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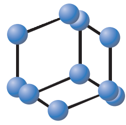
RESEARCH ARTICLE





# M918: A Novel Cell Penetrating Peptide for Effective Delivery of HIV-1 Nef and Hsp20-Nef Proteins into Eukaryotic Cell Lines

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**Abstract:** ***Background***: HIV-1 Nef protein is a possible attractive target in the development of therapeutic HIV vaccines including protein-based vaccines. The most important disadvantage of protein-based vaccines is their low immunogenicity which can be improved by heat shock proteins (Hsps) as an immunomodulator, and cell-penetrating peptides (CPPs) as a carrier.

***Methods***: In this study, the HIV-1 Nef and Hsp20-Nef proteins were generated in *E.coli* expression system for delivery into the HEK-293T mammalian cell line using a novel cell-penetrating peptide, M918, in a non-covalent fashion. The size, zeta potential and morphology of the peptide/protein complexes were studied by scanning electron microscopy (SEM) and Zeta sizer. The efficiency of Nef and Hsp20-Nef transfection using M918 was evaluated by western blotting in HEK-293T cell line.

***Results***: The SEM data confirmed the formation of discrete nanoparticles with a diameter of approximately 200-250 nm and 50-80 nm for M918/Nef and M918/Hsp20-Nef, respectively. The dominant band of ~ 27 kDa and ~ 47 kDa was detected in the transfected cells with the Nef/ M918 and Hsp20-Nef/ M918 nanoparticles at a molar ratio of 1:20 using anti-HIV-1 Nef monoclonal antibody. These bands were not detected in the un-transfected and transfected cells with Nef or Hsp20-Nef protein alone indicating that M918 could increase the penetration of Nef and Hsp20-Nef proteins into the cells.

***Conclusion***: These data suggest that M918 CPP can be used to enter HIV-1 Nef and Hsp20-Nef proteins inside mammalian cells efficiently as a promising approach in HIV-1 vaccine development.

**Keywords:** HIV, Cell-penetrating peptide, Heat shock protein 20, Nef, M918, Transfection.

## 1. INTRODUCTION

Human immunodeficiency virus (HIV) is an important public health issue. In 2016, it was estimated that approximately 36.7 million people were living with a global HIV prevalence of 0.8% among adults [1]. The HIV genome includes three main genes encoding 15 viral proteins involved in the viral life cycle: a) *env, gag and pol* genes expressing proteins with the envelope, enzymatic and structural roles, respectively; b) *tat* and *rev* genes expressing the regulatory proteins; c) *vpr, vip, vpu* and *nef* expressing the accessory proteins [2, 3]. Among viral proteins, HIV-1 Nef is a protein with a molecular weight varying from 27 to 35 kDa which is expressed early in the viral life cycle. The Nef protein has major roles including inhibition of CD4 activity, stimulation of virus replication, and induction of apoptosis in both uninfected and infected cells [4]. Generally, the advantages of HIV-1 Nef as a good antigen candidate for HIV vaccine development include its expression in the first step of the infection, its presence during the period of viral infection, and possessing T-cell epitopes [5-10]. An effective vaccine should induce antibody response along with an increased cellular immunity against the viral infection. Among various vaccination strategies, the protein-based vaccines need to increase their potency due to poor immunogenicity [11]. Therefore, the researchers are investigating for the proper combination of antigens with effective adjuvants or carrier molecules in subunit vaccines such as protein-based vaccines. Heat shock proteins (HSPs) were suggested to increase immune responses against infectious diseases as an effective adjuvant [5, 12]. Among HSPs, there is a family of proteins with an average molecular weight of 20 kDa, known as Hsp20 that is mainly expressed in the brain and heart. It acts as a molecular chaperone which binds to protein kinase 1 (PDK1) and leads to its nuclear transport [12]. On the other hand, biological membranes are usually resistant to pass

