Original Article

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Toxicity of Hexyl Acrylate Modified PAMAM Dendrimer

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Abstract

The aim of this study was to evaluate the effect of the hexyl acrylate conjugated polyamidoamine (PAMSM) dendrimer on different cell lines in the presence and absence of serum. Although the positively charged dendrimers including PAMAM has been considered as efficient gene carriers, their cytotoxicity is a major barrier for their clinical applications. The conjugation of hexyl acrylate on PAMAM dendrimer was carried out to improve the hydrophilic-hydrophiobic balance of the dendrimer as well as the modulation of the positive charge on the dendrimer structure. The cytotoxicity of PAMAM derivatives was evaluated using MTT test on HepG2, MCF-7 and A549 cell lines in the presence and absence of serum. The results revealed that the modified PAMAM dendrimers induced less toxic effects on the cell lines in the presence of serum. Therefore, conjugation of acrylate on the PAMAM structure could be an efficient way to reduce the dendrimer toxicity allowing the administration of more dendrimer for gene delivery.

Keywords: Polyamideoamine (PAMAM), Cytotoxicity, Hexyl acrylate, Dendrimers, Gene delivery.

1. Introduction

Gene delivery using polymeric nanoparticles have attracted great attention in recent years (1-3). Although viral gene carriers have been widely used for transferring genetic materials into the cells, the disadvantages including insertional mutagenesis, immunogenicity, limited gene capacity as well as expensive industrial production procedures have limited their wide clinical applications (4). Therefore, alternative carriers such as liposomes and polycationic polymers have been considered to transfer various oligonucleotide therapeutics into the cells (5-9). Polycationic polymers or dendrimers interact electrostatically with the negatively charged backbone of the nucleic acid materials and condense them into nanoparticles (10-12). The nucleic acid condensation is a crucial step in gene delivery protecting genetic materials from degradation by different enzymes. Also, the formation of nanoparticles leads to the efficient uptake of the carriers into the cells through adsorptive endocytosis (13, 14). The positively charged nanoparticles interact with the negatively charged components on the cell membrane and enter the cells. Therefore, the presence of positive charge on the surface of carriers not only leads to the formation of nanoparticles but also facilitate their uptake (15). The positive charge of the polycationic polymers comes from the presence of various amine groups on the surface of polymers or dendrimers. The high amine content of the polycationic compounds play a crucial role in the induction of early escape from endosomes through

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a mechanism called proton sponge effect (15-17). High buffering capacity of these compounds results in the adsorption of the protons inside the endosomal compartment followed by the influx of chloride ion and water which finally leads to osmotic burst and rupture of the endo/lysosomal vesicles before starting the enzymatic degradation (18). Hence, the amine content of the polycations has shown significant impact on its gene transfer efficiency. On the other hand, the cytotoxicity of these compounds is one of the major concerns limiting their clinical applications in human gene therapy (13, 15, 18, 19). The major reason for their high toxicity is the high density of positive charge results from the high amine content of the materials. Several various strategies have been applied to modulate the surface positive charge of polycations in order to reduce their toxicity including the conjugation of targeting ligands, amino acids, peptides, carbohydrates and hydrophobic moieties via the surface amines on the polycation structure (20-26). In our previous investigation (16), various acrylates were conjugated on PAMAM structure at different substitution degrees. The transfection efficiency experiments revealed that the modified PAMAM dendrimer was able to transfer plasmid DNA with high efficiency to the cells. In order to evaluate the toxicity of the PAMAM derivatives, the most efficient conjugate (i.e.; hexyl acrylate derivative of PAMAM at the conjugation degree of 30%; HA-PAMAM30%) was selected and its cytotoxicity was tested on three cell lines in the presence and absence of serum by MTT test.

