Tetraiodothyroacetic acid-conjugated polyethylenimine for integrin
receptor mediated delivery of the plasmid encoding IL-12 gene

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ABSTRACT

Targeted delivery by polymer-based nanoparticles has been considered as an efficient approach to transfer genetic materials into cells. Considering the over expression of integrin $\alpha_v\beta_3$ receptor on tumor cells and the presence of the binding site for tetraiodothyroacetic acid (tetrac) on integrin receptor, we hypothesized that the conjugation of tetrac to polyethylenimine (PEI) might be an effective strategy for pDNA delivery into the cells over-expressing integrins on their surfaces. In order to test the hypothesis, tetrac conjugated PEI/plasmid DNA complexes were prepared and their ability in the delivery of plasmid encoding IL-12 gene was investigated. Moreover, the conjugates were characterized with respect to plasmid DNA condensation ability, particle size and zeta potential as well as cell-induced toxicity and plasmid protection against DNase degradation. The results demonstrated that tetrac conjugated derivatives of PEI were able to condense the plasmid and protect it against enzyme degradation. The results of dynamic light scattering (DLS) and atomic force microscopy (AFM) revealed that the formed nanoparticles were in the size range of 85-125 nm. The highest level of IL-12 gene expression was achieved by tetrac-conjugated PEIs at the carrier to plasmid ratio of 8 where they could increase the level of gene expression up to 4 fold in the cell lines over-expressing integrin $\alpha_v\beta_3$ receptor whereas no increase in the level of IL-12 expression in the cell lines lacking integrin receptors was observed. Also, the results of the competitive inhibition of the receptors demonstrated the specificity of transfection for the cells over expressing $\alpha_v\beta_3$ receptor. On the other hand, tetrac conjugation of PEI significantly reduced the polymer-induced apoptotic effects. The results obtained in this investigation suggest the potential of tetrac as a small molecule mimicking the binding properties of integrin binding peptides (e.g., RGD) for targeted gene delivery.

Keywords: Gene delivery, Integrin, Polyethylenimine, Interleukin-12, Targeted delivery.

1. Introduction

The different roles of cell adhesion molecules such as integrin family include the regulating of cell proliferation and migration as well as cell survival and apoptosis [1-5]. The involvement of integrin receptor $\alpha_v\beta_3$ in various biological processes including tumor metastasis and angiogenesis has been shown in previous investigations [6, 7]. These so-called vitronectin receptors bind to the extracellular matrix ligands (*e.g.*, vitronectin, laminin and fibronectin) and provide the connection between the extracellular environment and the signaling pathways inside the cells [8]. Overexpression of $\alpha_v\beta_3$ receptors have been reported in prostate carcinoma, pancreatic tumors, glioblastoma, and particularly on the vessels of tumor tissues [9-11]. Tumor targeting reduces the side effects associated with the systemic administration of therapeutic agents such as small molecules and nucleic acid materials. Also, there are some reports indicating the use of integrin receptors for enhanced cell entry by adenoviruses [12-14]. In other words, the targetability improves the cell internalization via a receptor-mediated mechanism. Therefore, targeting tumor sites using the integrin receptors might be a useful strategy to direct the molecules into the target malignant cells and overcome the barriers limiting the wide clinical application of chemotherapeutics as well as oligonucleotides [15, 16].

Previously, it was believed that the entrance of thyroid hormone (L-thyroxine, T_4) into the cells occurs via simple diffusion in which the hormone crosses the membrane as a result of the difference between the concentration inside and outside the plasma membrane [17, 18]. Interestingly, it has been recently shown that there is a recognition site for thyroid hormone on the integrin receptor which is located at or near the RGD (Arginine-Glycine-Aspartic acid) binding site [7, 19]. The RGD-based targeting approaches have been widely utilized for the efficient and targeted delivery of various therapeutics including nucleic acid materials and small molecules [7, 19-21]. On the other hand, the capability of thyroid hormone for binding to a

specific recognition site on the $\alpha_v\beta_3$ integrin receptor has been proved [7, 19]. These make integrin $\alpha_v\beta_3$ as an attractive site which could be targeted by thyroid hormone and its analogues [22]. As the binding capacity of tetraiodothyroacetic acid (tetrac), deaminated analogue of T_4 hormone, to the $\alpha_v\beta_3$ integrin receptors has been demonstrated in previous investigations [23-25], this small molecule could be considered as promising non-peptide ligand mimicking RGD properties for targeted delivery which allows the uptake of large molecules into the cells through a zippering mechanism [26].

The delivery of nucleic acid based-therapeutics such as plasmid DNA, siRNA, aptamers and ribozymes as well as CRISPR/Cas9 genome editing platforms has been remained the primary limiting factor in their wide clinical applications [27-29]. Therefore, vectorization of nucleic acids has been considered as an effective route to overcome the barriers hampering the efficient delivery into the target cells. Among the non-viral gene delivery systems, polyethylenimine (PEI) has shown reasonable transfection efficiency; however its remarkable cytotoxicity, non-specific interactions with the positively charged cell surface components as well as the interaction with serum proteins have limited its broad application [30, 31]. For these reasons, conjugation of targeting moieties onto PEI structure is an efficient strategy to direct the cargo-containing nucleic acid materials into the precise site of action which in turn reduces the undesirable adverse reactions attributed to their systemic distribution. Moreover, the attachment of targeting ligands may reduce the PEI toxicity due to the reduction of its positive surface charge [22, 32-36].

In recent years, cancer immunotherapy has been proposed as an outstanding breakthrough with the ability to open up new horizons for the treatment of various types of cancers [37]. According to the results of different cytokine-based gene therapy approaches, interleukin-12 (IL-

12) has been particularly considered as a powerful antitumor agent with the ability to activate natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) as well as the induction of IFN- γ secretion [38-40]. Following the administration of IL-12 recombinant protein, severe systemic toxic reactions have been reported which is mainly associated with the elevated levels of IFN- γ . Therefore, localized production of IL-12 protein in tumor cells could be an alternative approach to decrease the toxic adverse effects associated with the secretion of IFN- γ in the whole body [36, 41, 42].

