RESEARCH PAPER

Synthesis of L-DOPA conjugated doxorubicin-polyethylenimine nanocarrier and evaluation of its cytotoxicity on A375 and HepG2 cell lines

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ABSTRACT

Objective(s): Polyethylenimine (PEI) is one of the most-extensively investigated cationic polymers for gene and drug delivery. Recently, great attention has been directed to design of carriers for co-delivery of nucleic acids and small molecules. These delivery systems are able to overcome the limitations of gene or drug delivery alone. The aim of this study is to prepare a targeted nano-carrier for co-delivery of doxorubicin (Dox) and gene using polyethylenimine.

Materials and Methods: In order to prepare the ligand-containing polymer conjugates, succinic anhydride was conjugated onto the hydroxyl group of Dox through an ester bond following the protection of Dox amines by Fmoc. Drug-polymer conjugates were then coupled with L-DOPA in order to prepare the targeted nanocarriers to the cells through Large Amino Acid Transporter-1 (LAT-1). The PEI derivatives were characterized using ^{1}H-NMR. The toxicity of conjugated polymer, Dox and PEI was assessed on HepG2 and A375 cell lines with different expression level of LAT-1 transporters using MTT assay.

Results: The chemical structure of PEI conjugate was confirmed by ^{1}H-NMR. The cytotoxicity measurement demonstrated a cell line-dependent toxicity profile at the concentrations tested in this study. It was shown that there was no significant difference in cell-induced toxicity between conjugated polymer and its parent form in A375 cell line while the cytotoxicity of conjugated polymer was significantly lower than the parent PEI in HepG2 cells.

Conclusion: These results provide promising evidence for further evaluation of PEI conjugate for co-delivery of drug and gene via LAT-1 transporters.

Keywords: *Cytotoxicity, Doxorubicin, L-DOPA, Polyethylenimine*

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INTRODUCTION

Doxorubicin as is one of the key chemotherapeutic agents in the most chemotherapy regimens [1]. Despite its wide application for chemotherapy, there are some adverse reactions hampering its applications such as dose-dependent cardiotoxicity and multi drug-resistance following repeated doses [2].

Recently, co-delivery systems utilizing oligonucleotides with an anticancer drug has been considered a novel platform to overcome the problems associated with the administration of chemotherapeutic agents alone. These platforms provide drug and gene protection, targeted cell internalization and controlled release of the therapeutic agents as well as reversion of multi-drug resistance [3].

A major obstacle hampering the clinical application of co-delivery is the lack of efficient and safe carriers to transfer both drug and genetic materials. Due to the poor stability and solubility of nucleic acid materials and small molecule drugs, suitable carriers are needed for efficient delivery of such materials into the site of action in the human body [4]. Currently viral and non-viral vectors are

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being used for gene delivery. There are several advantages for non-viral gene carriers including simple preparation methods and scalability. These delivery systems can be modified by the addition of targeting moieties facilitating their targeted delivery into the precise site of action [5]. Polyethylenimine (PEI) is the most extensively studied cationic polymer for gene delivery due to its high gene transfection efficiency. It has been demonstrated that the high efficiency of PEI is related to its capacity to buffer the endosomes [6]. PEI shows toxicity in certain concentrations due to high density of positive charge on the surface. Therefore chemical modifications are needed to modulate the positive charges and its cytotoxicity. This positive charge also leads to non-specific interactions between polymer and plasma proteins resulting in the aggregation of complexes in biological media [7]. The addition of targeting moieties on the carriers not only direct the complexes to the specific cells but also make the polymer less toxic [8].

Large neutral amino acid transporter (LAT) is a major route to transport essential amino acids to different organs such as the brain. There are several studies showing that LAT-1 is the most commonly expressed transporter of LAT family in a variety of cancer cells [9]. Levodopa (L-DOPA; L-3,4-dihydroxyphenylalanine) is an amino acid that can easily cross blood brain barrier through LAT-1 transporter. This amino acid can be used as a ligand in targeted drug delivery systems [10].

In the current study, Dox conjugated PEI polymer was prepared and coupled with L-DOPA. Following the chemical characterization of these conjugates, the cytotoxicity of the PEI derivatives was evaluated. This conjugate may provide a suitable nanocarriers system for co-delivery of Dox and desired nucleic acid materials.

