Estimating the Two Graph Dextran-Stearic Acid-Spermine Polymers Based on Iron Oxide Nanoparticles as Carrier for Gene Delivery

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Research article

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Abstract

Background

Non-viral gene carriers because of their limited side effects, biocompatibility, simplicity and taking the advantages of electrostatic interactions have shown noticeable potential in gene delivery. The low transfection rate of non-viral vectors under physiological conditions is a significant issue.

Methods

We investigated the efficacy of hydrophilic and hydrophobic groups on gene carriers such as two synthesized amphiphilic polymer of dextran-stearic acid-spermine (DSASP) with verified lipid and amine conjugations that associated with Fe$_3$O$_4$ superparamagnetic nanoparticles to promote the target delivery and decrease the transfection time using static magnetic field.

Results

Our findings illustrate that magnetic nanoparticles are spherical with positive surface charges and superparamagnetic behaviors. The DSASP–pDNA/MNPs offered a strong pDNA condensation, protection against DNase degradation, significant cell viability in HEK 293T cells. Although conjugations of spermine play a critical role in transfection efficiency, amphiphilic polymer with more derivatives of stearic acid showed better transfection yields.

Conclusion

DSASP amphiphilic magnetic carriers offer new insights for gene delivery due to the amine contents and ameliorate the uptake of complexes via cell membrane based on its hydrophilic surface.

Background

The undeniable side effects of traditional approaches such as surgery, chemotherapy and radiotherapy in treatment of cancers have led to valuable research in the field of gene therapy to avoid the killing of some normal cells, DNA damaging, emergence of secondary cancer, etc. Small particles are sent to tumor cells based on selective treatment to repair and modify missing cellular functions and genes, which prevent the expression of unwanted genes production before translation and regulate proteins [1–3]. The ideal gene delivery system must comprise fundamental criteria. First, attending to the importance of the cellular uptake of nanocarriers and prevention of charge repulsion in the delivery process, polycations should be able to neutralize negative charge of DNA due to negative charges of plasma membrane [4, 5]. The relationship of cellular clearance and size of nanoparticles could be assumed as a second parameter. Additionally, biocompatibility and immunogenicity are other substantial parameters. Therefore, nanoparticles should be stabled enough in the biological conditions not only before reaching the targeted-tumor cells, but also in the process of expression in the nucleus [1, 6]. Moreover, opsonization
based on the surface characteristics of nanoparticles, is deemed as a crucial factor in their stabilization [7].

The carriers are classified into two major groups of viral and non-viral vectors, which each represent the gene delivery process as transduction and transfection, respectively [8]. Although the efficiency of viral vectors have been shown in clinical research, it is hard to elicit an effective therapeutic response due to their inherited substantial disadvantages like difficult production process, risk of mutagenesis and limited cargo capacity [9]. Furthermore, immunogenic responses to viruses will intensively suppress the effect of viral vectors and subsequently restrict the rate of transduced vectors to targeted cells [10]. Non-viral vectors are able to circumvent some of delivery-associated limitations of viral vectors with some constructive biological advantages such as low immune responses, affordable cost of production in large scale, safety or non-cytotoxicity, and more targeted transfection through designed-surface ligands. However, in vivo transfection efficiency of non-viral vectors is lower than viral vectors [3, 11].

Polymeric and cationic lipids as non-viral carriers condense the string-like DNA into nanoscale dimension to become appropriate for cellular uptake [12]. Polycations like polysaccharides have potential opportunities to change their physicochemical attributes in order to improving power of binding, reaching the least toxicity and increasing cellular release. [1, 13]. The polycations are all polyamines that contain the first, second, third, or fourth amine groups [1]. Polymeric macromolecules with high cationic charges can operate as an endosomal buffering system in gene delivery which suppresses the endosomal enzymes activity and prevents degradation and accumulation of trapped-DNA molecules in cytosol [1, 14].

Dextran is a linear and non-polyelectrolytes polysaccharide, consists of β-1,6 glycosidic linkages and also few branch of β-1, 3 glycosidic linkages [15–17]. Additionally, the presence of this neutral surface alongside other properties like low immunogenicity, water-solubility, lower rate of plasma protein absorption and more specific cellular uptake lead to dextran widespread use in the different gene transfection of tumor targeted-therapy [15, 18]. Spermine is known as a natural tetra-amine, which involves in two primary and two secondary amino groups [19]. Azzam et al., shown that only polycations of dextran grafted with spermine has high efficiency in the in vitro cells transfection between 300 cationic polysaccharides in different molecular weights [20].