Considering the overexpression of integrin $\alpha_v\beta_3$ receptors on tumor cells and the importance of targeted delivery of plasmid encoding IL-12 gene into these cells, we hypothesized that conjugation of tetrac onto PEI may provide the opportunity to create an efficient targeted non-viral gene carrier to transfer the plasmid encoding IL-12 into the $\alpha_v\beta_3$ over-expressing cells. The tetrac-conjugated PEI derivatives were characterized with respect to plasmid condensation ability, protection against nuclease degradation and buffering capacity as well as particle size and zeta potential. The ability of PEI conjugates in transferring the plasmid encoding IL-12 gene and their cytotoxicity was evaluated in A375 cells, a melanoma cell line overexpressing integrin $\alpha_v\beta_3$ [25], and compared with HepG2 cells lacking these receptors.

2. Materials and methods

2.1. Materials

Branched polyethylenimine (PEI; average MW 25kDa), 3,3',5,5'-tetraiodothyroacetic acid (tetrac), N-hydroxybenzotriazole, 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-[2-hydroxyethyl] piperazine-N0-[2-ethanesulfonic acid] (HEPES) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma-

Aldrich (Munich, Germany). 3,5,3',5'-tetraiodo-L-thyronine (L-thyroxine, T₄) was kindly donated by Iran Hormone Pharmaceutical Company (Tehran, Iran). Human IL-12 plasmid (pUMVC3-hIL12) and mammalian vector with independent tGFP expression (pCMV6-A-GFP) were obtained from Aldevron (Madison, WI) and Origene (Rockville, MD), respectively. Plasmid pRL-CMV-luc under the control of cytomegalovirus (CMV) promoter was purchased from Promega (Madison, USA). Human IL-12 (p70) ELISA Kit and IFN- γ ELISA kit were purchased from BD Bioscience (Heidelberg, Germany). EndoFree Plasmid Mega Kit was obtained from Qiagen (Hilden, Germany). FITC Annexin V Apoptosis Detection Kit was purchased from BioLegend (Fell, Germany). Ethidium bromide, DNase I and DNA ladder 1 kb were purchased from Cinnagen (Tehran, Iran). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Gibco (Gaithersburg, MD, USA). All solvents and chemicals were obtained from Sigma-Aldrich (Munich, Germany) and were of the highest purity available. Dialyses were performed by Spectra/Por dialysis membranes (Spectrum Laboratories, Houston, TX USA).

2.2. Plasmid Preparation

The plasmid (pUMVC3-hIL12) was propagated in selective Luria-Bertani (LB) medium containing kanamycin following the transformation into *Escherichia coli* bacterial strain DH5 α . The amplified plasmids were purified using the Qiagen EndoFree Mega Plasmid Kit according to the manufacturer's instructions. A UV spectrophotometer was used to determine the concentration and purity of the preparations containing the plasmid encoding IL-12 gene. Also, the size and integrity of the plasmids were confirmed using agarose gel electrophoresis.

2.3. Carrier preparation

To prepare tetrac-conjugated PEI derivatives, 25 kDa branched PEI (0.5 g) was dissolved in water. In order to achieve 1, 2 and 4 % of PEI primary amines substitution, desired amounts of tetrac were added dropwise over a period of 3 h to a vigorously stirred PEI solution followed by the addition of 1 mol equivalents of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxybenzotriazole to activate the carboxylate groups of tetrac for the formation of amide linkage between tetrac and PEI amines. The reaction mixture was allowed to proceed at room temperature for another 24 h with constant shaking. In order to remove the unreacted materials, the solutions containing products were poured into dialysis tubes (10,000 Da cut-off, Spectra/Por membrane) and dialysis was carried out against ethanol for the first 24 h followed by double distilled water: ethanol (50:50) and double distilled water for the second and third days, respectively. Finally, the aqueous solutions were lyophilized to yield fluffy materials. The formation of amide bond between tetrac and PEI was confirmed using FT-IR (VERTEX 70, Bruker, Germany). The structure of tetrac-conjugated PEI derivatives and the conjugation degrees were determined by ¹H-NMR (D₂O) spectroscopy using a Bruker Avance DRX-500 MHz NMR spectrometer (Bruker, Ettlingen, Germany).

2.4. Polyplex preparation

Tetrac-PEI/pDNA polyplexes were prepared in HBG buffer (HEPES buffered glucose solution; 20mM HEPES, 5% glucose, pH 7.2) by adding 50 µl of a solution containing tetrac-PEI conjugates at various concentrations to the same volume of pDNA (40 µg/ml) in the same buffer followed by pipetting up and down and finally incubation at room temperature for 20-30 min to form stable complexes. The composition of the formulated polyplexes was defined by C/P ratio

in which C is the weight of PEI and its derivatives and P represents the weight of plasmid DNA used for polyplexes formation.

2.5. Polyplex Characterization

2.5.1. Determination of plasmid DNA binding affinity to the modified polymers

In order to qualitative assessment of the binding affinity of tetrac-conjugated PEI derivatives with plasmid DNA, gel retardation assay was carried out. Polyplexes were prepared as described earlier at different C/P ratios ranging from 0.25:1-8:1. Ten microliters of the prepared solution was mixed with the loading buffer (KBC power load, Kawsar Biotech Company, Tehran, Iran) and loaded onto 1% agarose gel. The electrophoresis was performed in TAE buffer at 100 V for 30 min. Eventually, DNA bands were visualized by a UV transilluminator.

The quantitative association of the plasmid DNA with the tetrac conjugated PEIs was evaluated by a DNA intercalating dye (ethidium bromide). The exclusion of EtBr from pDNA structures upon the addition of PEI and its conjugates leads to a decrease in fluorescence intensity indicating the ability of PEI and its derivatives to condense pDNA. A solution of plasmid DNA (400 ng/ml) and EtBr (0.4 mg/ml) was prepared in HBG buffer and its fluorescence intensity was measured and set to 100% using a spectrofluorometer (FP-6200; Jasco, Japan). The polymer solution was added stepwise to the plasmid DNA solution and the reduction of fluorescence intensity was recorded following each addition (excitation: 510 nm, emission: 590 nm). The graph represents the quantitative condensation ability of tetrac conjugated PEI derivatives at various C/P ratios. All measurements were done in triplicate.

