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Modification of SPION Nanocarriers for Sirna Delivery: A Therapeutic Strategy Against HIV Infection

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A R T I C L E I N F O

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A B S T R A C T

Introduction: To date, while many studies have investigated antiviral agents or vaccines against HIV, success has been limited. In this field, mutagenesis of the viral genome mostly contributes to the viral escape from the antiretroviral therapies as well as the emergence of resistant strains of HIV-1 to pharmaceutical therapy. Therefore, developing alternative methods, including more effective vaccines, antiviral therapies (such as RNAi therapy) and delivery systems, seem to be necessary to compensate for these issues. The aim of this research was to establish an efficient system for siRNA delivery as a safe anti-HIV therapeutic approach. **Methods:** Chitosan-coated superparamagnetic iron oxide nanoparticles (SPION) was investigated as a method for RNA delivery. After generating HEK293 stable cells (expressing HIV-1 tat), a potent siRNA against HIV-1 tat was designed and the effectiveness of the modified SPION in siRNA delivery to HEK293 cells was evaluated. **Results:** The optimal concentration (50 µg/mL) of the modified SPION-containing anti-tat siRNA (with a range size of 50-70 nm and average zeta potential of +25 mV) was significantly internalized into the cells and decreased the expression of HIV-1 tat, more than 80%. Moreover, the nanoparticles showed no considerable toxicity on the cells. **Conclusion:** SPION could be optimized as a probable RNA/vaccine delivery system into target cells. Therefore, this study offers a therapeutic strategy against HIV or other infectious diseases.

INTRODUCTION

To date, antiviral therapeutics and vaccines have been successfully used against infectious diseases; however unfortunately, there is no currently approved vaccine or cure for human immunodeficiency virus (HIV)/AIDS [1]. HIV has unique ways of escaping the immune system, and it seems that the human body is incapable of presenting an effective immune response against it. As a result, developing alternative methods, including more effective vaccines and treatments, seem to be imperative to provide protection against HIV infection [2]. On the other hand, one of the important issues in a successful vaccination or antiviral therapy (such as RNAi therapy) is developing an efficient delivery system to protect the nucleic acids from the nuclease degradation, as well as to deliver and release the nucleic acids into the cells [3, 4, 5]. In this way, non-viral vectors have been shown to be more useful and safer alternatives compared to the viral vectors [3].

Superparamagnetic iron oxide nanoparticles (SPIONs) have attracted attention in nucleic acid delivery applications due to their low cost, low toxicity, and potential for direct targeting by using external magnets. Therefore, SPIONs could be used as both diagnostic and therapeutic agents because of their unique characteristics [6]. Moreover, the mechanism of magnetic hyperthermia of SPIONs can be applied to suppress the HIV viral load, since hyperthermic temperatures increase the activity of cytotoxic T lymphocytes (CTLs), which can inhibit HIV replication [7]. On the contrary, due to the non-reproducibility of synthesis and agglomeration of the SPION colloidal suspensions, their clinical use have been limited [8, 9]. To tackle these issues, the SPION surface can be coated with synthetic or natural polymers such as polyethylene glycol, alginate, dextran, chitosan and polyethylenimine. Therefore in our study, the surface of SPION was coated with trimethyl chitosan (TMC) and polyethylenimine (PEI) to prevent the agglomeration and to enhance the solubility of SPION [7]. These polymers could also promote the cell uptake through condensation of nucleic acids (DNA or RNA) into nanoparticles [3].

TMC is a derivative of chitosan (a biocompatible and non-toxic polymer) that displays a positive charge and great water solubility in a broad range of pH [10]. The positive

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charge of TMC can enhance the loading quantity of nucleic acids into nanoparticles [7]. Moreover, PEI is the most efficient polymer for nucleic acid delivery due to its high capacity of proton-buffering; although it is dose-dependent toxic [11, 12]. Therefore, the combined use of PEI and chitosan with SPION could suggest a promising strategy to accomplish a highly effective nanocarrier with low cytotoxic effect.