2. Material and methods

2.1. Materials

Polyamidoamine dendrimer (1,6-diaminohexane core, PAMAM G4) was purchased as a methanolic solution from Sigma-Aldrich (Munich, Germany). N-[2-hydroxythyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (Munich,Germany). EndoFree Plasmid Mega Kit was purchased from Qiagen (Valencia, CA, USA). Plasmid pUMVC3-hIL12 (human interleukin-12 under control of the cytomegalovirus enhancer/promoter) was purchased from Aldevron (Madison, WI, USA). Fetal bovine serum (FBS), cell culture media, and antibiotics were purchased from Gibco (Gaithersburg, MD, USA). All solvents were obtained from Sigma-Aldrich (Munich, Germany) and were of the highest purity available.

2.2. Plasmid preparation and nanoparticle formation

Plasmids pUMVC3-hIL12 were transformed into Escherichia coli bacterial strain DH5α and amplified in selective Luria-Bertani (LB) media at 37 ºC. The propagated plasmids were extracted and purified using the Qiagen Endo-Free Plasmid Mega Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. The concentration and purity of the plasmid preparations were determined by UV spectrophotometer at 260 and 280 nm. The purification procedure resulted in the preparation of 3 mg of pDNA. The purity of the plasmid DNA was measured using the following formulae: Purity=A260/A280. pDNA preparations with purities higher than 1.8 were used in this study. Also, agarose gel electrophoresis was used to confirm the purity and size of the plasmid.

Different concentrations of PAMAM and its modified derivative were prepared in HBG solution (HEPES buffered glucose; 20 mM HEPES, 5% glucose pH=7.2). In order to prepare the dendrimer/plasmid complex (dendriplex), 50 µl of dendrimer solution were added to 50 µl of plasmid solution in the same medium. Then, the mixture was incubated for 30 min to form stable complexes. The composition of nanoparticles was characterized by weight/weight ratio of PAMAM and its derivatives (C) to the plasmid (P) in the final formulation.

2.3. Cell culture and cytotoxicity assay

Human HepG2 hepatocellular carcinoma cells (NCBI C158, Tehran, Iran), human MCF-7 breast cancer cells (NCBI C135) and human A549 lung cancer cells (NCBI C197, Tehran, Iran, Tehran) were maintained at 37 ºC, 5% CO_2 and 100% humidity in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), streptomycin at 100 µg/ml and penicillin at 100 U/ml. Cells were seeded at a density of 1×10^4 cells/well in 96-well

plates 1 day prior to cytotox¬icity tests, and grown in the appropriate medium with 10% FBS. Different dendrimer/plasmid DNA ratios (C/P) were used to prepare the dendrimer/plasmid complexes. nanoparticles were prepared by adding 50 µl of a solution of dendrimer at varying concentrations in HBG to 50 µl of a solution of plasmid DNA (40 µg/ml) in HBG. Cytotoxicity assay was performed by adding 10 µl (equivalent of 200 ng pDNA) of dendriplex solution to the wells of 96-well plates containing 60%-90% confluent cultures of cells in complete medium containing 10% FBS. The same procedure was performed without serum in order to assess its effect of toxicity. The cytotoxicity of the dendrimer itself was also evaluated by adding diggerent concentration of PAMAM and its derivative ranging from 100-1000 µg/ml to the cells at the same condition. In order to assess the cytotoxic effects of the PAMAM and its derivatives as well as the nanoparticles formed by these compounds, MTT assay was performed as described elsewhere. Briefly, the nanoparticles or dendrimer itself was added to the medium as described above and after 4 h the medium was replaced by a fresh one and the plates were incubated in 37 ºC for 48h. Subsequently, 10µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added to each well and incubated for another 2 h. Then, the supernatant of all wells were aspirated and 100 µl DMSO was added and allowed to dissolve the formazan crystals. The absorbance was measured using a microplate reader at 590 nm with background correction at 630 nm. Data are presented as mean±SD, n=3.

2.4. Statistical analysis

Data are presented as the mean±SD. The statistical significance was determined using Student's t-test and *P* values <0.05 were considered as significant.