2.5.2. Protection of pDNA against enzymatic digestion

DNase I protection and release assay was carried out to demonstrate the capability of PEI and its conjugates to protect pDNA against enzymatic degradation. The prepared polyplexes at different C/P ratios (ranging from 0.25:1 to 8:1) were mixed with 1 ml of DNase I or PBS in DNase/Mg²⁺ digestion buffer (50 mM Tris-Cl, pH=7.6 and 10 mM MgCl₂) and incubated at 37 °C for 30 min. Instant inactivation of DNaseI following the incubation period was carried out by adding 4 ml of EDTA (250 mM) and mixing with 1% sodium dodecyl sulphate (SDS), dissolved in 1M NaOH (pH=7.2). The samples were incubated at room temperature for approximately 2 h to allow the dissociation of the plasmid from the modified PEI derivatives. The location of plasmid bands was analyzed by agarose gel electrophoresis at 70 V for 1 h.

2.5.3. Measurement of the buffering capacity of PEI and its conjugates

In order to evaluate the buffering capacity of unmodified PEI and its conjugated derivatives, the pH of each polymer solution (400 µg/ml) was adjusted to 12 using 1 M NaOH. Following each addition of the aliquots (5 µl) of 1 M HCl, the pH value was recorded by a pH meter (Mettler Todelo, Greifensee, Switzerland). The addition of HCl was stopped while the pH of around 2 was reached. The slope of the plot of pH versus the amount of added HCl demonstrates the buffering capacity of different conjugates.

2.5.4. Particle size and ζ potential measurements

The sizes and ζ potentials of the polyplexes were measured using Malvern Nano ZS instrument and DTS software (Malvern Instruments, Malvern, UK) by adding the desired amounts of PEI and its conjugates prepared in HBG buffer (pH 7.4) into the pDNA solution

prepared in the same buffer. The measurements were carried out in automatic mode and the results are presented as **mean $\pm$ SD, n=3**. Each mean represents the average value of 30 measurements.

2.5.5. Scanning electron microscopy (SEM) and Atomic force microscopy (AFM)

The size and morphological properties of the polyplexes at the highest C/P ratio tested (i.e., C/P=8) were also studied using scanning electron microscopy (VEGA TESCAN instrument, Brno, Czech Republic) and atomic force microscopy (JPK NanoWizard II instrument, Berlin, Germany). The SEM analysis was performed by dispersing the polyplexes on the copper tapes followed by coating with gold in an ionization chamber. The AFM analysis was carried out by deposition of complexes onto the split untreated disk and incubation at room temperature for drying before imaging.

2.6. Biological studies

2.6.1. Cell culture and transfection procedure

HepG2 hepatocellular carcinoma cell (C158, NCBI, Tehran, Iran) and A375 melanoma cells (C136, NCBI, Tehran, Iran) were cultured in DMEM media (1g/l glucose, 2 mM glutamine), supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G and 100 μ g/ml streptomycin. Cultures were incubated at 37 °C in a humidified 5 % CO₂ before starting the gene transfer experiments. In vitro transfection experiments were carried out using the plasmid encoding human IL-12 gene (pUMVC3-hIL12) and **the GFP expressing plasmid (pCMV6-A-GFP)** at the final concentration of 200 ng plasmids/well. The cells were seeded at a density of 1×10^4 cells/well in 96-well trays one day prior to the transfection assay. Polyplexes

were prepared at various C/P ratios ranging from 0.25:1-8:1 (*vide supra*). Then, each well was treated by the addition of 10 μ l of the polyplex solution followed by the incubation at 37 °C for 4 h. After the incubation period, the medium was replaced with fresh DMEM containing 10% FBS and the cells were incubated for an additional 48 h. In order to perform the transfection procedure by the plasmid encoding green fluorescent protein, the A375 cells were seeded into a 6-well plate at the density of 1×10^6 cells/well. The cells were treated with the polyplexes containing pCMV6-A-GFP plasmid as described above. The transfected cells were visualized under a fluorescent microscope and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Mountain View, CA) using FlowJo software (TreeStar Inc., San Carlos, CA). Plasmid pRL-CMV-luc was used as the control for all transfection experiments.

2.6.2. Specificity of transfection

In order to show the impact of tetrac conjugation in improving the gene transfer efficiency of PEI through the integrin receptors, inhibition of transfection was carried out using free targeting ligand of $\alpha_v\beta_3$ integrin receptor (i.e., T₄). A375 and HepG2 cells were seeded as described above and pretreated with T₄ at different concentrations ranging from 10^{-10} to 10^{-3} mol/ml of the free ligand for 30 min at 37 °C. All the cultures were also treated with propylthiouracil (1mM) to inhibit tetraiodothyronine 5' deiodinase and prevent the conversion of T₄ to T₃. Then, the polyplexes prepared of unmodified PEI and its tetrac conjugates were added into the medium and incubated for 4 h. Following the medium replacement, the incubation continued and the transgene expression was evaluated 48 h later.

2.6.3. Expression of hIL-12 and tGFP and evaluation of IFN- γ level

The expression of hIL-12 gene and induction of IFN- γ were assessed using the human IL-12 (p70) and IFN- γ ELISA sets (BD Bioscience, Heidelberg, Germany), respectively according to the manufacturer's instructions. Briefly, 96 well ELISA plates were coated by the capture antibody and maintained overnight at 4 °C. In order to prevent non specific bindings, assay diluent was added to the wells followed by the addition of controls, standard and samples. After incubation for 2 h at room temperature, detection monoclonal antibody containing streptavidin-horseradish peroxidase reagent was added followed by the addition of substrate solution. At the end, 50 μ l of H₃PO₄ (1M) was added to each well to stop the reaction and the absorbance was recorded with an ELISA plate reader at 450 nm within 30 min of stopping the reaction. The levels of hIL-12 and IFN- γ were presented as (pg/ml)/seeded cells. Also, the efficiency of pCMV6-A-GFP delivery was determined by flow cytometry 48 h after transfection.