MATERIALS AND METHODS

Materials

Doxorubicin hydrochloride was purchased from ACTOVER Company (Iran). Branched polyethylenimine with an average molecular weight of approximately 25000 Da (25kDa PEI), dimethyl formamide, N-(9-Fluorenylmethoxycarbonyloxy) succinimide (Fmoc-Osu), N-Hydroxysuccinimide (NHS), N,N-dicyclohexylcarbodiimide (DCC),3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) and L-3,4-dihydroxyphenylalanine (L-DOPA) were obtained from Sigma-Aldrich (Germany). N,N-diisopropylethylamine (DIPEA), succinic anhydride and trifluoroacetic acid (TFA) were obtained from Merck (Germany). Fetal bovine serum and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Biowest (France). All solvents were purchased from Merck (Germany). Dialyses were carried out using Spectra/Por dialysis membranes (Spectrum Laboratories, USA).

Preparation of Dox-Suc-PEI-L-DOPA conjugate

The synthesis of Dox-Suc-PEI-L-DOPA has been shown in Scehme1. In order to prepared Fmoc-Dox, Dox hydrochloride (20 mg, 36 µmol) was dissolved in 1 ml of dimethyl formamide (DMF), then Fmoc-Osu (12 mg, 35 µmol) and 11 µl of N,N-diisopropylethylamine (DIPEA) were added. The reaction was stirred at room temperature in the dark for 24 hr. Then, the solvent was evaporated and the residue was crystallized by aqueous trifluoroacetic acid (TFA). The obtained red solid was washed once with cold diethyl ether to remove traces of excess Fmoc-Osu and then separated from impurities by high speed centrifugation.

In order to conjugate succinic acid linker to Dox, the intermediate material was then reacted with succinic anhydride (45 mg, 449 µmol) in 1 ml of DMF in presence of DIPEA (26.1 µl) overnight. The solvent was evaporated and the residual oil was again solidified by aqueous TFA [11]. The reaction was monitored using thin layer chromatography (chloroform-methanol 70:30). This crude product was purified by column chromatography.

In order to prepare Fmoc-Dox-Suc-PEI, esterified Dox was dissolved in 1 ml DMF. NHS (7.9 mg, 68 µmol) and DCC (14 mg, 67 µmol) were added and stirred 24 hr to form the active ester of Dox (Dox-NHS). Then, PEI (48 mg, 2 µmol) was added to mixture and allowed to react for 12 hr while being protected from light. The reaction efficiency was monitored as mentioned above.

The final step was the preparation of L-DOPA conjugated PEI derivative. L-DOPA was dissolved in DMF. NHS (1.9 mg, 16 µmol) and DCC (3.4 mg, 16 µmol) were added to L-DOPA to activate its carboxylic acid group. After stirring, Fmoc-Dox-Suc-PEI was added to L-DOPA and stirred for additional 24 hr. At the final step, deprotection of fmoc-protected amine carried out. The red solid was dissolved in 3 ml of DMF and 300 µl of piperidine. After 5 min, the reaction mixture was placed in an ice bath and acidified by the addition of a mixture containing 300 µl of TFA and 700 µl of

pyridine. The resulting solution was purified using a dialysis membrane (1000 Da cut-off spectra/por membrane) and dialyzed against distilled water for 3 consecutive days to remove impurities. After the dialysis, the aqueous solution was lyophilized to yield fluffy powder.

Characterization of L-DOPA conjugated doxorubicin-polyethylenimine

^{1}H-NMR was used to characterize the final product. ^{1}H-NMR spectra of products obtained in each step were recorded in D_2O and DMSO at room temperature. (BRUKER, AVANCE III-400, GERMANY).

The Dox content in the conjugated PEI derivative was determined by measuring the absorbance at 488 nm using UV spectrophotometry [12]. Calibration curve was prepared for the Dox concentrations ranging from 0.5 to 25 µg/ml. Dox calibration curve was validated at the concentrations of 0.25, 1.25, 2.5, 5 and 10 µg/ml. A serial dilution method was used to prepare Dox concentrations in phosphate buffer solution, pH 7.4.