The serum medium has high inhibitory effects on the transfection rate of water-soluble dextran-spermine–DNA polymer up to 95% based on high hydrophilic property. Moreover, covalent attachment of hydrophobic residues to some dextran–spermine NH2 moieties could ameliorate the transfection efficiency in the serum containing media [3, 21]. In addition, based on the resemblance of structure of hydrophobic molecules with the plasma membrane, amphiphilic modifications of polycations tend to enhance or even ease carriers’ cellular uptake via endocytosis [21]. Stearic acid is a saturated-fatty acid, with appropriate biocompatibility and lower cytotoxicity has been utilized a lot in delivery systems. An amphiphilic dextran-stearic acid-spermin (DSASP) preparation takes place through esterification between the carboxyl group of stearic acid and hydroxyl group of the glucose units in dextran [15, 22].
Magnetofection is known as an effective approach for transfection to overcome restrictions like inactivation of some carriers under cell culture conditions and acceleration of diffusion [8, 23]. Superparamagnetic iron oxide (Fe$_3$O$_4$) nanoparticles are cubic inverse spin structures with well discretion in original solvent. These nanoparticles are considered as substantial magnetic materials based on their widespread usage in various biomedical applications. Fe$_3$O$_4$ nanoparticles are often undergone surface changes or coated with polymers in order to reduce complex agglomerations and being more prolong in biological conditions [17, 24–26].

In this study, we have investigated the aspects of magnetic DSASP polymers based on magnetofection for gene delivery in the HEK 293T cells (Fig. 1). Fe$_3$O$_4$ nanoparticles are encapsulated inside two amphiphilic DSASP polymers with different grafted rates of stearic acid and spermine, which well condensed pDNA. Our findings show that interaction of DSASP@pDNA with Fe$_3$O$_4$ represents a novel biodegradable and biocompatible non-viral carrier which can be utilized for gene delivery.

Materials And Methods

Chemical reagents

Dulbecco's modified eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco. Penicillin-streptomycin and trypsin-EDTA were obtained from Bioidea. The 25 kDa branched polyethyleneimine (PEI) and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich. FeCl$_3$.6H$_2$O, FeSO$_4$.7H$_2$O and NaOH were purchased from Merck. Dimethyl sulfoxide (DMSO) was obtained from Daejung Chem., Korea. Two prepared polymers of dextran-stearic acid-spermine polymers (DSASP) were obtained from Dr. Ebrahim Vasheghani-Farahani lab.

Synthesis of magnetic nanoparticles

Chemical co-precipitation method is defined as one of the Fe$_3$O$_4$ nanoparticles synthesized protocol [27]. In brief, FeCl$_3$.6H$_2$O and FeSO$_4$.7H$_2$O with a molar ratio of 2:1 were mixed under continuous stirring and nitrogen gas bubbling to inhibit oxidation. Subsequently, to reach the pH up to 10, NaOH (2.0 M) was slowly added into the mixture under continuous stirring. Moreover, the black precipitate as a result of NaOH addition, indicates the formation of Fe$_3$O$_4$ nanoparticles. Then, the mixture was vigorously stirred for 15 min and incubated at 70°C for 30 min, after that it was cooled down at room temperature. Next, Fe$_3$O$_4$ nanoparticles were magnetically separated and frequently washed by deionized-water and ethanol solution to remove the impurities. Finally, MNPs were dried in an oven at 60°C and collected by magnetic separation and stored under N$_2$ gas condition [28].

Plasmid purification

Plasmid DNA (pDNA) used in this study was pDB2 plasmid encoding luciferase, was propagated and isolated from DH5α E. coli overnight. Qiagen Maxi kit-25 (Qiagen K.K., Tokyo, Japan) was applied for
extraction and purification of pDNA. The concentration and purification of pDNA was measured using Nanodrop (Thermo, Wilmington, DE, USA) at an absorbance ratio of 260/280 nm ranging from 1.9 to 2.2.

**Preparation of polymer-based magnetic complexes**

The pDNA (1 µg/µl) and DSASP polymer at different mass ratios were diluted in deionized-water and incubated for 30 min at room temperature to form DSASP–pDNA complexes. Then, 1 µg/µl Fe₃O₄ magnetic nanoparticles (MNPs) were added to the mixture of DSASP–pDNA complexes and reincubated for extra 30 min.