2.6.4. MTT assay

Cytotoxicity assay was carried out with the same polyplex formulations and the cell lines used in the transfection experiments. Cells were seeded at a density of 1×10^4 cells/well and treated with polyplexes. After 4 h, the medium was exchanged with fresh DMEM supplemented with FBS and incubated for 48 h. Following the incubation time, the cell viability assay was performed using MTT solution. Twenty microliters of the solution (5mg/ml) was added to each well and incubated at 37 °C for 1.5-2 h. Finally, dimethyl sulfoxide (100 μ l/well) was added to dissolve the formazan product and the absorbance was recorded at 590 nm with background correction at 630 nm using an ELISA plate reader. Cell viability was presented as a percentage absorbance relative to that in untreated control cells. Values of metabolic activity are expressed as the mean $\pm$ SD of triplicates.

2.6.5. Flow cytometry assay of apoptosis

In order to evaluate the induction of apoptosis following the treatment of the cells by the polyplexes formed by PEI and its conjugates, FITC Annexin V Apoptosis Detection Kit with 7-AAD was used according to the manufacturer's instruction. Briefly, cells were seeded into a 6-well plate at the density of 1×10^6 cells/well. The cells were treated with the polyplex formulations as described above. After 48 h, the cells were detached, aspirated and suspended in Annexin V binding buffer. Then, 5 μ l of FITC Annexin V and the same amount of 7-AAD viability staining solution was added and incubated in room temperature in dark for 15 min. Finally, 400 μ l of Annexin V binding buffer was added and apoptosis was measured by flow cytometry (FACSCalibur, Becton Dickinson, CA) using FlowJo software (TreeStar Inc., CA). All measurements were done in triplicate and the cells treated with no plasmid or polyplex (i.e., with medium only) were considered as negative control.

2.7. Statistics

Data are presented as the mean $\pm$ SD. The statistical significance was determined using Student's *t*-test, and *p* values of ≤ 0.05 were considered significant.

3. Results and discussion

3.1. Synthesis of tetrac conjugated PEI derivatives

Surface primary amines of PEI were conjugated to the carboxylic group of tetrac using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxybenzotriazole through the amide bond formation (Scheme 1). The conjugation of tetrac onto the PEI structure and the formation of amide linkage were confirmed by ^1H -NMR and FT-IR. The ^1H -NMR

spectra revealed that PEI protons appeared between 2.7-3.2 ppm, whereas tetrac protons were found between 7.2-7.9 ppm (supplementary Fig.1). The formation of amide bond was demonstrated by FT-IR spectra in which the amide peak at 1650 cm^{-1} was detected (supplementary Fig. 2). The modification degree was analyzed by ^1H NMR from the ratio between the peaks of PEI (δ 2.7-3.2 ppm) and the aromatic rings of tetrac (δ 7.2-7.9 ppm). The conjugation degree was expressed as a percentage of modified amines of PEI under the assumption that each amine is able to react. Depending on the tetrac/PEI amine ratio in the initial feed (mol %), three substitution degrees of 1, 2 and 4 % were achieved. The PEI conjugates were coded as PEI-Tet X% in which Tet represents tetrac and X% shows the PEI substitution degree.

3.2. Biophysical characterization of PEI conjugates and the polyplexes

3.2.1. Measurement of the polymer binding affinity to the pDNA

Substantial cationic charges on the surface of PEI structure results in electrostatic interaction between the positively charged polymers and the negatively charged backbone of the nucleic acid materials such as plasmid DNA [27, 32, 33]. This process leads to the formation of compact complexes called polyplexes. This condensation of pDNA into nano-sized structures is a prerequisite step in efficient transfer of pDNA into the cells. In other words, PEI forms the compact complexes by wrapping around nucleic acids leading to pDNA packaging [43, 44]. One of the most commonly used techniques for the analysis of PEI binding strength to pDNA is ethidium bromide exclusion assay in which the addition of polycationic polymers results in the displacement of ethidium bromide from the stacked base pairs of pDNA and subsequently the reduction of fluorescence intensity occurs. Therefore, more decrease in the fluorescence intensity demonstrates more affinity of PEI and its derivatives to plasmid DNA. The fluorescence

quenching analysis demonstrated that PEI and its conjugates were able to condense pDNA at the C/P ratios higher than 1 (Fig. 1A). Although a similar pattern for the decrease in the fluorescence intensity of PEI and its derivatives was observed, the unmodified PEI reduced the fluorescence intensity by around 70% at the lowest C/P ratio tested (i.e., C/P=0.25), while the reduction of EtBr fluorescence intensity for PEI-Tet 4% was approximately 35% at the same C/P ratio. In other words, higher degrees of conjugation resulted in the formation of looser complexes than those formed by the unmodified PEI or its conjugates at lower grafting degrees of 1 and 2% (*vide infra*). The addition of more polymer (e.g., C/P $\geq$ 1.5) resulted in a plateau state.

Since the electrostatic interaction between the positively charged polymer and the negatively charged pDNA results in the formation of polyplexes showing less mobility than free nucleic acid materials such as plasmids, the gel retardation assay can demonstrate the affinity of PEI and its conjugates for pDNA condensation. As shown in Fig. 1B, the complexes prepared by unmodified PEI and the conjugates of PEI-Tet 1% and PEI-Tet 2% could retard the plasmid DNA at all the C/P ratios tested (lanes 3-5 and 7-14). Although the polyplexes based on PEI-Tet 4% could optimally condense pDNA at C/P ratios of 4 and 8, the weakest association between the modified polymer and the plasmid occurred at the lowest C/P ratio tested (i.e., C/P=0.25). This observation is consistent with the results obtained in the ethidium bromide quenching assay in which PEI-Tet 4% reduced the fluorescence intensity just around 35% compared to a reduction of approximately 75% for the rest of the polymers at the C/P ratio of 0.25:1 (Fig. 1A).