In the present study, we aimed to establish an effective delivery system for siRNA (or other nucleic acids) as a safe anti-HIV therapeutic approach. Thus, after generating a stable cell line of HEK293 (to express HIV-1 tat), we designed a potent anti-tat siRNA, and then the effectiveness of our nanoparticle (SPION-TMC-PEI) in siRNA delivery to HEK293 cells was evaluated. The inhibition of tat RNA expression was also studied in the stable cells.

MATERIALS and METHODS

Materials

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), Opti-MEM medium and trypsin-EDTA were supplied by Gibco (NY, USA). Cell proliferation kit II (XTT) was purchased from Roche (Mannheim, Germany), and the other chemicals were provided from Sigma-Aldrich (MO, USA).

Generation of Stable Cells (Expressing HIV-1 tat)

HEK293 cell line was provided by the Pasteur Institute of Iran (Tehran, Iran) in order to create stable cells (expressing HIV-1 tat). The cells were seeded in DMEM containing 10% FBS, 100 µg/mL of streptomycin (Biochrom, Germany), and 100 U/mL of penicillin (Biochrom, Germany). HIV-1 tat (GenBank accession number GQ473128.1) was synthesized by Biomatik Corporation (Ontario, Canada) and then subcloned into pEGFP-N1 vector (Clontech, USA; Fig. 1). The pEGFP-N1-tat was transfected into the cells (70% confluent) using Lipofectamine 3000 reagent (Invitrogen, NY, USA), according to its manufacturer's protocol. The stable cells were finally derived from a single cell (by the method of limiting dilution), and the recombinant protein expression of Tat-GFP was verified through flow cytometry and qRT-PCR (data not shown)

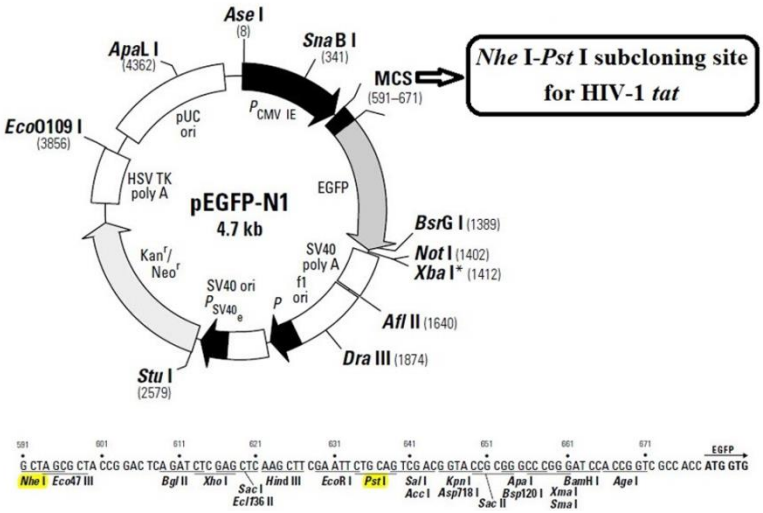

Fig. 1. Schematic illustration of pEGFP-N1 expression vector (GeneBank U55762). The HIV-1 tat gene was subcloned into the MCS (between two restriction enzymes Nhe I and Pst I).

Preparation of siRNA

A total of 15 siRNAs to target HIV-1 tat, as well as a siRNA scrambled control, were designed by siDirect (http://sidirect2.rnai.jp/) and Oligo walk (http://rna.urmc.rochester.edu/cgi-bin/server_exe/oligowalk/oligowalk_form.cgi). Next, 3 siRNAs with higher scores, namely siRNA-63, siRNA-100 and siRNA-102, were picked. All siRNAs were investigated for their thermodynamic properties [13]. Moreover, BLAST analyses were performed to avoid any non-specific effects of the siRNAs (Table 1).

Table 1. siRNA sequences against HIV-1 tat.