**Characterization of magnetic nanoparticles and complexes**

The size and charge of MNPs and complexes were performed by dynamic light scattering (DLS). The size distribution and zeta potential were evaluated with a Zetasizer Nano-ZS instrument, Model ZEN3600 (Malvern Instruments, UK). Briefly, the dispersions of Fe₃O₄ were sonicated for 30 s in deionized-water and then incubated with DSASP–pDNA complex as previously mentioned. The particle size was measured in the scattering angle 173° at 25°C. The zeta potential was measured using a universal zeta dip cell. DLS measurements were taken for DSASP–pDNA/Fe₃O₄ in the presence of increasing concentrations of DSASP. In addition, scanning electron microscopy (SEM) analysis was carried out to indicate the morphology and the size of both MNPs and complexes. Moreover, the magnetic properties of dried-SPIONs and DSASP–Fe₃O₄ were measured at a maximum applied field of 10 kOe using VSM 7300 vibrating sample magnetometer (VSM) (Lakeshore, USA). Furthermore, the chemical component and molecular vibrations of samples were analyzed by Fourier transform infrared spectroscopy (FTIR, Thermo Scientific Nicolet IR100, Madison, USA) using KBr pellet. Three scans per spectrum (400–4000 cm⁻¹) at the resolution of 4 cm⁻¹ were measured for dried samples of Fe₃O₄, DSASP and DSASP–Fe₃O₄ mixed with KBr and made into pellets. The 6 mg/ml DSASP–Fe₃O₄ was used in SEM, VSM and FTIR measurements.

**Gel retardation assay**

The gel retardation or electrophoretic mobility shift assay is utilized to evaluate the DNA interaction with complexes [29] and ensure about formation of ternary complexes of pDNA–DSASP–Fe₃O₄. DNA molecules migrate from the negative electrode towards the positive electrode. Moreover, addition of the small molecules such as DSASP to pDNA, the mobility will be restricted through the gel based on the emergence of electrostatic interaction between pDNA and polymer [30]. DNA condensation and mobility after binding with DSASP–Fe₃O₄ complex was identified by gel retardation assay. Different weight-mixing ratios of DSASP–pDNA/Fe₃O₄ (1, 2.5, 5, 10, 25, 50 and 100) were prepared while the content of DNA to Fe₃O₄ was kept at 1 µg/µl and incubated at 37°C for 15 min. After that, 8 µL of mixture suspension was analyzed by 1% agarose gel electrophoresis (90 V, 45 min). Naked–pDNA was utilized as a control group. The gel was stained in 0.5 mg/ml ethidium bromide for 30 min to visualize the localization of pDNA by UV illumination.

**Serum stability assay**
In the process of gene transfection, the usage of naked-DNA due to effects of nucleases and reticuloendothelial system cannot be possible. So, the necessity of appropriate vectors for protecting DNA play a crucial role against the enzymatic conditions. Moreover, to have an efficient gene delivery system especially in the in vitro, genetic materials' carrier should protect DNA in front of serum enzyme during gene delivery, and also minimize the time association of pDNA with blood serum, which was investigated by DNasel activity at weight ratio of 10 in the gel electrophoresis. Briefly, 6.5 µl of each complex was exposed to 0.75 µl of DNasel (Promega, USA) in the final volume of 15 µL for 15 min at 37°C. The reaction was inactivated followed by transferring complex into −20°C. After the addition of 10% sodium dodecyl sulfate (SDS) to the solution, pDNA was released from complex and subsequently 8 µl of each sample comprising 3 µg DNA that was analyzed by 1% agarose gel electrophoresis (90 V, 45 min). The integrity of released-pDNA from the complex was compared with the naked-pDNA as control.

**Cell Culture**

The human embryonic kidney 293T cell line (HEK 293T) was obtained from Pasteur Institute of Iran (IPI). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) in the neutral PH (7.2–7.4) supplemented with 10% (v/v) heat-inactivated (50°C, 30 min) fetal bovine serum (FBS) and 2 mM glutamine, 100 units/mL of penicillin and 100 mg/mL of streptomycin at 37°C and 5% CO₂ in a humidity incubator. The cells were then trypsinized (0.025% trypsin, 0.02% EDTA) after they were grown up to 70–80% confluent. Prior to treatments, cells were allowed to reattach overnight.