According to the previous investigations, conjugation of various moieties via the PEI surface amines decreases the positive charge density of the polymer and leads to the formation of less tightly complexes. It is assumed that these looser polyplexes could be dissociated easier which leads to a more effective “vector unpackaging” process [45, 46]. However, our results

suggested that the PEI conjugates with the highest condensation ability were also the most effective ones in gene delivery. These controversial results have also been reported in several previous studies in which the formation of less tightly complexes led to an increase in transfection efficiency [45-47]; whereas the more tightly complexes enhanced gene delivery ability of PEI in some other investigations [44, 47, 48]. According to the results obtained in this study, facilitating the dissociation of PEI and plasmid inside the cytosol or cell nucleus will not necessarily lead to enhanced transfection efficiency unless the vector unpackaging is considered as the rate-limiting step in the whole process [22, 49, 50].

3.2.2. pDNA protection against DNase I digestion

Considering the fast digestion of naked nucleic acid materials such as pDNA by various enzymes including nucleases and their short half life in blood circulation, the polymer-based carriers must be able to protect genetic materials against degrading enzymes. While the interaction of the positively charged polymers and the negatively charged pDNA occurs, the process of plasmid condensation does not only result in the formation of compact nanosized particles, but also protects the plasmid against different degrading factors including nucleases. In the present investigation, DNase I was utilized as a model enzyme in order to demonstrate the ability of PEI derivatives to protect the plasmid against digestion induced by the enzyme. As an example, the results of the protection assay for the polyplexes prepared by PEI-Tet 1% have been shown in Fig. 2. The DNase I protection test for the rest of the PEI conjugates led to the similar pattern. The treatment of the naked pDNA by the enzyme led to the complete digestion of the nucleic acid material (lane 3). At the lowest C/P ratio tested ($C/P=0.25$), neither of the polyplexes formed by unmodified PEI or PEI-Tet 1% could protect the plasmid DNA (lanes 7

and 8) while the complete protection occurred at the highest carrier to plasmid ratio of 8 (lanes 16 and 17). As shown in Fig. 2, unmodified PEI was able to induce an optimal protection effect at the C/P ratio of 4 while a partial protection was observed by the polyplexes prepared by PEI-Tet 1% at same C/P ratio (lanes 12 and 13). According to the results of gel retardation assay (Fig. 1B), PEI-Tet 1% could condense pDNA at all the C/P ratios tested in this study. On the other hand, this complete condensation did not lead to an optimal resistance against degradation by DNase I (Fig. 2, lanes 8 and 13). In other words, a substantial protection against degrading enzymes could not be necessarily achieved by a full condensation. This behavior might be associated with the other physicochemical properties of the polyplexes including particle shape, size and charge. These characteristics would probably result in the formation of nanoparticles containing plasmid portions on their surface areas which in turn increases the susceptibility of the exposed pDNA segments to be digested by nucleases [43, 44, 51].

3.2.3. Evaluation of the buffering capacity of the PEI derivatives

Proton sponge effect has been considered as the main mechanism by which the polycation-based gene carriers induce an early escape from endo/lysosomal compartments before starting the lysosomal degradation of the nucleic acid materials by degrading enzymes inside the compartments [52]. According to this hypothesis, the high amine content of PEI is responsible for capturing the protons pumped into the endosomes during the process of endosomal acidification. This process is followed by the influx of chloride ion and water which in turn leads to the osmotic swelling and rupture of the endo/lysosomal compartments particularly at the pH range of 5.5-7 [52, 53]. In order to evaluate the buffering capacity of PEI and its derivatives, an acid-base titration method was utilized and the intrinsic buffering capacity of the conjugates was

393 demonstrated by plotting the pH versus added HCl (Fig. 3). The results shown in Fig. 3 indicated
394 that unmodified PEI had remarkable buffering capacity at pH range of 5.5-7 as well as 9-11.
395 Also, PEI derivatives exhibited substantial buffering capacity at the same pH range. In other
396 words, substitution of tetrac at the conjugation degrees of 1-4% could not decrease the capacity
397 of the modified polymers to act as a buffering agent over a broad pH range. The buffering
398 capacity of PEI and its conjugates at the pH range of 9-11 is associated with the primary and
399 secondary amines of the parent polymer with the pKa values of 8-9 while the polymer buffering
400 capacity at the pH range of 5.5-7 might be attributed with the tertiary amines with the reported
401 pKa values of 6-7 [49, 54]. Since PEI and its conjugates act as an abundant source of the primary
402 and secondary amines, the low conjugation degrees could not affect the buffering capacity of PEI
403 derivatives. This observation is consistent with the previous investigations which reported the
404 relationship between low conjugation degrees and transfection efficiency. [49, 50]. Also, the
405 conjugation of tetrac onto the PEI structure occurred through the amide bond formation. This
406 converts the primary amines on the surface of the parent polymer to the secondary ones with the
407 same pKa values. Therefore, the overall buffering capacity of the polymer did not changed. On
408 the other hand, the driving force for the osmotic burst mechanism results from the buffering
409 capacity of PEI and its derivatives at the pH range of 5.5-7 which induces the early escape of the
410 polyplex formulations from endolysosomal compartments [55]. Since the tertiary amines of PEI
411 are located inside the molecule and undergo protonation at acidic environments such as
412 lysosomal compartments, it is supposed that the major role of these amines is to promote the
413 early escape from endosomes whereas the primary and secondary amines are oriented toward the
414 exterior of the PEI molecules and their protonation occurs at higher pH values. Therefore, the
415 major responsibility of these functional groups is the electrostatic interaction with the negatively

charged nucleic acid materials and compacting them into the nanoparticles. Although there are some controversial reports indicating that no significant changes of pH occurs following the accumulation of PEI inside the endosomes [56], proton sponge effect is still the well accepted mechanism for the release of PEI-based nanoparticles into the cytosol.