Name	[a]Position (bp)	Strand	Sequences (5' -> 3')	[b]Modification
siRNA-63	63-84	Sense	GUACCAAUUGUUAUUGUAAAA	none
		Antisense	UUACAAUAACAAUUGGUACAA	none
siRNA-100	100-121	Sense	CCAAGUUUGUUUCAUAACAAA	none
		Antisense	UGUUAUGAAACAAACUUGGCA	none
siRNA-102	102-122	Sense	CCAAGUUUGUUUCAUAACAA	none
		Antisense	UUGUUAUGAAACAAACUUGG	none
[c]Scrambled siRNA	–	Sense	GCCGAUCUAAUCAGAGUUA	3' TAMRA
		Antisense	UAACUCUGAUUAGAUCGGC	none

a Position of siRNA on the HIV-1 tat mRNA
b Fluorescently modification of siRNA strands
c Negative control of siRNA

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The scrambled siRNA (a negative control) was labeled with TAMRA dye (yellow) [3]. The selected siRNAs were manufactured by Bioneer Corporation (Daejeon, Korea). To determine the optimal siRNA (and its suitable concentration) and to inhibit the expression of HIV-1 tat, various concentrations of siRNAs were transfected in Opti-MEM medium, using the commercial reagent Lipofectamine RNAiMAX (Invitrogen, Karlsruhe, Germany) according to manufacturer's directions. The levels of gene expression were assessed through qRT-PCR assay (data not shown), and siRNA-102 with a final concentration of 100 pmol/mL was selected as the most effective siRNA to prepare the nanoparticles.

Preparation of Nanoparticles

SPIONs were prepared through a co-precipitation approach [7]. In brief, 0.298 g of $FeCl_2.4H_2O$ and 0.810 g of $FeCl_3.6H_2O$ were dissolved in deionized water (in a nitrogen environment). Next, 25% (v/v) of ammonium hydroxide solution (as the size-controlling agent) was drop-wisely added at 70 °C. The mixture was stirred for 1 h (at 70 °C) and then washed a few times through precipitation by a magnet and re-dispersion in water. The resulted SPIONs were dried at 40 °C in a vacuum oven. TMC was synthesized as described previously [14]. Briefly, 1 g of chitosan with Mw of 100-150 kDa (95% deacetylation degree, Primex, Norway) was dissolved in 40 mL of N-methylpyrrolidone solvent (Merck, Germany), and then 2.4 g of sodium iodide (Merck, Germany) was dissolved into the mixture. Next, 6 mL of methyl iodide and 5.5 mL of 15% (w/v) sodium hydroxide solution (as the precursor) were added to the mixture and stirred at 2000 rpm for 1 h (60 °C). After adding 250 mL ethanol in order to separate the methylated chitosan, the mixture was centrifuged at 20,000 RCF (5 min). In order to dissolve the precipitated product, 20 mL of 10% (w/v) sodium chloride solution was added into the mixture. The solution was dialyzed for 24 h against deionized water (cut-off: 12 kDa) and then freeze-dried. TMC-coated SPIONs were finally provided by using electrostatic forces between anionic SPION and positively charged TMC. For this purpose, 5 mg of the SPIONs were dispersed into 1 mL of deionized water and then sonicated for 30 min at room temperature (to create a homogeneous dispersion). The amount of 125 µl of the TMC solution (50 mg/mL) was then added to the solution of SPIONs. After shaking for 24 h at room temperature, the TMC-covered SPIONs were separated by centrifugation for 15 min at 100,000 RCF. Herein, according to our experiment results, the N:P ratio (the molar ratio of polymer amine groups to siRNA phosphate groups) equals to 40 was shown to be the optimum ratio. Therefore, the optimal amounts of siRNA solution (200 nM of siRNA-102), PEI 25 kDa solution (15 µg/mL) and SPION-TMC suspension (50 µg/mL) were added to deionized water, up to 500 µl final volume in a 1.5 mL tube. The suspension was stirred for 40 s (2500 rpm) and then incubated for 60 min at room temperature. The size distribution and zeta potential of the nanoparticles were measured using DLS (dynamic light scattering).

Moreover, the nanoparticles surface morphology was evaluated by scanning electron spectroscopy (SEM, JEOL-JSM-6700F, Japan).