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Current HIV Research

large and hydrophilic molecules which cause difficulties for their biomedical applications indicating the importance of improving their uptake into cells. Recently, several peptides with the ability to deliver polypeptides and oligonucleotides into the living cells have been now named as "cell-penetrating peptides" (CPPs) [13, 14]. CPPs are able to form the covalent or non-covalent complexes with different cargoes [15-18]. Generally, CPPs were determined as short (~30-35 amino acids residues), water-soluble and partly hydrophobic or polybasic peptides with a net positive charge at physiological pH. The main benefit of CPPs is that they are able to cross the cell membrane at low micromolar concentrations without causing significant membrane damage *in vivo* and *in vitro* [19-27]. Thus, it is of special importance to find novel and non-toxic CPPs with more extensive delivery properties. M918 peptide consists of 22 amino acids (MVTVLFRRLRIRRACGPPRVRV) derived from the C-terminal of the tumor suppressor protein p14ARF. This cationic CPP could be utilized for both covalent and non-covalent delivery of macromolecules in animal and plant cells [28]. For instance, M918 was efficiently transferred into HeLa and breast cancer cell lines, in a non-toxic approach up to concentrations of 25 μM [29]. Moreover, transduction of transposase sleeping beauty protein was reported using M918 [30]. In this study, we evaluated the potency of M918 peptide for antigen delivery in HEK-293T mammalian cell line. Herein, we utilized HIV-1 Nef accessory protein and Nef linked to Hsp20 as two antigenic models for HIV-1 vaccine development in the future.

## 2. METHODS

### 2.1. Peptides

The M918 cell penetrating peptide (MVTVLFRRLRIR-RACGPPRVRV), a highly hydrophobic and positively charged peptide, was synthesized by BioMatik Company (Canada).

### 2.2. Construction of the Recombinant Hsp20-Nef Plasmid

To construct the expression vector harboring HIV-1 Nef, the full length Nef gene (from HIV-1 vector pNL4-3, Accession No: AF324493.2, ~ 648) was previously cloned into the unique *Eco*RI/*Sal*I cloning sites of the pET-23a [31]. Herein, for generation of pET-Hsp20-Nef, HIV-1 Nef sequence was digested from pET-Nef with *Eco*RI/*Sal*I enzymes and ligated to the linearized pET23a-Hsp20 using T4 DNA ligase. It should be mentioned that for preparation of pET-Hsp20 construct, the full length of Mus musculus Hsp20 gene (Accession No: NM_001012401, ~ 581 *bp*) was previously subcloned from pQE-Hsp20 into the pET23a vector using *Nhe*I/*Hind*III restriction enzymes in our laboratory. The *Eco*RI/*Sal*I restriction sites were designed after *Hind*III site in Hsp20 sequence. The recombinant pET-Hsp20-Nef was provided in a large scale using DNA extraction Mega Kit (Qiagen) and confirmed by digestion and sequencing.

### 2.3. Expression, Purification and Assessment of the Recombinant Protein

The process was performed according to our previous protocols [31]. Briefly, two *E. coli* BL21 and Rosetta strains were transformed with the recombinant pET-Hsp20-Nef plasmid. The transformants were selected on Luria-Bertani (LB) agar plate and grown to an optical density of 0.7-0.8 at 600 nm in Ty2x medium. The induction of protein expression with 1mM IPTG was optimized at different times of incubation (*i.e.,* 2, 3, 4 and 16 h) and temperature scales (*i.e.,* 25˚C and 37°C) after induction. The cell pellets were harvested and analyzed by 12% SDS-PAGE and western blotting using anti-Nef monoclonal antibody (Abcam). The recombinant Hsp20-Nef protein was purified by affinity chromatography using a Ni-NTA agarose column according to the manufacturer's instructions (Qiagen). Further purification was performed by reverse staining method [32]. Next, the purified Hsp20-Nef protein was dialyzed against PBS1X, assessed by NanoDrop spectrophotometry, and stored at -70˚C until used. The endotoxin contamination was less than 0.5 EU/mg proteins as detected by LAL assay. It should be noted that the recombinant HIV-1 Nef protein could be successfully expressed, purified under denaturing conditions and dialyzed against PBS1X in our previous studies [31].

### 2.4. Physicochemical Characterization of the Non-covalent Peptide/ Protein Complexes

The M918/ Nef and M918/Hsp20-Nef complexes with molar ratios of 2:1, 5:1, 10:1, 15:1, 20:1 and 30:1 (1 µg Nef or Hsp20-Nef protein) were formed in a pyrogenic water and incubated for 30 min at room temperature. The formation of the complexes was confirmed by 12% SDS-PAGE electrophoresis. Their size and zeta potential were assessed by a Zetasizer Nano ZS instrument (Malvern Instruments, UK). In addition, their size and morphology were studied using a scanning electron microscope (SEM; KYKYEM3200 model, China).