3.2.4. Analysis of particle size and ζ potential

The PEI/pDNA complexes form upon the addition of positively charged polymer to the negatively charged plasmid. The formation of nano-sized polyplexes with favorable surface charge plays a key role in transfection efficiency as well as cytotoxicity and accessibility to the target tissues and cells. Having demonstrated that tetrac-conjugated PEI derivatives are able to electrostatically interact with plasmid DNA and condense it into nanoparticles, the complexes were formed at the C/P ratio of 8 in which the highest transfection efficiency was achieved and characterized by Dynamic Light Scattering (DLS) and Laser Doppler Velocimetry (LDV) in order to measure the particle size and ζ potential, respectively. The results illustrated that the tetrac conjugated PEIs formed complexes in the size range of 85-125 nm while the unmodified PEI resulted in the formation of polyplexes with size of 75 nm (Fig. 4A). The sizes of the complexes prepared by PEI-Tet 1%, PEI-Tet 2% or PEI-Tet 4% showed no significant difference ($p>0.05$). The larger sizes of the PEI conjugates compared to unmodified PEI might be associated with the substitution of relatively large tetrac molecules on the surface of the polymer which may alter the interaction between the negatively charged backbone of the pDNA and the positively charged surface of PEI. This observation was in a good agreement with the previous investigations in which the conjugation of large pendant moieties on the surface of polycationic polymers resulted in the formation of larger polyplexes [50, 51, 57]. The results of LDV

demonstrated that PEI-Tet 4% led to the polyplexes with lower surface positive charge compared to unmodified branched 25kDa PEI ($p<0.05$). As shown in Fig. 4A, substitution of tetrac molecule on the PEI surface reduced the positive charge of the polyplexes from 35 mV in the case of unmodified PEI to 23 mV for PEI-Tet 4%. Also, the results indicated that the polyplex ζ potential decreased on increasing the conjugation degree. There are several studies showing the surface charge reduction following the substitution of various pendant moieties through the PEI surface amines [35, 45, 49, 51, 57, 58]. Since the particle size measurements were also performed 1 h after preparation of the complexes and no significant increase in the particle size was observed, it seems that the polyplexes preserved enough positive charge on their surfaces preventing aggregation by charge repulsion. In other word, substantial amine content of PEI even following the tetrac conjugation contributed to the net positive charge which in turn may hamper aggregation and facilitate the cell entry [46].

3.2.5. SEM and AFM analyses

The morphology and particle size of the polyplexes at the C/P ratio of 8 was studied using SEM (Fig. 4B) and AFM (Fig. 4C). The images revealed that the polyplexes were spherical and in the relatively similar size ranges obtained by DLS analysis (Fig 4A). However, the limitations of these techniques such as the examination of individual nanoparticles and observation of a small fraction of polyplexes must be considered.

3.3. Biological investigations

3.3.1. Gene transfer experiments

The introduction of cancer immunotherapy as the breakthrough of the year for 2013 has raised hope that cytokine-mediated therapy could be utilized as an effective treatment for cancer [37]. On the other hand, the antitumor potential of IL-12 protein in cellular and preclinical models has led researchers to test this protein in human patients. However, the systemic administration of the recombinant IL-12 protein not only led to low efficacy but also resulted in a severe dose-dependent toxicity [40]. Therefore, targeted delivery of the plasmid encoding IL-12 gene into the tumor site might be an effective alternative strategy to achieve a persistent and non-toxic therapeutic response with IL-12 protein [59, 60]. In the present investigation, the gene transfer ability of tetrac-conjugated PEI polyplexes were evaluated on A375 cell line over expressing $\alpha_v\beta_3$ integrin receptor and on HepG2 cell line lacking this receptor. The transfection results obtained in this study demonstrated that the tetrac-conjugated PEI derivatives could not enhance the delivery of the IL-12 plasmid compare to unmodified PEI on HepG2 cell line at any C/P ratio tested ($p>0.05$) (Fig. 5A), whereas the tetrac-substituted PEI conjugates could increase the ability of the polymer to deliver the IL-12 plasmid into the A375 cells at the C/P ratio of 8 compared to unmodified PEI at the same C/P ratio ($p<0.05$) (Fig. 5B). In other words, tetrac substitution of amines of PEI enhanced hIL-12 expression by up to approximately 4 folds relative to the parent polymer, with the highest increase occurred at the conjugation degree of 1% at the C/P=8 (Fig. 5B). Also, PEI-Tet 2% and PEI-Tet 4% could enhance the hIL-12 expression levels up to three and two folds, respectively. The results indicated that the highest gene transfer efficiency occurred at the highest C/P ratio (i.e., C/P=8). The transfection tests with the plasmid pRL-CMV-luc resulted in undetectable hIL-12 production. The low transfection efficiency of the PEI conjugates at the C/P ratios of 0.25 and 4 could be explained by the method by which the polyplexes were prepared. In this study the polymer/pDNA complexes were prepared on a

weight/weight ratio (C/P) rather than on a nitrogen to phosphorus molar ratio (N/P). Hence, higher molecular weight of the tetrac conjugated PEI compared to the unmodified polymer could be considered as the main reason for the lower transfection activity at the C/P ratios of 0.25 and 4. By increasing the polymer concentration in the polyplex formulation, the gene transfer ability increased. Interestingly, the transfection efficiency decreased by increasing the conjugation degree ranging from 1% to 4%. This result is consistent with the previous studies in which the coupling of higher degrees of RGD peptides for integrin targeting reduced the transfection efficiency. This decrease might be associated with the fact that the strong binding of the targeted conjugates to the integrin receptors on the cell surface results in powerful extracellular adherence which in turn leads to low cellular internalization and consequently low transfection efficiency. Therefore, optimization of the density of ligands conjugated on the surface of the carrier has a pivotal role in enhanced targeted gene delivery [61].

Non targeted polymer-based carriers enter the cells through an adsorptive mechanism in which the electrostatic interaction between the positively charged polyplexes and the negatively charged components of the cell membrane results in the cell entrance [34, 62, 63]. Although this mechanism has been considered as an efficient route to cross the plasma membrane, the highly charged polyplexes may disrupt the plasma membrane and cause cell death (*vide infra*) [64]. Since these polyplexes interact non-specifically with different types of cells, incorporating targeting moieties such as integrin targeting ligands is an effective conjugation method to enhance the gene transfer ability in the cells possessing integrin receptors [22, 25].