**Cell viability assay**

The viability of cells which are treated by nanoparticles were measured by MTT assay. This is a colorimetric assay based on ability of viable cells to reduce the water-soluble yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) by mitochondrial NAD(P)H-dependent oxidoreductase enzyme (succinate dehydrogenase) to purple insoluble formazan crystal. The HEK 293T cells were seeded into 96-well cell culture plates (SPL Life Sciences Co., Ltd. Korea) at the final concentration of $10^4$ cells/well and incubated in 100 µL of DMEM containing 10% FBS and allowed to attach overnight. Then, cells were treated in the fresh supplemented DMEM with 10% FBS by DSASP–pDNA/Fe₃O₄ complexes including 1 µg/µL concentration of Fe₃O₄ nanoparticles and pDNA, and different weight-ratio of DSASP (0.5, 1, 2.5, 5, 10, 20, 25, 50 and 100) in presence and absence of homogenous static magnetic field (SMF, 20 mT) for 30 min. After 24 and 48 h treatments, the media was removed and MTT solution (0.5 mg/ml in FBS-free DMEM) was added and incubated at 37°C for extra 4 h in the dark. After that, the media was removed and 100 µL DMSO was added to each well. Finally, the absorbance of formazan intensity was quantified by a microplate reader (uQuant MQX200, BioTek, USA) at 570 nm.

**Magnetofection**

In this study, the gene delivery process, the magnetic ternary complexes were transferred to cells. The complexes comprised the pDNA and MNPs in the similar mass ratio of 1 µg. The extracted pDNA encoding the luciferase reporter gene that handles based on kinase activity, which converts luciferin to oxyluciferin and light using ATP. The ATP is converted to AMP and release the energy of the phosphate
bonds in the form of light that was measured by luminometer [31]. The intensity of light indicates the activity of luciferase being equivalent to the amount of delivered nucleic acid to the cells. Briefly, $5 \times 10^4$ cells/well of HEK 293T cells were seeded in the 24 well cell culture plates and allowed to attach overnight. Then, 1 µg/µl pDNA was mixed with a DSASp solution at various mass ratios (0.5, 1, 2.5, 5, 10, 20, 25, 50 and 100) and incubated for 15 min. After that, the prepared-complexes gently were mixed with 1 µg/µl Fe$_3$O$_4$ nanoparticles and reincubated for extra 15 min. Each prepared complex’s DSASp–pDNA/Fe$_3$O$_4$ were diluted with FBS-free DMEM to a final volume of 300 µL and incubated at room temperature for extra 30 min. Next, polyplex solutions were added to cells in presence and absence of homogenous SMF (20 mT) at 37°C for 30 min. After that, the media was replaced with DMEM containing 10% FBS, and cells were incubated for 48 h. Moreover, the 4.8 µg PEI at N:P ratio of 12 was utilized as control. Then, the media was completely removed and cell lysates were analyzed for luciferase activity that is followed by injection of assay buffer to a tube containing cell lysate and luminescence was evaluated using a luminometer (Berthold detection systems, GmbH, Germany).

**Statistical Analysis**

GraphPad Prism v5.07 (GraphPad Software Inc., San Diego, CA) was used for statistical analysis and data graphing. We applied one-way followed by post-hoc analysis using Newman–Keuls multiple comparison test to compare between independent variables. The error bars represented the mean ± standard deviation (mean ± SD) of at least three independent experiments (n = 3). P-values including *p < 0.05; **p < 0.001; ***p < 0.0001 were considered as a statistical significance difference.