### 2.5. Cell Culture

Human embryonic kidney cells (HEK-293T) were cultured in RPMI medium supplemented with 5% Fetal Bovine Serum (FBS, Gibco) at 37˚C in the presence of 5% $CO_2$ atmosphere [5]. After several passages using trypsin-EDTA, the proliferated cells were counted and seeded into a 24-well plate for transfection and 96-well plate for cell viability assay.

### 2.6. Cell Viability Assay

For cell viability assay, HEK-293T cells ($10^4$ cells/ well) were seeded into 96-well microtiter plates in RPMI-1640 supplemented with 5% FBS and cultured for 16 h at 37°C in the presence of 5% $CO_2$ atmosphere. After replacement of culture medium with fresh RPMI-1640, different concentrations of M918 peptide (2, 5, 10, 15, 20 and 30 μM), and various molar ratios of Hsp20-Nef/ M918 and Nef/ M918 complexes (2:1, 5:1, 10:1, 15:1, 20:1 and 30:1) were added to the cells and left for 48 h without exchanging the media. After 48 h of incubation, the media was removed and 20 μL of sterile filtered MTT stock solution (5 mg/ mL, sigma) in fresh RPMI-1640 media was added to each well. After 3 h, the media was removed and 100 μL dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan products. The color intensity (which is proportional to the number of viable cells) was assessed by an ELISA reader at

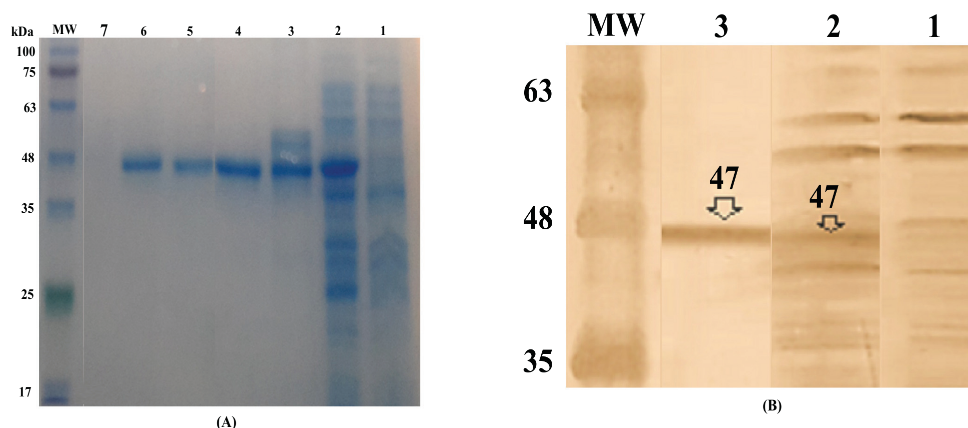


**Fig. (1).** Expression and purification of Hsp20-Nef protein in *E. coli* expression system as shown in SDS-PAGE (**A**) and western blotting (**B**); **A**) Lane 1: Before induction, Lane 2: After induction, Lane 3: Purified protein using affinity chromatography, Lane 4-6: Further purification using reverse staining; **B**) Lane 1: Before induction, Lane 2: After induction, Lane 3: Purified protein; MW is the molecular weight marker (10-180 kDa, Fermentas).

570 nm. The non-treated cells were used as a negative control (100% cell viability) as well as ethanol 70% as a positive control. MTT assay was performed in triplicate [33].

