetrating peptide was used to transfer the Nef protein into the HEK-293T cell. Spider venom peptides usually affect the permeability of the membrane [32]. LDP-NLS peptide was utilized for the effective delivery of the β-galactosidase enzyme into the HeLa cells [16]. Herein, the LDP-NLS at a molar ratio of 1:10 formed stable nanoparticles through non-covalent binding with the Nef protein. The size of ~100-200 nm and non-spherical shape were observed for Nef/LDP-NLS complexes. The SEM results showed that the addition of LDP-NLS peptide to Nef protein reduced the size of the complexes. On the other hand, zeta potential analysis indicated that the combination of positively charged LDP-NLS with negatively charged Nef protein formed positively charged complexes that could pass through the plasma membrane. MTT assay revealed that the Nef/LDP-NLS complexes at a molar ratio of 1:10 did not represent any considerable cytotoxic effect compared to untreated cells. Moreover, *in vitro* delivery of Nef/LDP-NLS complexes was confirmed by western blot analysis using the anti-His antibody. The dominant band of ~30 kDa was detected in the transfected cells with the Nef/LDP-NLS nanoparticles similar to the Nef/TurboFect complexes as a control.

In conclusion, HR9 peptide with pEGFP-N1-*nef*, and LDP-NLS with Nef protein formed the stable nanoparticles that can efficiently penetrate to mammalian cells. The small size and the positive charge of these complexes were two crucial factors for their transfection into the cells. Moreover, MTT assay showed that there is no toxicity for the complexes at the selected doses on the HEK-293T cell line. Therefore, the desired CPPs can be used as a practical approach for the delivery of DNA and protein into cells. However, further studies are required to evaluate the *in vivo* transfection efficiency of these complexes and their role in stimulating the humoral and cellular immune responses.

ACKNOWLEDGMENT

The authors thank from Elnaz Agi (Iranian Comprehensive Hemophilia Care Center, Tehran, Iran) for her technical assistance.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

REFERENCES

1. Alves BM, Siqueira JD, Prellwitz IM, Botelho OM, Da Hora VP, Sanabani S, et al. Estimating HIV-1 genetic diversity in Brazil through next-generation sequencing. Front Microbiol. 2019; 10.

2. Seitz R. Human Immunodeficiency Virus (HIV). Transfus Med Hemother. 2016; 43 (3): 203-22.

3. Li G, Piampongsant S, Faria NR, Voet A, Pineda-Peña A-C, Khouri R, et al. An integrated map of HIV genome-wide variation from a population perspective. Retrovirology. 2015; 12 (1).

4. Khairkhah N, Namvar A, Kardani K, Bolhassani A. Prediction of cross-clade HIV-1 T-cell epitopes using immunoinformatics analysis. Proteins: Structure, Function, and Bioinformatics. 2018; 86 (12): 1284-93.

5. Bråve A, Gudmundsdotter L, Gasteiger G, Hallermalm K, Kastenmuller W, Rollman E, et al. Immunization of mice with the nef gene from Human Immunodeficiency Virus type 1: Study of immunological memory and long-term toxicology. Infect Agent Cancer. 2007; 2 (1).

6. Jafarzade B, Sadat S, Yaghobi R, Bolhassani A. Improving the potency of DNA vaccine encoding HIV-1 Nef antigen using two endogenous adjuvants in mouse model. Bratisl Lek Listy. 2017; 118 (09): 564-569.

7. Quaranta MG, Mattioli B, Giordani L, Viora M. Immunoregulatory effects of HIV-1 Nef protein. BioFactors. 2009; 35 (2): 169-174.

8. Felli C, Vincentini O, Silano M, Masotti A. HIV-1 Nef signaling in intestinal mucosa epithelium suggests the existence of an active inter-kingdom crosstalk mediated by exosomes. Front Microbiol. 2017; 8.

9. Pandey RK, Ojha R, Aathmanathan VS, Krishnan M, Prajapati VK. Immunoinformatics approaches to design a novel multi-epitope subunit vaccine against HIV infection. Vaccine. 2018; 36 (17): 2262-72.

10. Lema D, Garcia A, De Sanctis J. HIV vaccines: A brief overview. Scand J Immunol. 2014; 80 (1): 1-11.

11. Kadkhodayan S, Jafarzade B, Sadat S, Motevalli F, Agi E, Bolhassani A. Combination of cell penetrating peptides and heterologous DNA prime/protein boost strategy enhances immune responses against HIV-1 Nef antigen in BALB/c mouse model. Immunol Lett. 2017; 188: 38-45.

12. Alhakamy NA, Nigatu AS, Berkland CJ, Ramsey JD. Noncovalently associated cell-penetrating peptides for gene delivery applications. Ther Deliv. 2013; 4 (6): 741-57.

13. Farkhani SM, Valizadeh A, Karami H, Mohammadi S, Sohrabi N, Badrzadeh F. Cell penetrating peptides: Efficient vectors for delivery of nanoparticles, nanocarriers, therapeutic and diagnostic molecules. Peptides. 2014; 57: 78-94.