**Results**

Formation of MNPs and DSASP–pDNA/MNPs

The formation of ternary nanocomplexes is fundamental in transferring nucleic acids comprising magnetic cores with polymeric shells that are conjugated to pDNA. In the delivery systems, the size of nanocarriers have significant roles that were obtained from DLS about 62 nm for the synthesized Fe$_3$O$_4$ and 100–300 nm for the SPIONs formulated in DSASP–pDNA complex at weight ratios of 10–100 w/w (Fig. 2a,c). The zeta potential of the superparamagnetic iron oxide nanoparticles was −27 mv (Fig. 2b). The stronger negative surface charge distribution of nanoparticles can facilitate the electrostatic interaction with positive charge particles. Additionally, results demonstrated that increasing the positive charge and decreasing the particle size as a result of rise in the mass ratio of DSASP (0.5, 1, 2.5, 5, 10, 25, 50, 100) at the a concentration of Fe$_3$O$_4$/pDNA (1 mg/ml), can efficiently affect the electrostatic interactions with pDNA and lead to more condensed molecules (Fig. 2c,d). Successful synthesis of DSA$_6$SP–pDNA/Fe$_3$O$_4$ nanoparticles sustained due to the positive zeta potential of +35 mv value at weight rate of 50 w/w. In addition, DSASP 6 and 8 have a relative difference in amine contents as a result of divers' conjugation of stearic acid, in which the amount of 20% and 15% were evaluated for their hydrophobic part, respectively.
Boosting the graph rate of stearic acid can decrease the micelles particle size. There is a harmony with this phenomenon that the ratio of hydrophobic to hydrophilic chain length, remarkably impresses the particle size in linear amphiphilic polymers (Fig. 2c). So, smaller particle size with higher surface/volume ratios will have higher zeta potential, which could efficiently surround negative charge of nucleic acids (Fig. 2d). Negative surface charge of DSASP–pDNA/Fe₃O₄ complexes at w/w ratio below 10, were gradually transferred into positive potential as result of an increase in w/w rate of polymer. Furthermore, in this study the negative surface charge almost up to 5 w/w demonstrated insufficient formation of complexation between polymer and DNA though electrostatic repulsion which indicated complex inability to condense nucleic acids negatively charged phosphate backbone properly (Fig. 2d) [12, 32].

As shown in Fig. 3a, the SEM image of MNPs declares a homogeneously spherical shape for Fe₃O₄ particles with diameter around 12–18 nm, which has proper agreement with the result of DLS assay. Obtained SEM results also represent roughly spherical form for agglomeration of DSASP–Fe₃O₄ nanoparticles with desirable dispersibility and no significant size difference in comparison with Fe₂O₄ MNP (Fig. 3b). Therefore, due to optimum size of delivery vectors from 50 to 100 nm [12], the MNPs derived from DSASP–pDNA/Fe₃O₄ seems to be efficient in gene delivery. Size comparison between SEM imaging and zetasizer measurement is attributed to dried and hydrated state of samples, respectively. It means that the DLS information represents the hydrodynamic size of nanoparticles being naturally larger than their actual size [33].

Moreover, VSM was used to investigate the magnetic behavior of nanoparticles and the formation of DSASP–Fe₃O₄ complexes. The maximum size for superparamagnetic behavior is generally taken to be almost 30 nm [34]. The hysteresis loops of bare Fe₃O₄ and coated MNPs of DSASP–Fe₃O₄ was shown in Fig. 3c,d. Reduction in hysteresis magnetization from plateau level to zero was remarkably shown at room temperature (~ 298 K) for both curves through removal of MFs confirming their superparamagnetic property. In addition, the magnetic saturation of MNPs complexes is estimated to be 30.160 emµ·g⁻¹, being lower than bulk Fe₃O₄ at 65.3 emµ·g⁻¹. The lower rate of magnetic saturation of DSASP–Fe₃O₄ compared to Fe₃O₄ could be attributed to the attachment of DSASP to nanoparticles. Indeed, cationic surface spines of polymer as modification, affect the core spin of Fe₃O₄ from along the MFs that decrease the complex saturation magnetization values [35].

The IR spectrum being obtained from the FTIR spectrometer is located in the mid-IR region (666–4000 cm⁻¹). Considering the mid-IR region, many functional groups' transition energy due to their alternation in vibrational energy state could be estimated to determine whether specific functional groups exist in the structure of molecules [36]. The FTIR spectrum shows striking information on the chemical structure of Fe₃O₄ nanoparticle and DSASP–Fe₃O₄ complexes in the region of 400–4000 cm⁻¹ (Fig. 3e). The first gradual absorption pick at 3413.89 cm⁻¹ originated from strong stretching vibrations of hydrogen band with hydroxyl groups (OH) that were absorbed by the sample from the medium. The evaluated peak at 1617 cm⁻¹ is attributed to the O–H bending [37]. In addition, the second absorption peak at 567.06 cm⁻¹
is assigned to Fe-O bond in Fe$_3$O$_4$ nanoparticles which indicates the synthesis of nanoparticles. Changing the peak location of Fe–O from 567 cm$^{-1}$ to 583 cm$^{-1}$ showed relative interaction of stearic acid with SPIONs. Although the intensity of this peak replacement was expected to be stronger, decline in Fe$_3$O$_4$ absorption peak spectrum, demonstrated its sufficient entrapment by biopolymer. Moreover, decline in COO– vibration from 1709.29 to 1699.78 could be considered as another evidence for stearic acid interactions. Furthermore, both binding groups of C-N at wavelength peak of 1650 – 1620 cm$^{-1}$ and noticeable decline in absorption peak of 580 to 631 cm$^{-1}$ in Fe$_3$O$_4$ could be related to dextran-spermine entrapment [18, 38, 39]. So, results showed a successful synthesis of MNPs grafted to polymers.