### 2.7. Transfection Assay of the Complexes into HEK-293T Cells

HEK-293T cells (6 × $10^4$ cells/well) were seeded onto 24-well microtiter plates in complete RPMI-1640 medium (Sigma) supplemented with 5% FBS at 37°C in an atmosphere containing 5% $CO_2$. After growth of the HEK-293T cells to 80% confluency, the medium was replaced by serum-free medium and then 100 µl of each protein and complex (at a molar ratio of 20:1) was applied to each well. After 1 h incubation at 37°C, the cells were supplemented with fresh RPMI, 5% FBS in a total volume of 200 µL medium without removal of the proteins and complexes overlay, and were further incubated for 3 h. Then, the cells were treated with trypsin-EDTA, harvested by centrifugation and resuspended in PBS 1X. The delivery of Nef and Hsp20-Nef proteins was confirmed by western blotting [33]. Briefly, for western blot analysis, protein bands were separated in 12% (w/v) polyacrylamide gel and transferred to nitrocellulose membrane (Millipore, USA). The anti-Nef-HRP monoclonal antibody (Abcam, 1:10000 v/v) was used to confirm the expression of Nef and Hsp20-Nef proteins under standard procedures. The immunoreactive protein bands were visualized using peroxidase substrate 3, 3'-diaminobenzidine (DAB, Sigma) [31].

### 2.8. Statistical Analysis

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

## 3. RESULTS

### 3.1. Generation of the Recombinant Hsp20-Nef Protein

The Hsp20-Nef gene was successfully cloned in pET23a expression vector. The presence of Hsp20-Nef gene was confirmed using digestion as a clear band of ~ 1230 bp migrated in agarose gel and also sequencing. The expression of Hsp20-Nef protein was analyzed in BL21 and Rosetta *E.coli* strains. The recombinant Hsp20-Nef protein could be expressed in Rosetta strain at 37°C and 4 h post-induction. The data indicated that Hsp20-Nef could be successfully purified under denaturing conditions (Urea, pH= 4.5) and dialyzed against PBS1X. We used the reverse staining method after affinity chromatography due to the existence of additional bands in the purified protein and removing urea. The purified Hsp20-Nef protein migrated as a clear band of ~ 47 kDa in SDS-PAGE as indicated in Fig. (**1A**). Furthermore, the recombinant Hsp20-Nef protein was detectable using anti-Nef antibody in western blotting (Fig. **1B**). The recombinant Hsp20-Nef protein had a concentration range between 0.6 and 0.8 mg/ml determined by NanoDrop spectrophotometry.

### 3.2. Formation of M918/ Nef and M918/ Hsp20-Nef Complexes

The formation of M918/ Nef and M918/ Hsp20-Nef complexes was confirmed by SDS-PAGE analysis. As observed in Fig. (**2**), a chemical dissociation was detected as a dominant band of ~ 27 kDa and ~ 47 kDa related to Nef and Hsp20-Nef proteins, respectively, along with M918 peptide band in SDS-PAGE indicating the formation of complexes over a range of molar ratios of 1:2, 1:5, 1:10, 1:15, 1:20 and 1:30 (protein cargo: peptide carrier). Moreover, the size and morphology of nanoparticles were analyzed by SEM and Zeta sizer as shown in Fig. (**3**). These complexes formed the particles with an average diameter of 200-250 nm for M918/Nef and 50-80 nm for M918/Hsp20-Nef complexes at a molar ratio of 20:1. These data were in agreement with the particle hydrodynamic diameter and zeta potential analysis of M918/Nef (Surface charge/ Z-average: 13 mV; size: 464.9 nm) and M918/Hsp20-Nef (Surface charge/ Z-average: 17.87 mV; size: 294.2 nm) complexes at a molar ratio of 20:1 with dynamic light scattering. Moreover, the particle hydrodynamic diameter and zeta potential analysis of Nef protein, Hsp20-Nef protein and M918 peptide were approximately 359.1 nm & -8.55 mV, 241.9 nm & -9.15 mV, and 100 nm & 13.7 mV, respectively. The addition of M918 CPP to Nef or Hsp20-Nef proteins increased the diameter of the complexes