3.3.2. Evaluation of IFN- γ production

The wide clinical application of IL-12 protein as an antitumor agent has been limited due to its highly toxic adverse reactions which are mainly associated with the elevated levels of IFN- γ [65]. On the other hand, IFN- γ has been considered as the most powerful mediator of antitumor action associated with IL-12. The potent antiangiogenic effects of IL-12 results from the increased level of IFN- γ and consequently the production of IFN- γ -inducible protein 10 and monokine induced by IFN- γ (MIG, CXCL9) [66]. As an alternative route of administration, the production of IL-12 protein inside the tumor tissues or cells may reduce the systemic toxic reactions attributed to the increased level of IFN- γ in the whole body. In other words, the limited production of IL-12 and subsequently IFN- γ in the target tumor sites increase the antitumor effects while the systemic adverse effects will not be expected. In the present investigation, the level of IFN- γ was evaluated to determine whether the hIL-12 was able to upregulate the production level of IFN- γ (Fig. 5C and D). As shown in Fig. 5C, the levels of IFN- γ slightly increased following the treatment of HepG2 cells with PEI and its derivatives. However, this enhancement was not significant ($p>0.05$). On the other hand, the treatment of A375 cells with tetrac-conjugated PEI derivatives resulted in an increased level of IFN- γ by up to around 2.5 fold, with the highest increase occurring at the highest C/P ratio tested in this study (C/P=8) (Fig. 5D). The results of the IFN- γ production confirmed that the produced hIL-12 in the cells was functional and able to induce the production of further mediators used in the process of tumor suppression.

3.3.3. Competitive inhibition of transfection

In order to determine the role of integrin receptors in the elevated transfection efficiency of tetrac-conjugated PEIs containing hIL-12 plasmid, the receptors were competitively inhibited

by the free targeting ligand of T₄ at the concentrations ranging from 10⁻⁹-10⁻³ mol/ml. The results of the competitive inhibition in A375 cell line using the concentration of 10⁻³ mol/ml of T₄ indicated that the transfection efficiency of tetrac-conjugated PEI derivatives was not significantly different from that of unmodified PEI at the same C/P ratios (Fig 6A). The similar results were obtained using the concentrations of 10⁻⁷ and 10⁻¹⁰ mol/ml (data not shown). On the other hand, the gene delivery experiments and the inhibition of transfection were carried out using pCMV6-A-GFP plasmid at the highest C/P ratio of 8 (Fig 6B and 6C). The results obtained in this study revealed that the transfection efficiency of PEI-Tet 1% was significantly higher when performed on A375 cells (34%) than HepG2 cells (8.9%). Following the treatment of the integrin over-expressing cells by the free targeting ligand of T₄, the transfection efficiency decreased to around 5% which was consistent with the results obtained with hIL-12 plasmid. On A375 cells, PEI-Tet 2% and PEI-Tet 4% resulted in the transfection efficiency of 18.5% and 14%; respectively whereas their gene transfer ability on HepG2 cells was around 5% (Fig. 6B). The decrease of the transgene expression by competitive inhibition of the integrin receptor clearly demonstrated the role of these receptors in elevated transfection efficiency of the tetrac conjugated PEIs. Since the competitive inhibition was also carried out on HepG2 cells and the results revealed that the treatment of the cells using the free ligand had no effect on the transfection efficiency, it could be concluded that the polyplexes enter the HepG2 cells via non-receptor mediated endocytosis.

3.3.4. MTT and apoptosis assays

The cytotoxicity of the polyplexes prepared with tetrac conjugated PEIs were assessed in A375 and HepG2 cell lines using MTT metabolic activity assay. Cells were treated with the

552 conjugated PEI derivatives at the same C/P ratios used in gene transfer tests. The cell viability
553 profile on HepG2 cells indicated that the viability was around 80% at the lowest C/P ratio tested
554 (i.e., C/P=0.25), while the highest cytotoxicity was observed at the C/P ratio of 8 (Fig. 7A). At
555 such carrier to plasmid ratio, the viability decreased to around 20%. There was no difference in
556 cell-induced toxicity between the conjugated PEI and the parent unmodified polymer at the same
557 C/P ratios. A significant C/P ratio-dependent increase in cell toxicity of the polyplexes over the
558 C/P ratio range of 0.25:1-8:1 was observed in HepG2 cell line. In A375 cell line, unmodified PEI
559 induced the highest toxic effects particularly at the highest C/P ratio (Fig 7B). In other words, by
560 increasing the polymer concentration in the final polyplex formulation, the toxicity of the
561 polyplexes prepared with unmodified PEI elevated in A375 cell line. Interestingly, tetrac
562 conjugated PEI derivatives resulted in less cytotoxicity compared to unmodified PEI at the same
563 C/P ratio ($p<0.05$) (Fig.7B). All the conjugated PEI derivatives induced less toxic effects at the
564 highest C/P ratio of 8 while the unmodified polymer exhibited the highest cytotoxicity at the
565 same C/P ratio. According to the results of cell-induced toxicity on HepG2 cells, the conjugation
566 of 1-4% of tetrac on the PEI and the modulation of charge density on the polymer surface could
567 not improve the cell viability. According to the previous investigations, high positive charge
568 density on the PEI surface has been considered as the major factor inducing cytotoxicity [62].
569 The high positive charge of the polymer interacts with the negatively charged components on the
570 cell membrane which leads to the disruption of plasma membrane and consequently cell death
571 [45, 49, 50, 57, 58, 62, 64]. The results of cell toxicity assay on HepG2 cells indicated that the
572 charge modulation was not sufficient to reduce the toxic effects associated with the high positive
573 charge on the PEI surface. In other words, there was still high charge density on the PEI inducing
574 cytotoxicity. In the present investigation, tetrac-conjugated derivatives of PEI demonstrated

575 substantially less toxic effects with A375 cells bearing integrin receptor than with HepG2 cells
576 which lack the receptor. This difference may merely be explained by the cell to cell variations.
577 However, another explanation for the less toxic effects of tetrac conjugated PEIs on A375 cell
578 line is that these cells contain the integrin receptors on their surfaces which reduce the
579 concentration of the PEI derivatives in the medium by receptor-mediated endocytosis, whereas
580 the HepG2 cells are not able to uptake the polyplexes via the similar mechanism causing the
581 accumulation of the complexes in the medium which results in more toxic effects [34, 35, 64].