**Estimating the protection capability of nanocomplexes**

Protection capability for both 6 and 8 DSASP polymers in different complexes charge and mass ratios at 1, 2.5, 5, 10, 20, 25, 50, 100 w/w were prepared in 1% agarose gel electrophoresis. As shown in Fig. 4a,b, for both of DSA$_6$SP and DSA$_8$SP at mass ratio above 1 w/w, with increasing concentration, no migration was observed for complexes since they remained in the well. Turning to 1 w/w for both polymer weak interactions was shown between DNA and DSASP and the positive charge of complexes could not completely prevent the plasmid from migration toward the positive electrode. In addition, at this mass ratio compared to polymer 8, it seemed that DSA$_6$SP had better electrostatic interaction with DNA due to the appearance of a more inconspicuous bond on agarose gel. Therefore, these differences indicated that in equal proportions of these polymers, the DSA$_6$SP ability in pDNA capsulation was greater than DSA$_8$SP polymer. The instability of pDNA in presence of DNasel was incubated with pDNA-DSASP–Fe$_3$O$_4$ ternary complexes. As seen in Fig. 4c,d, the fragment smear at the bottom of agarose gel indicated that naked DNA is degraded within 30 min while duplex and ternary complexes with pDNA were completely protected from enzymatic degradation. Furthermore, the plasmid bands after the addition of SDS to incubated complexes with DNasel indicates the pDNA complexes with DSASP or DSASP–Fe$_3$O$_4$ particles remained intact whereas naked pDNA was almost digested.

**The effects DSASP on cell viability in presence of SMF**

The cell viability based on the efficacy of MNPs with DS$_6$ASP and DS$_8$ASP was performed through MTT assay at different mass ratios in presence and absence of SMF. As shown in Fig. 5, the MFs do not show any excess cytotoxicity in both nanocomplexes compared to the absence of SMF. Moreover, increasing the mass ratios of DS$_8$ASP complexes, toxicity did not surge significantly (P ≥ 0/05) except 100 w/w in presence of SMF, and also in some mass ratios such as 10 w/w increase in cell proliferation was estimated compared to control. In addition, in all treatments of DS$_6$ASP polymer except the 100 w/w, by increasing the mass ratios, no toxicity was significantly observed (P ≥ 0/05). In the following treatments, the concentration of DSASP and also MNPs were prepared at 10 w/w and incubated in presence and absence of SMF. The results indicated no significant cytotoxicity in presence and absence of SMF compared to control after 48 h.
Transfection efficiency of DSA\textsubscript{6}SP and DSA\textsubscript{8}SP polyamines in presence SMF

We transfected nucleic acids based on the magnetofection process, the DSA\textsubscript{8}SP–pDNA/MNP and DSA\textsubscript{6}SP–pDNA/MNP ternary complexes were transferred to cells using an external SMF 20 mT. The ratio of polymer to DNA was determined due to different mass ratios including 10, 20, 25, 50 and 100 w/w. Moreover, the amount of nanoparticles in the complex was optimized based on the weight ratio of pDNA. In different mass ratios of magnetic DSA\textsubscript{8}SP, an increase in luciferase activity was recorded compared to treated cell with pDNA alone. The transfection rate of the PEI as a positive control did not increase significantly compared to most mass ratios of DSA\textsubscript{8}SP–pDNA/MNP complex (P > 0.05). In addition, the SMF enhanced the transfection efficiency compared to control, especially in the mass ratios of 10 and 50 w/w (P < 0.05) (Fig. 6a). The magnetic DSA\textsubscript{6}SP polymer with different mass ratios surged the activity of luciferase significantly and also the SMF affected the transfection efficacy of polymer specifically at the mass ratio of 20 and 50 w/w (P < 0.05). Moreover, transfection rates of magnetic nanocomplexes were higher than PEI polymer (P < 0.05) (Fig. 6b). As seen in Fig. 6c, comparison of both complexes illustrated appropriate cellular internalization as a result of a composed hydrophobic core of stearic acid, but based on a slight difference in the amount of fatty acids in polymers, the transfection efficiency of DSA\textsubscript{6}SP was higher than DSA\textsubscript{8}SP polymer.