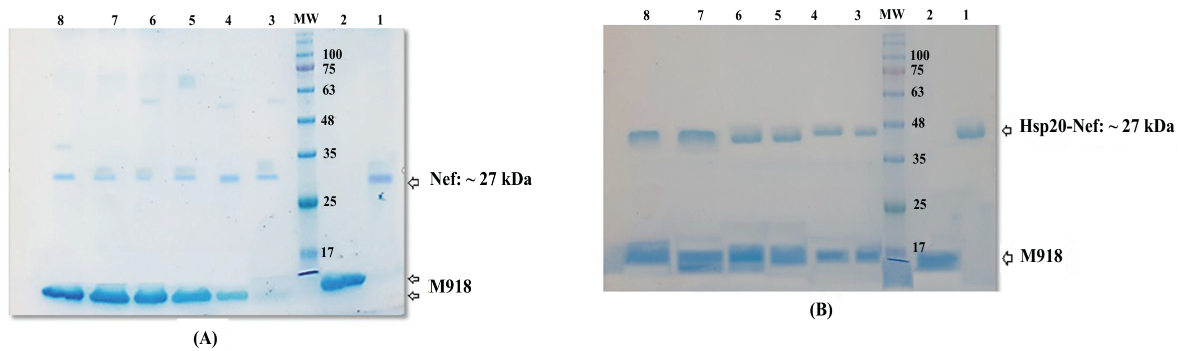


**Fig. (2). A**) Analysis of M918/Nef (**A**) and M918/Hsp20-Nef (**B**) complexes at different molar ratios in SDS-PAGE: Lane 1) purified Nef or Hsp20-Nef protein as a control, Lane 2) M918 peptide, Lane 3) M918/ Nef or M918/Hsp20-Nef complexes at ratio of 2:1, Lane 4) M918/ Nef or M918/Hsp20-Nef complexes at ratio of 5:1, Lane 5) M918/ Nef or M918/Hsp20-Nef complexes at ratio of 10:1, Lane 6) M918/ Nef or M918/Hsp20-Nef complexes at ratio of 15:1, Lane 7) M918/ Nef or M918/Hsp20-Nef complexes at ratio of 20:1, Lane 8) M918/ Nef or M918/Hsp20-Nef complexes at ratio of 30:1; MW is the molecular weight marker (10-180 kDa, Fermentas). The lanes 2-8 describe a chemical dissociation detected as a dominant band of ~ 27 kDa and ~ 47 kDa related to Nef and Hsp20-Nef proteins along with M918 peptide band in SDS-PAGE indicating the formation of protein/ peptide complexes.

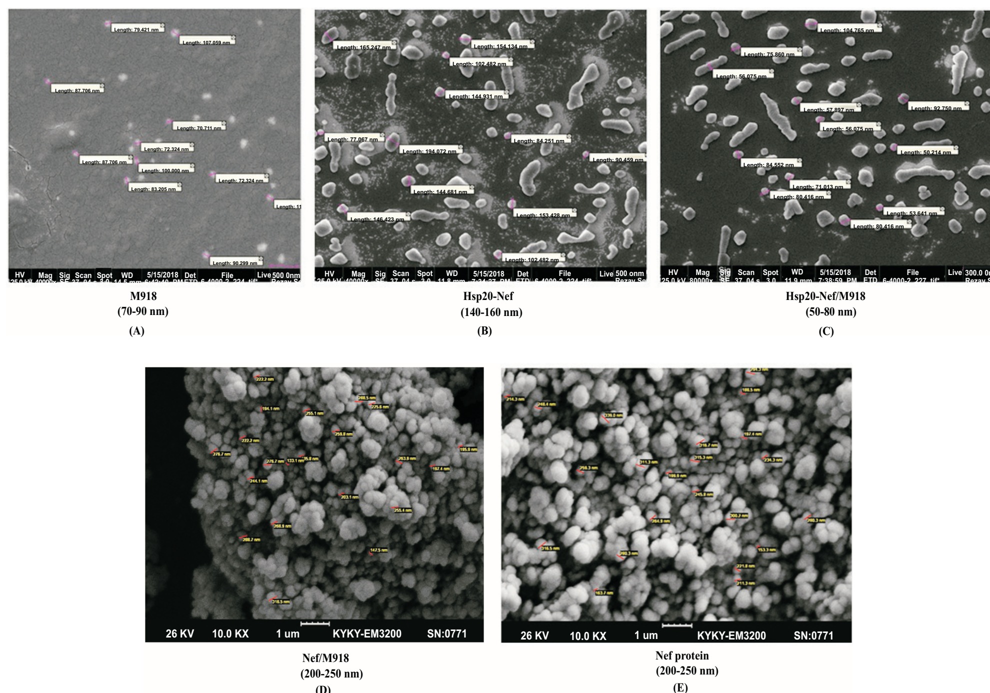


**Fig. (3).** The SEM micrograph of the spherical nanoparticles: **A**) M918 peptide, **B**) Hsp20-Nef, **C**) Hsp20-Nef/M918, **D**) Nef/M918, **E**) Nef; A size of ~ 200-250 nm was observed for M918/Nef nanoparticles and ~ 50-80 nm for M918/Hsp20-Nef nanoparticles at 25°C.