582 The induction of apoptosis was determined using annexin V/7-AAD assay. As
583 phosphatidylserine (PS) has been considered as a marker for early apoptosis, the FITC
584 conjugated annexin V attaches to PS and demonstrates early apoptosis whereas the late apoptosis
585 is detected by the binding of 7-AAD to the nuclear DNA. As shown in Fig. 7C, the viability of
586 A375 cells was 99.7% after 48 h of incubation, while the addition of unmodified PEI at the C/P
587 ratio of 8 reduced the cell viability to around 3.5% (Fig. 7C). In this case, the majority of the
588 treated cells were in the early stage of apoptosis (annexin V +/7-AAD-). While the cells were
589 treated with PEI-Tet 1% at the same C/P ratio (i.e., C/P=8), the cell viability significantly
590 increased to the level of 99%. **Competitive inhibition of integrin receptors by the free targeting**
591 **ligand of T₄ (10⁻³ mol/ml) resulted in a remarkable shift of the cells into the early apoptosis stage**
592 (Fig 7C). By inhibition of receptor-mediated endocytosis, the polyplexes may remain in the
593 medium and subsequently induce apoptosis similar to the unmodified PEI (Fig. 7C). According
594 to the previous findings, the PEI cytotoxic effects occur during two different phases including an
595 early necrotic stage which begins around 30 min after exposure resulting from the perturbation of
596 the cell membranes. This stage might be followed by an apoptotic phase which occurs 24 h later
597 [64, 67]. The results of our study showed that the unmodified PEI was able to induce remarkable

apoptosis on the A375 cells whereas the conjugation of the targeting ligand of tetrac substantially reduced its apoptotic effects. Since the inhibition of the receptor mediated delivery of the polyplexes resulted in a significant increase in the apoptosis induction, it could be suggested that the remaining of the positively charged polyplexes in the medium were responsible for the induction of cell death.

4. Conclusion

Using a simple conjugation method such as the formation of amide bond, a small non-peptide molecule mimicking the RGD properties to bind to the integrin receptor was grafted to the PEI surface amines. The results revealed that the substitution of PEI amines by tetrac led to a greater expression of the plasmid encoding IL-12 gene in $\alpha_v\beta_3$ integrin receptor over-expressing cells. Hence, tetrac-conjugated PEI derivatives described in this study might serve as a nanocarrier for targeted delivery of various nucleic acid materials into the cells over expressing integrin receptors.

Acknowledgments

This work was funded by the Shiraz University of Medical Sciences (SUMS), Shiraz, Iran. Financial support from Iran National Science Foundation (INSF) (#89000844) and Iranian Nanotechnology Initiative Council (INIC) is acknowledged. This work was a part of Fatemeh Sheikhsaran Ph.D. thesis. Also, I would like to thank Dr Samira Hossaini Alhashemi for her helpful assistances.

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Graphical Abstract: Integrin receptor-mediated delivery of plasmid encoding IL-12 gene

Scheme 1. Synthesis of tetrac grafted PEI conjugates.

Fig. 1. Plasmid DNA binding affinity by 25 kDa PEI and PEI-Tet conjugates determined by (A) ethidium bromide exclusion assay and (B) gel retardation assay at various C/P ratios.

Fig. 2. DNase I protection assay for PEI-Tet 1% series.

Fig. 3. Titration curves for aqueous solutions of unmodified PEI and its tetrac-conjugated derivatives. Water has been considered as a control.

Fig. 4. (A) Particle size and ζ potential of the polyplexes formed with PEI and its conjugates in HBG buffer at C/P=8. (B) Scanning electron microscopy and (C) atomic force microscopy micrographs of the polyplexes at C/P ratio of 8.

Fig. 5. The levels of hIL-12 and induced IFN- γ in HepG2 cells (A and C) and A375 cell line (B and D) following the treatment with PEI and its derivatives complexed with pUMVC-IL12 plasmid at C/P ratios of 0.25 (black bars), 4 (gray bars), and 8 (white bars) determined in triplicate. The level of hIL-12 expression and the induced IFN- γ were presented as the concentration of the protein (pg/ml) per seeded cells. * $p < 0.05$, conjugated PEI derivative compared to unmodified parent polymer at the same C/P ratio (N = 3; error bars represent $\pm$ standard deviation).

Fig. 6. (A) Competitive inhibition of transfection efficiency of PEI and its tetrac conjugated derivatives complexed with pUMVC3-hIL12 in A375 cell lines at the concentration of 10^{-3} mol/ml of the free targeting ligand of T₄. (B) GFP expressed in A375 and HepG2 cell lines in the absence and presence of the free targeting ligand of T₄ at the C/P=8 determined by flow cytometry. * $p < 0.05$ and ** $p < 0.01$, conjugated PEI in the absence of T₄ compared to the same derivative in the presence of T₄. † $p < 0.01$, conjugated PEI compared to the unmodified polymer in the same condition (N = 3; error bars represent $\pm$ standard deviation). (C) Fluorescence microscopy image of A375 cells treated by the PEI-Tet 1% conjugates complexed with pCMV6-A-GFP plasmid at C/P ratio of 8.

Fig. 7. (A and B) Cytotoxicity of PEI and its tetrac conjugates complexed with pUMVC3-hIL12 plasmid at C/P ratios of 0.25 (black bars), 4 (gray bars), and 8 (white bars) assayed by MTT determined in triplicate in HepG2 (A) and A375 (B) cell lines. * $p < 0.05$, conjugated PEI derivative compared to unmodified parent polymer at the same C/P ratio. (N = 3; error bars represent $\pm$ standard deviation). (C) FITC-Annexin V/7-AAD flow cytometry demonstrated apoptosis induction 48 h post-transfection by untreated cells, unmodified PEI, PEI-Tet 1% and PEI-Tet 1% pre-treated with the free targeting ligand. Shown are the mean values of three independent experiments and their standard deviations.

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