Discussion

Several methods are used for synthesis of superparamagnetic iron oxide nanoparticles that are generally developed through two mechanisms including thermal decomposition and co-precipitation. The latter is represented to be more appropriate as it is considerably biocompatible in the in vivo conditions. Iron oxide nanoparticles are coated with biocompatible polymer for cellular transfection [27, 40]. Here, we investigated the properties of DSA\textsubscript{6}SP and DSA\textsubscript{8}SP–Fe\textsubscript{3}O\textsubscript{4} for gene delivery in the in vitro transfection systems.

In cells, spermine due to having a considerably cationic charge, ameliorates DNA packaging into nucleus through neutralizing anionic phosphate backbone charge which leads to DNA condensation. Spermine could be applied as a valuable factor in gene delivery applications [21]. Therefore, a decrease in the size of DSASP–Fe\textsubscript{3}O\textsubscript{4} complex at weigh rate above 10 nm tend to be attributed to higher spermine rate (Fig. 2c). On the other hand, different stearic acid grafted rates in DSASP amphiphilic polymer of 6 and 8 tend to be another efficient factor in polymer size rate that leads to more DNA condensation. Indeed, the natural chemical structure of stearic acid enabled molecules to be more flexible to move inward considering its free rotation of saturated carbon atoms [21]. Moreover, the surface charge of dextran-stearic acid polymers is often negative and close to zero due to the absence of ionizing groups in the dextran chains and stearic acid molecules [15, 19, 22]. Furthermore, zetasizer measurement revealed that grafts of spermine converted the zeta potential of Fe\textsubscript{3}O\textsubscript{4} nanoparticles from negative into positive
charges (Fig. 2d). In aqueous environment, spermine residues in DSASP–NPs are localized outside the micelle formation due to its hydrophobicity property. Considering to cell membrane negatively charged, the positively charged complexes facilitate its cellular uptake [41].

Nanoparticles with critical diameter less than 25 nm have superparamagnetic behaviors [42]. VSM magnetization curves indicated superparamagnetic behavior with superior magnetic response for having efficient magnetofection process in both unmodified Fe₃O₄ nanoparticle and DSASP–Fe₃O₄ complexes (Fig. 2c,d). Magnetic properties of nanoparticles strongly depends on the size and shapes [42, 43], because their single magnetic domain below critical diameter possess spherical structure which has parallel direction with magnetic spins that would be a reliable confirmation on SEM data and successful modification of the Fe₃O₄ MNPs by DSASP. The magnetism saturation of magnetic complexes is measured for 30.160 emu.g⁻¹ relatively, being 35% lower than Fe₃O₄ MNPs at 65.3 emu.g⁻¹, reflecting the thick amphiphilic shells covering Fe₃O₄ nanoparticles. This superparamagnetism feature is significantly recommended in the in vivo systems that prevents agglomeration of particles in the blood cycle through the removal of MFs and vanish magnetization, subsequently [42]. Results of FTIR spectra as shown in Fig. 3e, represent different interactions such as coordination between COO⁻ and Fe³⁺ (or Fe²⁺), hydrogen bonds, Van der Waals force and electrostatic interactions which keep DSASP on the surface of magnetite nanoparticles [38, 44].