Cell lines and cell culture

All cells were incubated at 37˚C in a humidified by 5% CO_2 atmosphere. HepG2 and A375 cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, streptomycin at 100 µg/ml and penicillin at 100 U/ml. All cells were seeded 24 hr prior to treatment in 96-well plates. These two cell lines were chosen to compare the cytotoxicity in cells expressing LAT-1 and cells lack this transporter.

Cytotoxicity of conjugated polymer

Cytotoxicity of conjugated polymer, Dox and PEI was assessed in HepG2 and A375 cell lines. In all experiments, treatment was performed in 96-well plates with 1×10^4 cells per well in triplicate. Cells were treated with 10 µl of conjugate at various concentrations ranging from 0.1 to 200 nmol/ml based on PEI concentration. After incubation for 4 hr, the medium was replaced with fresh one. Cell survival in each well was measured after 48 hr using MTT assay. Ten microliter of MTT reagent (0.5 mg/ml) was added to each well, the plates were incubated for 2 hr and the absorbance measured at 590 nm.

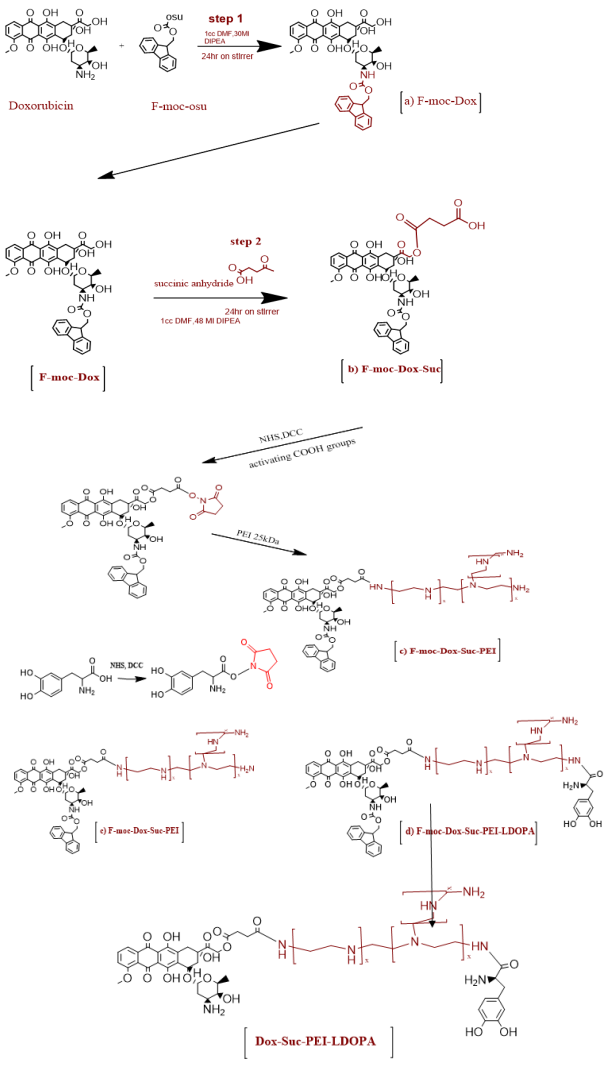
Statistical analysis

Cell-toxicity profile study of conjugated polymer was assessed using GraphPad prism. *P*-value<0.05 was considered as statistically significant.

RESULTS

Synthesis and characterization of conjugated polymer

Scheme 1 shows the strategy used for the preparation of Dox-Suc-PEI-L-DOPA conjugate. The peaks of Dox were found at 1-1.6 and 7-8.5 ppm, and the peaks of PEI were observed in 2.7-3.2 ppm. In addition, the peak of succinic acid was appeared at 2.5-3.6 ppm. In detail, the peaks of aromatic rings of Dox were found at 7-8.5 ppm, while the peaks of methyl and methylene of Dox were found at 1 and 1.1-1.6 ppm; respectively. [12]. The ^{1}H-NMR confirmed that Dox was successfully