3. Results and discussion

3.1. Cell culture and cytotoxicity assay

3.1.1- Cytotoxicity of dendrimer and its derivative

The major problem hampering wide clinical application of polycationic compounds including dendrimers is substantial toxicity (6, 15, 18, 20). The toxic effects of such materials results from the high amine content of dendrimer which leads to the accumulation of high positive charge on the surface of the dendrimers (27). The high charge density is essential for electrostatic interaction with negatively charged materials such as nucleic acids and consequently facilitates the interaction of the carrier/nucleic acid complexes with the negatively charged components on the cell membrane. On the other hand, high positive charge density on the dendrimer or the dendriplexes disrupts the cell membrane and disturbs the plasma cell integrity (28). Therefore, the modulation of charge density on the dendrimer structure has been considered as an effective strategy to reduce its cytotoxicity (11, 13, 20). In this investigation, hexyl acrylate conjugated PAMAM dendrimer at the substitution degree of 30% (HA-PAMAM 30%) was selected based on our previous study (16). The conjugate showed significant buffering capacity and plasmid condensation ability and formed nanoparticles with the size of around 100 nm and zeta potential of 14 mV. The HA-PAMAM30% demonstrated significant protection effect on preventing the plasmid to be digested by nuclease I enzyme. The modified PAMAM dendrimer was able to condense plasmid DNA at carrier to plasmid ratios higher than 4 which is comparable with unmodified dendrimer or the golden standard of unmodified branched polyethylenimine (PEI) (16). However, the ability of PAMAM derivative to transfer the plasmid encoding interleukin-12 (IL-12) into the cells was lower than the unmodified parent dendrimer (16). Considering the impact of cytotoxicity on low transfection efficiency of various polycationic compounds, the cytotoxic effects of the PAMAM derivative was investigated in different cell lines in order to determine whether the low transfection was the result of toxicity or not. The results of toxicity assessment on A549, HepG2 and MCF-7 cells (Figure 1A, B and C) revealed that the cell viability following the treatment of the cells with unmodified PAMAM dendrimer decreased to around 70% whereas the viability of the cells did not change after treatment with modified PAMAM derivative in the serum free medium. In other words, the HA-PAMAM30% was almost non toxic even at the highest concentration of 1000 µg/ml. However, in the absence of serum

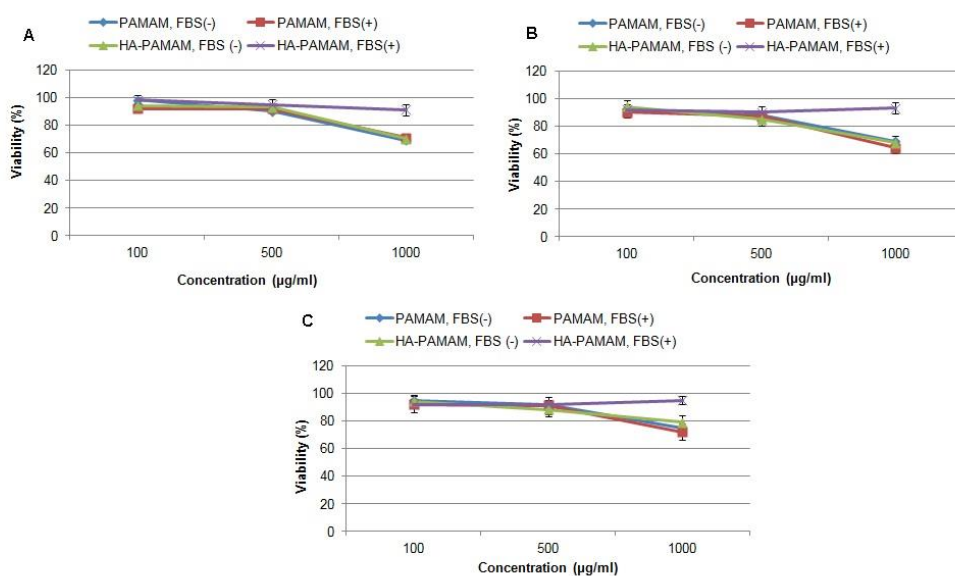

Figure 1. Viability of PAMAM and its conjugate on A549 (A), HepG2 (B) and MCF-7 (C) cells at three concentration measured by MTT determined in triplicate. **P*<0.05, PAMAM at the concentration of 1000 µg/ml compared to same dendrimer at the concentration of 100 µg/ml at the same condition (n=3; error bars represent ± standard deviation).