Gel Retardation Analysis

The ability of the nanoparticles (SPION-TMC-PEI) to protect siRNA was investigated through gel retardation assay in a 2% agarose gel (for 45 min at 100 V), and then siRNA was stained with 1X Gel Red (Biotium, USA). Naked siRNA was used as a control.

Characterization of Nanoparticle Structure

In order to investigate the dynamic surface processes of the optimal nanoparticle, their FTIR (Fourier transform infrared) spectra was measured by an FTIR spectrometer (Varian, California, USA). Here, samples were freeze-dried and mixed with potassium bromide (KBr) to prepare pellets required for analysis of FTIR spectra.

In Vitro Cytotoxicity Study

The impact of various concentrations of the optimal nanoparticle on the viability of HEK293 cells was evaluated by using XTT method. In brief, 1×10^4 cells per well were cultured in a 96-well microplate in a volume of 100 µL of DMEM with 10% FBS. The cells were incubated at 37 °C for 24 h (with 5% CO_2), and then the media were exchanged with culture media containing various concentrations of the optimal nanoparticle formulation (i.e. 10, 20, 50 and 100 µg/mL). The cells were incubated at 37 °C for an additional 6 h under 5% CO_2 atmosphere, and the media were then exchanged with fresh media supplemented with 10% FBS. Cells were incubated for an additional 48 h at 37 °C in 5% CO_2. Herein, non-treated cells, cells treated with 100 pmol/mL of naked siRNA, and cells transfected with siRNA-Lipofectamine RNAiMAX (following the manufacturer's protocol) were used as controls. The fluorescence intensity of the cells was measured at the wavelength of 450 nm (with a 630 nm reference wavelength), using a micro-plate reader (Bio-Rad, USA). The samples in the experiment were in triplicate, and the results were indicated as the average values ± standard deviation.

Nanoparticles Cellular Uptake

The cellular uptake of the optimal nanoparticles was monitored by Nikon confocal microscope A1 (Nikon Inc., Japan). HEK293 cells were seeded for 24 h at 5×10^5 cells per well on coverslips in 6-well plates, then washed and treated with nanoparticles labeled with TAMRA in media supplemented with 20% FBS at 37 °C for 6 h. After washing and fixation in 4% paraformaldehyde (for 10 min), the cells were stained with Hoechst 33342 and observed under a confocal microscope.

qRT-PCR Examination of HIV-1 tat Expression

HIV-1 tat mRNA expression was investigated by SYBR Green qRT-PCR (quantitative real-time PCR). The stable HEK293 cells capable of expressing HIV-1 tat were cultured at a density of 2×10^5 cells per well on 6-well plates, and then were incubated in DMEM for 24 h. The cell media were replaced by the various concentrations of optimal nanoparticle (i.e. 10, 20, 50 and 100 µg/mL) in DMEM supplemented with 20% FBS. Cells treated with the scrambled siRNA (100 pmol/mL), and cells treated with 100 pmol/mL of naked siRNA, as well as cells transfected with siRNA-Lipofectamine RNAiMAX (following the manufacturer's protocol) were considered as controls. The cells were incubated at 37 °C for 6 h in a 5% CO_2 and then the media were replaced by fresh DMEM with 10% FBS. Next, the cells were incubated for an additional 48 h at 37 °C (5% CO_2). Using a cDNA synthesis kit (Yekta Tajhiz Azma, Iran), the total RNA (extracted from the cells) was reverse-transcribed, and the HIV-1 tat mRNA expression was measured by qRT-PCR assay. The mRNA expression was normalized for HPRT1 expression. The sequences of primers for PCR amplification of HIV-1 tat were designed as follows: 5´-CAAAAGGCTTAGGCATCTCC-3´ and 5´-CTCCACCTTCTCCTTCGATT-3´ for forward and reverse primers, respectively. In addition, the forward and reverse primers for HPRT1 PCR amplification were respectively:

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5′-GCTTGCTGGTGAAAAGGACCTCTCGAAG-3′ and 5′-CCCTGAAGTACTCATTATAGTCAAGGGCAT-3′, obtained from a reference [3]. All samples were in triplicate and the results were indicated as the average values ± standard deviation.