Scheme 1. Synthesis of Dox-Suc-PEI-L-DOPA

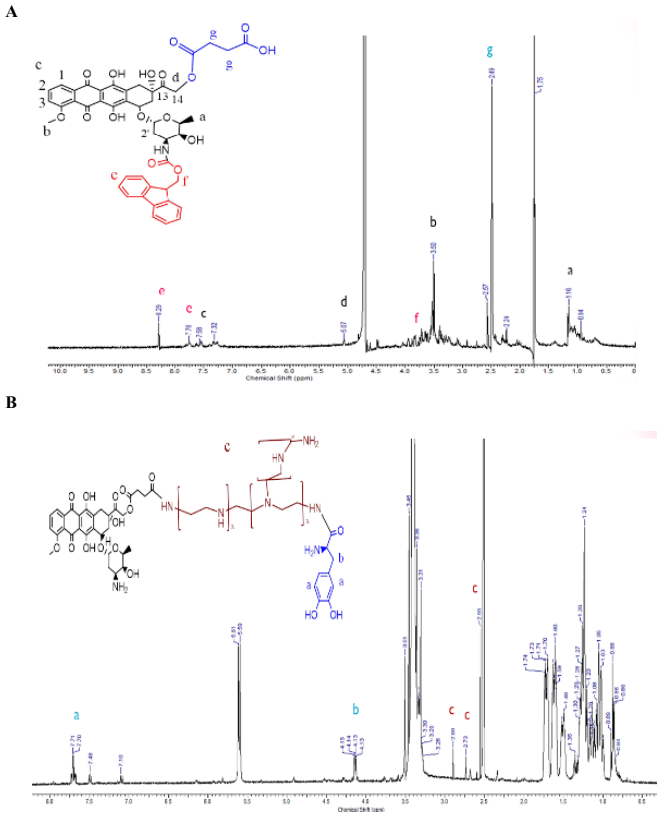


Fig 1. ^{1}H-NMR of Fmoc-Dox-Suc (A) and Dox-Suc-PEI-L-DOPA (B)

conjugated to PEI with succinate linker. The signal for aromatic ring of L-DOPA was appeared at 4.13-4.15 ppm [13]. The ^{1}H-NMR spectra of Fmoc-Dox-Suc and Dox-Suc-PEI-LDOPA are presented in Fig 1.

In order to determine Dox content in the final conjugate, the absorbance of the conjugate was measured at 488 nm using the validated analysis method. According to the results, Dox content in the conjugated polymer was calculated as 3.3 ±0.2%.

Cytotoxicity assay

HepG2 and A375 tumor cell lines were used for cytotoxicity assessment of the conjugated polymer. As it is demonstrated in Fig 2, the cytotoxicity of synthesized conjugated polymer was concentration dependent. According to these results, the cytotoxicity of conjugated polymer was lower than the parent unmodified polymer. By comparing the toxicity patterns in two cell lines, it seems that the cytotoxicity profile at these concentrations is cell line-dependent. As demonstrated in Fig 2 and 3, there is no significant difference in cell-induced toxicity between conjugated polymer and its parent form in A375

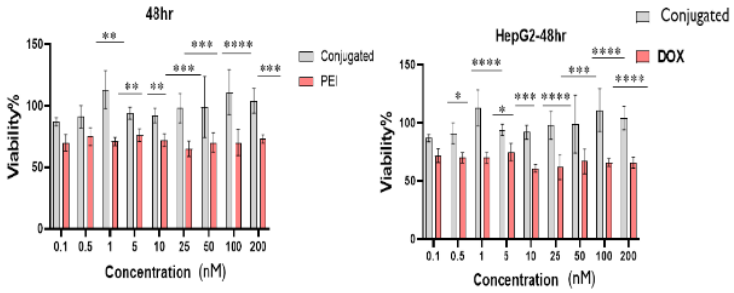


Fig 2. Cytotoxicity of conjugated polymer compared to free polymer and free drug in HepG2 cell line. Cell survival was assayed by the MTT method, and expressed as the percentages of cell viability. *P<0.05, PEI derivative compared to unmodified parent polymer or free Dox (n=3; error bars represent mean ± standard deviation)