in water. Their surface charges showed that Nef and Hsp20-Nef proteins exhibited negative charges (-8.55 and -9.15 mV), while their complexes displayed positive charges (13 mV and 17.87 mV). This suggests that the positive charges of M918 CPP/Nef or Hsp20-Nef complexes can be considered as an important factor for transport across the cell membrane of HEK-293T cells with the negative charges. Also, the Hsp20-Nef nanoparticles showed a significantly small size (50-80 nm) and positive charge as compared to the Nef nanoparticles (200-250 nm).

### 3.3. *In Vitro* Cytotoxicity of M918 Peptide, M918/Nef and M918/Hsp20-Nef Complexes

According to the cell viability results, M918 peptide, M918/ Nef and M918/ Hsp20-Nef complexes at molar ratios of 2:1, 5:1, 10:1, 15:1, 20:1 and 30:1 did not represent any considerable cytotoxic effect compared to untreated cells over a period of 48 h (Fig. **4**). Treatment of the cells with these compounds showed cell viability of 90-97% ($p > 0.05$). The cells treated with ethanol 70% significantly indicated a high decrease in viability (~10 %) compared to other groups ($p < 0.05$).

### 3.4. Cell Transfection Assay

The efficiency of Nef and Hsp20-Nef protein delivery was investigated in the HEK-293T cells transfected by the Nef/M918 and Hsp20-Nef/M918 nanoparticles at a molar ratio of 1: 20 as compared to the cells transfected by Nef and Hsp20-Nef protein, alone using western blotting. The

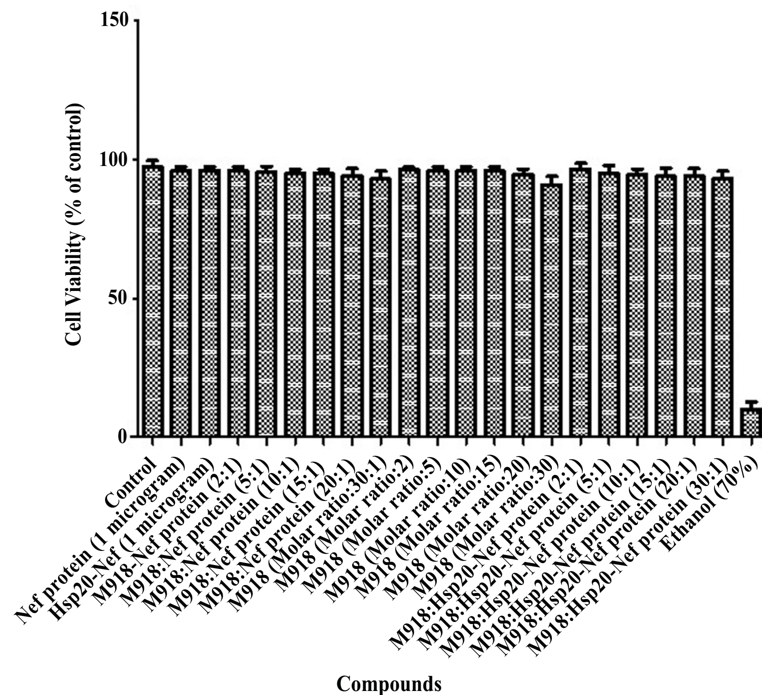


**Fig. (4).** Cell viability of the Nef, Hsp20-Nef, M918, M918/Nef and M918/Hsp20-Nef treatments: HEK-293T cells were treated with 1µg of the purified proteins, the complexes at different molar ratios of 2:1, 5:1, 10:1, 15:1, 20:1 and 30:1 (peptide: protein), M918 peptide (2, 5, 10, 15, 20 and 30 µM) as well as 70% ethanol as a positive control. The MTT assay was used to assess cytotoxicity. Data were presented as mean ± standard deviations from two independent experiments.

dominant bands of ~27 kDa and ~47 kDa were detected in the transfected cells with M918/Nef and M918/Hsp20-Nef nanoparticles using the anti-HIV Nef monoclonal antibody at 3 h post-transfection. These bands were not revealed in the un-transfected cells (Fig. **5**). Moreover, the transfected cells with Nef or Hsp20-Nef proteins alone did not detect any Nef/ Hsp20-Nef-related bands (data not shown) indicating that M918 could transfer Nef and Hsp20-Nef proteins into the cells.