Statistical analyses

Statistical analyses was carried out by the paired t test and ANOVA. The results were measured as mean ± standard deviation (SD), and differences at $p < 0.05$ were considered as significant.

RESULTS

Analysis of Particle Size and Zeta Potential

The size of optimal nanoparticles (SPION-TMC-PEI), as determined by DLS, was between 70 and 90 nm (Fig. 2a), and the nanoparticles' zeta potential was about +25 mV (Fig. 2b). Moreover, the SEM image revealed that the optimal nanoparticles were spherical in shape with diameters roughly between 50 and 70 nm (Fig. 2c).

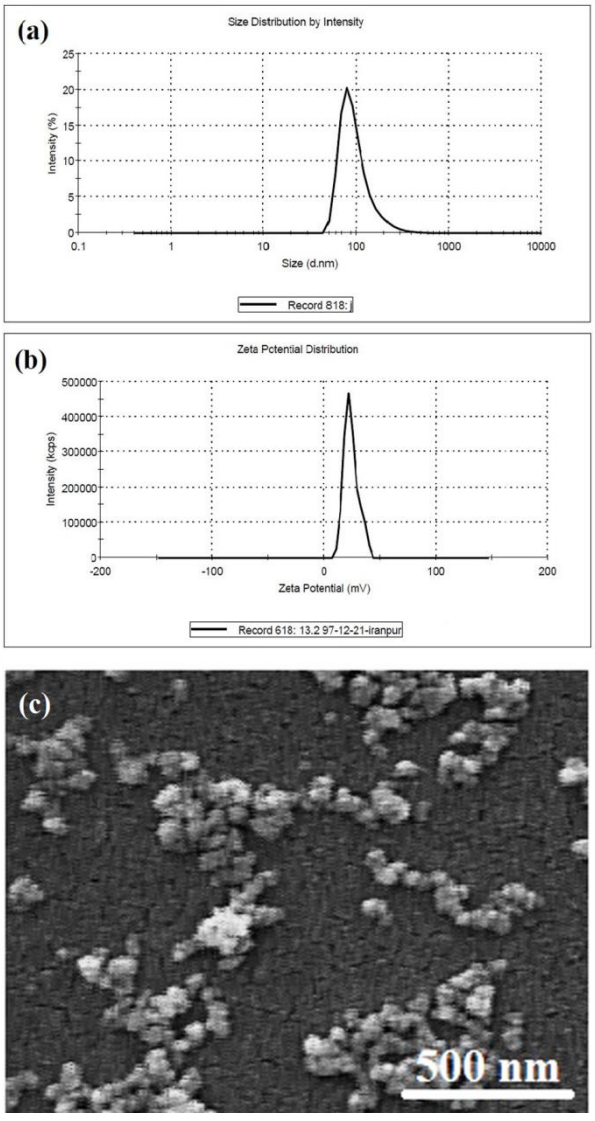

Fig. 2. Nanoparticles size and zeta potential. (a) Size distribution by intensity, measured by DLS, (b) Zeta potential distribution measured by DLS (c) SEM image of the optimal nanoparticles (SPION-TMC-PEI).

Gel Retardation Analysis

The results of gel retardation assay showed that the optimal nanoparticles (SPION-TMC-PEI) could retard siRNA completely (Fig. 3).

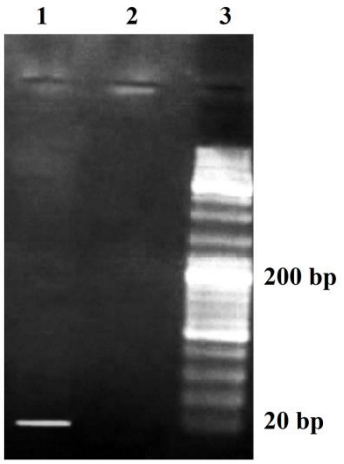

Fig. 3. Agarose gel retardation assay. Lane 1: naked siRNA (control), lane 2: siRNA-loaded nanoparticle, lane 3: RNA marker.