in the medium, the viability decreased to around 70%. Also, a dose-dependent toxicity for unmodified PAMAM was observed in which the viability was around 100% at the concentration of 100 µg/ml while the viability decreased to 70% at the concentration of 1000 µg/ml (*P*<0.05). The results also revealed that the toxic effect of the dendrimer and its derivative was not cell line dependent at the concentration tested in this study (Figure1). Serum is a rich medium in anionic compounds such as proteins with the ability to electrostatic interaction with the positive dendrimers or dendriplexes (29). This interaction may reduce the charge of the dendrimer or dendriplex which consequently leads to the lower ability to interact with the negatively charged components on the cell membrane. Therefore, these interactions could result in the lower adsorptive endocytosis which leads to decreased transfection efficiency (29).

3.2. Cytotoxicity of nanoparticles

The cytotoxicity of the dendrimer/plasmid

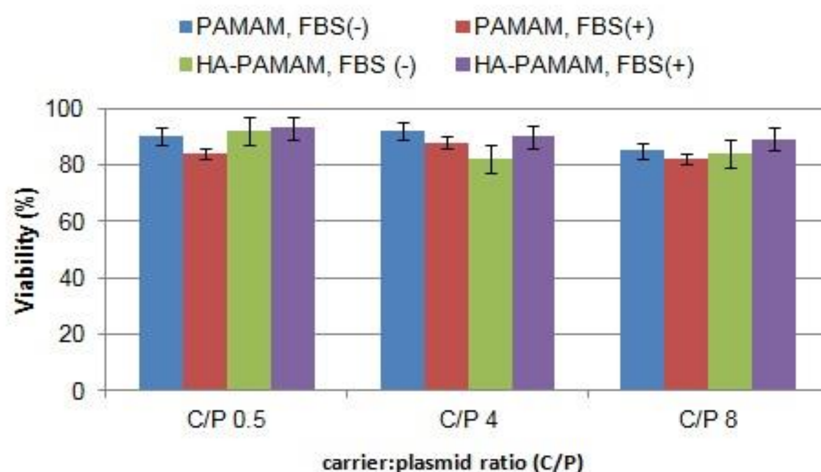

Figure 2. Viability of PAMAM and its conjugate complexed with pUMVC3-hIL12 plasmid at C/P ratios of 0.5, 4 and 8 assayed by MTT (n = 3; error bars represent ± standard deviation).

DNA complexes was also evaluated by MTT assay (Figure 2). The complexes were prepared at the same carrier:plasmid ratio used for transfection assessment in the previous study. The complexes were prepared at C/P ratios of 0.5, 4 and 8 and the cell viability was measured 24 h after treatment in the presence and absence of serum. Since the results of the toxicity assessment on different cell lines was similar and no cell line dependency was observed, the cytotoxicity of the dendriplexes was evaluated on A549 alone. The results revealed that the viability of the cell lines did not change even at the highest C/P ratio of 8. Also, the serum free medium could not change the toxicity of the dendriplexes. This observation might be the effect of the dendrimer concentration used for complex preparation. In other words, the concentration of dendrimer used for dendriplex preparation was in the range of non-toxic. Therefore, the viability of around 90% was observed even at the C/P ratio of 8. The serum free medium could not alter the cytotoxicity due to the low concentration of the dendrimer used for polyplex preparation. Since serum is rich of negatively charged material, the probability of the interaction of serum and polycations is higher when the high positive charged materials exist at higher concentrations. By decreasing the share of polycations in the preparation of nanoparticles the possibility of these interactions decreases. Therefore, the effect of serum is less important on toxicity of nanoparticles in comparison with dendrimer itself (29).

4. Conclusion

Conjugation of hexyl acrylate on PAMAM dendrimer could be considered as an efficient approach to decrease the dendrimer toxicity in presence of serum. Therefore, this conjugation strategy provides the opportunity to apply more dendrimer with less toxic effects for gene delivery.

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Conflict of Interest

None declared.

5. References

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