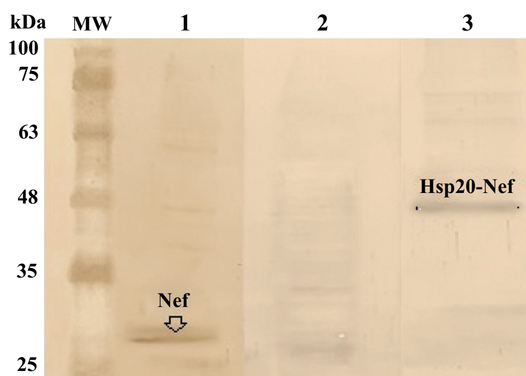


**Fig. (5).** Delivery efficiency of Nef and Hsp20-Nef proteins using M918 at a molar ratio of 1:20 (Nef/M918 and Hsp20-Nef/M918) in HEK-293 T cells for 3 h post-transfection: Western blot analysis showed delivery of the full-length Nef protein (~27 kDa, Lane 1) and Hsp20-Nef protein (~ 47 kDa, Lane 3) in transfected cells by M918 peptide at 3 h after transfection as compared to the un-transfected cells (Lane 2). The corresponding bands were not also detected in the transfected cells with Nef or Hsp20-Nef proteins alone similar to the un-transfected cells (data not shown). MW is the molecular weight marker (10-180 kDa, Fermentas).

## 4. DISCUSSION

In the current study, we used a short cationic peptide carrier, M918, for delivery of the full-length HIV-1 Nef and Hsp20-Nef proteins into mammalian cells and evaluated its cytotoxicity and penetration efficiency in different doses. Our data indicated that the Nef or Hsp20-Nef/M918 complexes formed stable nanoparticles through the non-covalent binding with an average size of 200-250 nm and 50-80 nm, respectively. The potent delivery of HIV-1 Nef or Hsp20-Nef protein by M918 peptide at a molar ratio of 1: 20 (protein: peptide) was detected in HEK-293T cell line at 3 h post-transfection in a non-toxic approach.

Recent studies have been successful for clarification of some problems involved in the host immune response to HIV infections, but novel infections still continue to occur without an effective treatment [34]. Nef is one of the HIV proteins involved in the infection regulation [35]. Nuclear magnetic resonance (NMR) studies suggested that HIV-1 Nef was composed of a globular core region (aa 58-149, 181-206), a flexible N-terminal region (aa 1-57), and a C-terminal loop (aa 150-180) [36]. Nef protein could be considered as a possible attractive target in the development of therapeutic HIV vaccines such as protein-based vaccines. As known, the most important drawback of protein-based vaccines is their low immunogenicity [11]. New strategies have been developed to overcome this problem such as the use of adjuvants (*e.g.,* Hsps) or carriers (*e.g.,* CPPs). The studies showed that the identity and range of cargoes depend on the type and diversity of Hsp present within a vaccine [37]. Different Hsps are potent inducers of innate and adaptive immunity [38]. Among heat shock proteins, small HSPs are highly conserved proteins among all species which have a