Analyses of Nanoparticles' Structure

The FTIR spectra in the wavenumber range from 4000 to 400 cm^{-1} verified the formation of nanoparticles. The Fe–O stretch band was observed in 598 cm^{-1} in spectra of SPION and SPION-TMC-PEI. In addition, the TMC and PEI characteristic peaks were observed in the nanoparticles which approved the successful coating of SPION with TMC and PEI (Fig. 4).

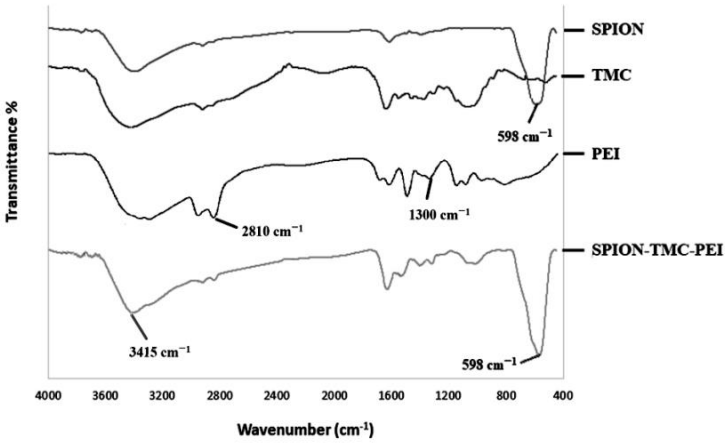

Fig. 4. FTIR spectra of the nanoparticle formulation.

Evaluation of Cytotoxicity of Nanoparticles

As shown in Fig. 6a, in group siRNA-loaded nanoparticles (100 µg/mL), the cell viability significantly ($p < 0.05$) decreased (to 61%) in comparison to that of the lower nanoparticle concentrations (i.e., 92, 88 and 85% cell viability for the nanoparticle concentrations of 10, 20 and 50 µg/mL, respectively). In addition, at the concentration of 50 µg/mL, the cell viability (> 85%) was not significantly ($p = 0.47$) different from transfected cells with the commercial reagent siRNA-Lipofectamine RNAiMAX (with a cell viability of 81%). Therefore, 50 µg/mL of the nanoparticle was used for cell uptake experiments.

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Cellular Uptake Evaluation

Evaluation of cellular uptake (in HEK293 cells) revealed a high uptake of TAMRA-labeled siRNA at the concentration of 50 µg/mL of the optimal nanoparticle (Fig. 5). In contrast, the cells treated with naked TAMRA-labeled siRNAs showed very low cellular uptake (data not shown).

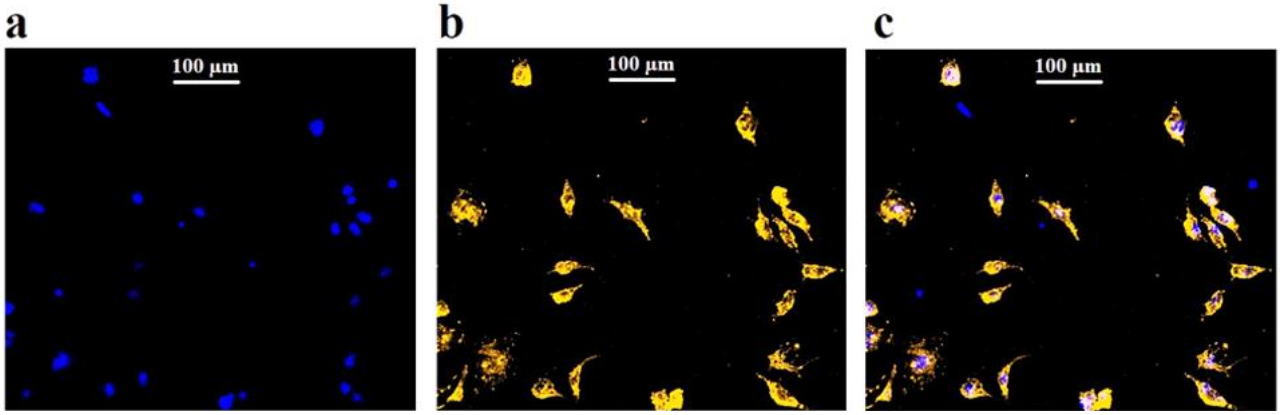

Fig. 5. Confocal microscopy images of HEK293 cellular uptake of TAMRA-labeled nanoparticles. (a) Cell nuclei stained with Hoechst 33342 dye (blue); (b) Cells treated with TAMRA-labeled nanoparticles (orange); (c) The merged image of the two fluorochromes used.