14. Rostami B, Irani S, Bolhassani A, Cohan RA. M918: A novel cell penetrating peptide for effective delivery of HIV-1 Nef and Hsp20-Nef proteins into eukaryotic cell lines. Curr HIV Res. 2018; 16 (4): 280-7.

15. Liu BR, Huang Y, Winiarz JG, Chiang HJ, Lee HJ. Intracellular delivery of quantum dots mediated by a histidine- and arginine-rich HR9 cell-penetrating peptide through the direct

[DOI: 10.29252/JoMMID.7.4.107]

membrane translocation mechanism. Biomaterials. 2011; 32 (13): 3520-37.

16. Ponnappan N, Chugh A. Cell-penetrating and cargo-delivery ability of a spider toxin-derived peptide in mammalian cells. Eur J Pharm Biopharm. 2017; 114: 145-53.

17. Milani A, Bolhassani A, Shahbazi S, Motevalli F, Sadat SM, Soleymani S. Small heat shock protein 27: An effective adjuvant for enhancement of HIV-1 Nef antigen-specific immunity. Immunol Lett. 2017; 191: 16-22.

18. Javanzad S, Bolhassani A, Doustdari F, Hashemi M, Movafagh A. Reverse staining method of polyacrylamide gels by imidazole-zinc salts for. J Paramed Sci. 2013; 4 (2).

19. Cosma A, Nagaraj R, Bühler S, Hinkula J, Busch D, Sutter G, et al. Therapeutic vaccination with MVA-HIV-1 nef elicits Nef-specific T-helper cell responses in chronically HIV-1 infected individuals. Vaccine. 2003; 22 (1): 21-9.

20. Billaut-Mulot O, Idziorek T, Ban E, Kremer L, Dupré L, Loyens M, et al. Interleukin-18 modulates immune responses induced by HIV-1 Nef DNA prime/protein boost vaccine. Vaccine. 2000; 19 (1): 95-102.

21. Asakura Y, Hamajima K, Fukushima J, Mohri H, Okubo T, Okuda K. Induction of HIV-1 Nef-specific cytotoxic T lymphocytes by Nef-expressing DNA vaccine. Am J Hematol. 1996; 53 (2): 116-117.

22. Harrer E, Bäuerle M, Ferstl B, Chaplin P, Petzold B, Mateo L, et al. Therapeutic vaccination of HIV-1-infected patients on HAART with a recombinant HIV-1 nef-expressing MVA: safety, immunogenicity and influence on viral load during treatment interruption. Antivir Ther. 2005; 10 (2): 285-300.

23. Madani F, Lindberg S, Langel Ü, Futaki S, Gräslund A. Mechanisms of cellular uptake of cell-penetrating peptides. J Biophys. 2011; 2011: 1-10.

24. Rathnayake PVGM, Gunathunge BGCM, Wimalasiri PN, Karunaratne DN, Ranatunga RJKU. Trends in the binding of cell penetrating peptides to siRNA: A molecular docking study. J Biophys. 2017; 2017: 1-12.

25. Keller AA, Mussbach F, Breitling R, Hemmerich P, Schaefer B, Lorkowski S, et al. Relationships between cargo, cell penetrating peptides and cell type for uptake of non-covalent complexes into live cells. Pharmaceuticals. 2013; 6 (2): 184-203.

26. Durzynska J, Przysiecka ucja, Nawrot R, Barylski J, Nowicki G, Warowicka A, et al. Viral and other cell-penetrating peptides as vectors of therapeutic agents in medicine. J Pharmacol Exp Ther. 2015; 354 (1): 32-42.

27. Chen Y-J, Liu BR, Dai Y-H, Lee C-Y, Chan M-H, Chen H-H, et al. A gene delivery system for insect cells mediated by arginine-rich cell-penetrating peptides. Gene. 2012; 493 (2): 201-10.

28. Liu BR, Lin MD, Chiang HJ, Lee HJ. Arginine-rich cell-penetrating peptides deliver gene into living human cells. Gene. 2012; 505 (1): 37-45.

29. Liu BR, Liou JS, Chen YJ, Huang YW, Lee HJ. Delivery of nucleic acids, proteins, and nanoparticles by arginine-rich cell-penetrating peptides in rotifers. Marine Biotechnol. 2013; 15 (5): 584-95.

30. Mandraccia L, Slavin G. Cell membrane. Hauppauge: Nova Science Publishers, Inc; 2013.

31. Laufer S, Restle T. Peptide-mediated cellular delivery of oligonucleotide-based therapeutics *in vitro*: Quantitative evaluation of overall efficacy employing easy to handle reporter systems. Curr Pharm Des. 2008; 14 (34): 3637-55.

32. Dubovskii P, Vassilevski A, Kozlov S, Feofanov A, Grishin E, Efremov R. Latarcins: versatile spider venom peptides. Cell Mol Life Sci. 2015; 72 (23): 4501-22.

Cite this article:

Namazi F, Bolhassani A, Sadat SM, Irani Sh. *In vitro* Delivery of HIV-1 Nef Antigen by Histidine-rich nona-arginine and Latarcin 1 peptide. J Med Microbiol Infect Dis, 2019; 7 (4): 107-115. DOI: 10.29252/JoMMID.7.4.107