conservative α-crystallin domain (~90 amino acid residues) [39]. Alvarez *et al.* showed that the *Leishmania* Hsp20 as DNA vaccine elicited ~ 62% of the humoral responses against Hsp20 [40]. The reports demonstrated that Hsp20 was a highly conserved protein between species and has B and T lymphocyte epitopes [41, 42]. Moreover, Ortiz *et al.* reported that DNA fragment containing B and T cell epitopes of the N-terminal fragment of Hsp20 (NT-Hsp20) with other *Babesia bovis* antigens induced high levels of specific IgG antibodies [43]. Milani *et al.* reported the efficiency of a small Hsp (*i.e.,* Hsp27) to increase Nef delivery in DNA vaccine [5]. Other finding showed that the use of Hsp27 in protein strategy could improve HIV-1 Nef-specific B- and T-cell immune [44]. Jafarzade *et al.* also indicated that HMGB1 DNA could be considered as a more efficient adjuvant for improvement of HIV Nef DNA-based immunization compared to Glycoprotein 96 (Gp96) DNA *in vivo* [45]. On the other hand, the therapeutic use of peptides and proteins was suppressed by the poor permeability of the plasma membrane which prevented the cellular uptake of large and hydrophilic molecules [33, 46]. Recently, a group of short, highly basic peptides known as cell penetrating peptides (CPPs) were used to carry and internalize efficiently the intact cargoes such as small molecules, plasmid DNA, small interfering RNA, proteins, viruses, and other various nanoparticles across the cellular membrane [33, 46]. These peptides were capable of internalizing non-covalently or covalently biological cargoes such as drugs with high efficiency and low toxicity [19]. Because CPPs provided a safe, effective and non-invasive method of transport for various cargos into cells, they were recently developed as vectors for the delivery of genetic and biologic products [47]. For instance, the amino acid 49-57 of HIV-1 Tat was able to transport therapeutic molecules into different cells [48, 49]. Several short amphipathic CPPs such as PEP and CADY families were also determined to deliver the therapeutic proteins or peptides through the formation of stable non-covalent complexes [50, 51]. In this line, the formation of Pep-1 and/ or Cady-2/ Nef protein complexes as discrete nanoparticles was confirmed by SEM analysis with an average size of ~150-250 nm [52]. Furthermore, the Pep-1 and Cady-2 delivery systems could increase the potency of Tat CPP conjugated to Nef (Tat-Nef) for cell penetration [52, 53]. Analysis of the immune responses demonstrated that the Tat-Nef + Gp96 DNA prime/Tat-Nef protein+Cady-2 boost regimen significantly enhanced the Nef-specific T cell responses [52]. On the other hand, a novel CPP, M918 could be used to translocate different cargoes inside various cells in a non-toxic fashion [29, 54]. M918 peptide contains seven arginine residues and was derived from the C-terminus of the tumor suppressor protein p14ARF. It was shown that secondary amphipathic cell penetrating peptides such as penetratin, pVEC, and M918 contain fewer amino acid residues as compared to the primary peptides [29, 55]. Holm *et al.* could increase the stability of M918 CPP as a potent transporter [56]. In addition, structural analyses of the peptide/lipid interactions indicated that peptides adopting a β-structure such as MPG and M918 were more sensitive to charges than the helical ones. In contrast, the interactions with the lipids involved both electrostatic and hydrophobic contributions for Pep-1 and Cady amphipathic peptides. Indeed, helical peptides showed a higher potential for peptide/lipids interactions than β-structured peptides [57-59]. Another study represented that FITC-labelled streptavidin was taken up by cells after formation of the non-covalent complexes with 5 μM M918. The authors were able to reduce this relatively high concentration to 1 μM and achieve much higher degrees of protein uptake without cell toxicity, when they biotinylated the peptide to improve streptavidin binding [29]. As similarly, in our study, no significant toxicity was detected in various doses of M918 peptide and different ratios of the nanoparticles (M918/Nef & M918/Hsp20-M918), thus this peptide may have a potential for *in vivo* studies.

## CONCLUSION

Our main goal of this study was to clarify the transfection potency of M918 complexed with Nef or Hsp20-Nef proteins without covalent binding as compared to proteins alone. At first, the recombinant Hsp20-Nef protein was expressed in *E.coli* as His-tagged protein and purified by affinity chromatography using Ni-NTA column and then reverse staining. It was shown that M918 forms stable nanoparticles with the Nef or Hsp20-Nef proteins. The Hsp20-Nef/M918 nanoparticles had a more positive charge and smaller size as compared to Nef/M918 nanoparticles. Moreover, MTT assay showed that there is no toxicity for M918 and the nanoparticles at the selected doses on HEK-293T cell line in comparison with the untreated cells. In general, protein uptake into mammalian cells using M918 cell penetrating peptide through the formation of non-covalent complexes will be an effective approach for protein vaccination. However, further studies are required to determine its therapeutic efficiency *in vivo*.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

## ACKNOWLEDGEMENTS

Declared none.

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