Gene Expression Evaluation

Fig. 6b indicates the tat mRNA expression in stable HEK293 cells after 48 h incubation with various treatment groups. The mRNA expression was down-regulated significantly in all groups of siRNA-loaded nanoparticles at various concentrations (i.e. 10, 20, 50 and 100 µg/mL), compared with the negative control groups (i.e. cells treated with scrambled siRNA and also cells treated with naked anti-tat siRNA; $p < 0.001$). Furthermore, in group with 50 µg/mL of the optimal nanoparticle, the tat mRNA expression (19%) was not significantly more than treated cells with 100 µg/mL of the optimal nanoparticle ($p = 0.4$).

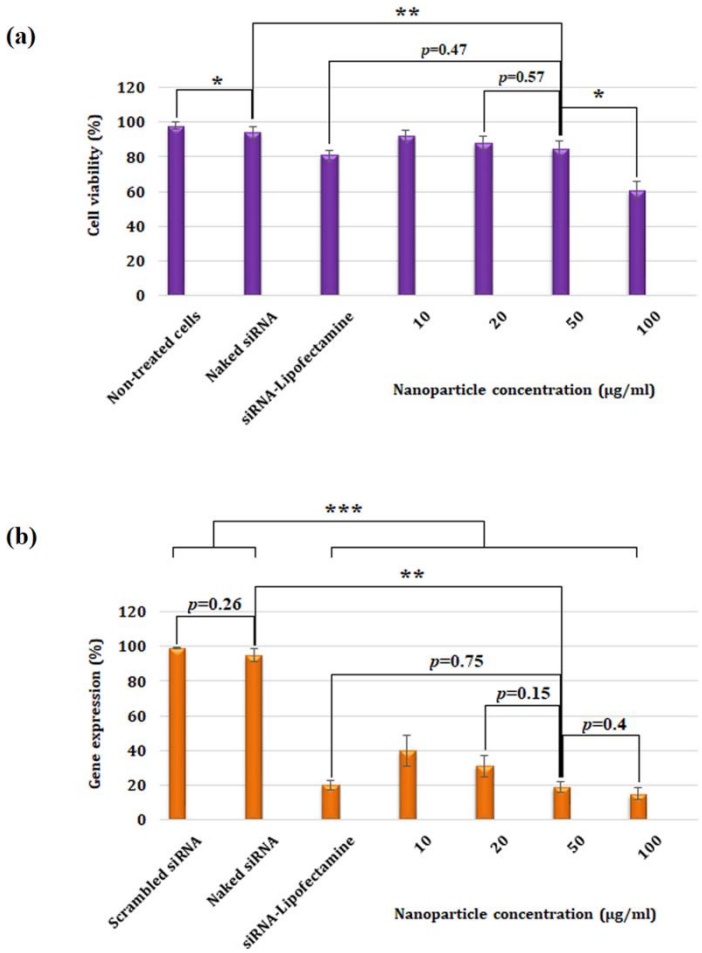

Fig. 6. The effect of various concentrations of the optimal nanoparticle (in comparison to the control groups) on (a) HEK293 cell viability and (b) HIV-1 tat expression. The results are indicated as mean values ± SD (n=3). Statistical significances were determined as $p < 0.05$, $p < 0.01$ and $p < 0.001$. Anti-tat siRNA-Lipofectamine RNAiMAX was utilized as a positive control.

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DISCUSSION

The main goal of this research was to develop a SPION-based RNA delivery system into cells because of the remarkable properties of SPIONs that can be applied in HIV treatment.