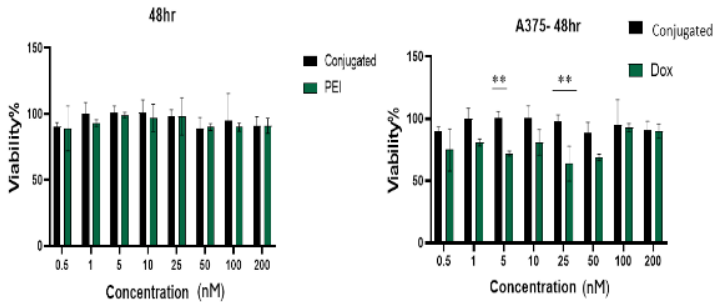


Fig 3. Cytotoxicity of conjugated polymer in A375 cell line compare to free polymer and free drug separately; viability bar-charts Cytotoxicity of conjugated polymer compared to free polymer and free drug in A375 cell line. Cell survival was assayed by the MTT method, and expressed as the percentages of cell viability. *P<0.05, PEI derivative compared to unmodified parent polymer or free Dox (n=3; error bars represent mean ± standard deviation)

cell line while the cytotoxicity of conjugated polymer is significantly lower than the parent PEI in HepG2 cells.

DISCUSSION

In this study we developed a targeted polymeric conjugate of PEI and evaluated its toxicity as a platform for potential application as a co-delivery system. The conjugation of polymer and doxorubicin carried out via an ester linkage. This ester bond prepared at C-14 hydroxyl group of doxorubicin. According to structure-activity relationship investigations, amino group at C-3` is a key functional group for intercalation into DNA and modifications at C-13 and C-14 of doxorubicin are associated with increased selectivity which results in greater efficacy at lower doses [14]. PEI is a polycationic polymer which has widely been studied as a carrier in drug and gene delivery. This polymer shows dose-dependent cytotoxicity due to high density of cationic charges resulting in non-specific interactions with proteoglycans on cell surfaces as well as blood components.

The MTT assay results showed a decreased cytotoxicity level in polymer conjugate compared with parent unmodified polymer. Conjugation of primary amines leads to modulation of cationic charges on the polymer surface and subsequently decreases the cytotoxicity [15]. Besides, the conjugation of targeting moieties not only modulates the cationic charge on the polymer surface, but also directs the polymeric vehicle to the precise site of action which in turn reduces non-specific interactions with undesired components in the blood circulation or extra-cellular matrix. Also, it has shown that targeted polymers enter the cells through receptor-mediated endocytosis increasing the speed of cell uptake. In the present study there is no significant difference between cell-toxicity of targeted conjugated polymer and unmodified one in HepG2 cell line, while the toxicity of conjugated polymer is lower than the unmodified PEI in A375 cells over-expressing LAT-1 transporter. There are several similar results demonstrating the lower toxicity of targeted polymers in the cells expressing specific receptors. It seems that the conjugation of targeting moieties alters the mechanism of cell entry. The non-targeted polymers enter the cells via adsorptive endocytosis. This process is based on the interaction between the positively-charged polyplexes and the negatively-charged components of cell membrane. On the other hand, the conjugation of targeting ligands leads to the cell entry through receptor-mediated endocytosis. This mechanism is based on the interaction between ligand and its receptor and occurs in a precise manner with higher speed rather than adsorptive endocytosis. Therefore, the remaining time for polyplexes in outer membrane media reduces and the positively-charged polyplexes have not extra time to induce damage on the cell membrane through non-specific interactions. Therefore, the conjugation of targeting moieties not only reduces the positive charge of the polymers but also reduces the toxicity by alteration in the cell entry mechanism [16-19].

CONCLUSION

In the present investigation, Dox was conjugated to PEI through a succinic acid linker followed by the addition of L-DOPA as targeting moiety. The result of toxicity studies demonstrated that the target delivery system induced lower toxic effects in the cells over-expressing LAT-1 transporter. This investigation showed a promising strategy to modify PEI to generate a vehicle in co-

delivery of doxorubicin and nucleic acid materials together.

ACKNOLEDGMENTS

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CONFLICT OF INTEREST

There is no conflict of interest in this study.

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