SPIONs were covered by two polymers (PEI and TMC) to obtain a stable and effective drug/ vaccine delivery nanocarrier system. In the literature, both polymers used here have indicated high potential for delivery of nucleic acids (DNA or RNA) into various cell types and have facilitated cell uptake by compacting nucleic acids into nanoparticles [7, 15]. Furthermore, they are shown to protect nucleic acids from degradation caused by extracellular enzymes, and also could help nucleic acids escape from endolysosomes [10].

Herein, the results of FTIR spectroscopy verified the formation of SPION-TMC-PEI. Moreover, according to our results (data not shown), the N:P ratio of 40 was considered to be the optimal ratio due to the appropriate size and positive zeta potential and also the high efficiency of RNA loading. It has been shown that the particle size below 150 nm together with a modest positive zeta potential are important for an effective cell uptake [7]. The positive surface charge of the nanoparticles can increase their stability and protects the nanoparticles from aggregation [16]. In addition, the positively-charged nanoparticles can efficiently interact with the negatively-charged plasma membrane of the cells, enhancing the uptake of the nanoparticles into the cells [17].

As shown in Figure 3, the optimal nanoparticles (SPION-TMC-PEI) could efficiently retard the siRNA. This indicates that the positively-charged PEI and TMC in the nanoparticles structure have an important role in siRNA condensation into the nanoparticles. Moreover, the SEM image revealed the spherical shape and dispersed distribution of the optimal nanoparticles. The XTT assay (Fig. 6a) confirmed an acceptable low-toxicity of the optimal nanoparticle. The results indicated that the viability of cells treated with PEI–TMC–SPIONs were significantly different from the cells treated with naked siRNA. However, the cells transfected with the concentrations $\leq$ 50 µg/mL of the nanoparticle showed cell viability of more than 80%, indicating a tolerable biocompatibility of the optimal nanoparticle at this concentration. Previous researches have also reported the biocompatibility and safety of SPIONs [7, 18].

The optimum formulation of SPION-TMC-PEI showed an effective cellular uptake. It has to be noticed that here the cell line HEK293 was used to generate the stable cells because of its simplicity to create stable cells and also its high gene expression. This provided a safer and easier approach to investigate the efficiency of the nanoparticles, compared to directly working with HIV-1 infected cells. Furthermore, the investigation of the cell transfection and gene silencing were accomplished in the medium supplemented by serum, implying the nanoparticle stability. The results of qRT-PCR analysis (Fig. 6b) indicated that the siRNA-loaded SPION-TMC-PEI (50 µg/mL of nanoparticle with a dose of 100 pmol/mL of siRNA) increased the siRNA quantity into the cells and thus dramatically decreased the expression of HIV-1 tat gene in comparison with the controls (i.e. cells transfected with Lipofectamine RNAiMAX containing scrambled siRNA (100 pmol/mL) and also cells treated with 100 pmol/mL of naked anti-tat siRNA; $p < 0.001$). Furthermore, the gene expression in the cells treated with 50 µg/mL of the nanoparticles was as low as that of the cells transfected with siRNA-Lipofectamine RNAiMAX which indicated the high efficiency of the optimal nanoparticles at this concentration to inhibit the gene expression. Therefore, considering the significant cytotoxicity of the nanoparticles at the concentration of 100 µg/mL (Fig. 6a) compared with that of 50 µg/mL ($p < 0.05$), the 50 µg/mL of SPION-TMC-PEI nanocarrier was presented as the optimal concentration to deliver siRNA into the target cells. It has to be noticed that the stable cells (expressing HIV-1 Tat protein) were only used to investigate the gene silencing while other methods of analysis, such as cell uptake, were achieved on unstable cells. This was because Tat as a transducing protein could affect the cellular uptake mechanism [3].

In conclusion, the focus in this study was to evaluate the nanoparticle efficiency for delivering anti-tat siRNA into target cells and silencing HIV-1 tat (a critical viral regulatory gene). It is envisaged that SION-TMC-PEI formulations could be evaluated as an inexpensive and promising delivery system in preclinical and clinical research against HIV infection. However, the effectiveness of the nanoparticle to deliver other therapeutic nucleic acids such as vaccines against HIV require further investigations.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

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