The issue of stability in formation of polycations-based nanocomplexes with negative phosphate backbone charge of DNA is considered as a substantial requirement in gene delivery applications to achieve proper transfection efficiency. Preparation of various weight ratio of polycations to DNA is assumed as one of the most significant parameters to achieve this process [41, 45]. So, a different mass ratio of DSASP was mixed with similar volumes of pDNA and then results were analyzed based on gel retardation assay. The pDNA band in mass ratio of 1 w/w illustrates amphiphilic polymer inability to have efficient electrostatic interaction between amino groups and phosphate groups of DNA to entrap plasmid. Whereas, the migration of DNA, above this mass ratio was completely retarded, indicating DSASP enough cationic charge to form sustained and strong complexation with negative charge of DNA. The spermine residue conjugation on dextran molecules in DSASP polymer is considered as a factor that neutralizes the negative surface charge of DNA [45]. Moreover, the content of the spermine moieties on the dextran chain in DSA₈SP (0.35 µmol / mg) is higher than the other formulation based on the lower conjugation of stearic acid to polysaccharide which results in less space barrier for binding of the primary amines. Additionally, in comparison with DSA₈SP, results show a more inconspicuous bond for gel retardation of DSA₆SP polymer in 1 w/w, which represents in an equal mass ratio of polymers, DSA₆SP was more effective in DNA encapsulation (Fig. 4a,b). Higher residues of non-cationic fatty acids seem to be more effective in nucleic acid retardation compared to amine positive charges. It means, the hydrophobicity of the amphiphilic polymer, even in lower density, could neutralize a considerable fraction of the DNA charges [21].
Enzymatic degradation discussed as a restriction factor, impressively inhibits the process of gene transfection applications. Serum stability results demonstrate that although free DNA was completely digested by DNaseI due to existence of smear/fragmented band, both DSASPs encapsulated DNA were efficiently protected pDNA under enzymatic conditions attending to bright wells as an evidence for the presence of DNA which was properly capsulated in amphiphilic biopolymer (Fig. 4c,d).

As DSASP polymer did not show any cytotoxicity, cell proliferation was reported in some weight ratio like 10 w/w compared to control. Additionally, by increasing the polymer mass ratio, except 100 w/w, no significant cytotoxicity was observed for any DSA_8SP and DSA_6SP magnetic complexes (Fig. 5a,b). It is noticeable that the iron oxide nanoparticles, even after amphiphilic surface modification (Fig. 5c), sustained their low cytotoxicity in presence and absence of MFs (Fig. 5c). The polyamines like spermine are fundamental factors in the cell proliferation cycle which naturally exist in cells [19, 46]. Indeed, although spermine's increasing rate provides grounds to accelerate cell proliferation and restrict apoptosis by inhibiting cytochrome c release, its intracellular depletion leads cellular growth to be arrested [46].

Decrease in time association of nucleic acids with enzymatic conditions in cells and/or serum by the aim of accelerating DNA agglomeration to targeted cells and intensifying its rapid physical transportation, is demonstrated as another impressive factor in DNA protection which is studied through magnetofection [32]. Magnetofection through applying the external MF is considered as a non-invasive therapeutic approach, leading to cell viability without disrupting plasma membranes [23]. It is clearly shown that incubation with the application of a magnetic field in cell culture media remarkably reinforces cellular efficiency of complex for all DSASP–pDNA/MNPs, especially at weight ratio of 50 and 10 w/w in DSA_6SP and also 50 and 20 in DSA_8SP (Fig. 6). However, DSA_6SP–pDNA/Fe_3O_4 nanoparticles show a preferable transfection efficacy presumably based on higher stearic acid residues which result in better condensation of complexes (Fig. 6b). DSA_6SP with higher grafted rate of stearic acid (20%), gradually restricted more spermine conjugation with dextran chain which its amine content was evaluated at 0.26 µmol/mg as well as 0.35 µmol/mg and also 15% stearic acid in DSA_8SP.

Spermine residues conjugated on dextran make it a more appropriate biodegradable polycations for gene delivery applications [47]. Moreover, based on our obtained results, the addition of both spermine and stearic acid conjugations along the dextran chain, affect the DNA-binding complex transfection efficiency and density, significantly. Hosseinkhani et al., considered stable interactions with various cationic polysaccharide derivatives and different grafts of oligoamines, only dextran-spermine polycations found active in transfection assay [47]. Additionally, the emphasis of grafted spermine moieties in transfection efficiency was represented properly by the study of Azzam et al., to point out the spermine residues high buffering capacity. Therefore, the more spermine incorporation in dextran-spermine compound, the more luciferase expression [14]. Furthermore, spermine could promote DNA condensation and simplify endoSOSomal escape of oligonucleotide with two primary and two secondary amino groups respectively which results in an efficient transfection rate [19].
The conjugation of fatty acids to polycations are utilized to protect the coated DNA-polymer complex from enzymes and ease the cellular uptake of amphiphilic complex into lipophilic plasma membrane based on hydrophobic surface [14]. Particle size in amphiphilic polymers, was directly affected by hydrophobic to hydrophilic chain ratios. So, the enhancement of stearic acid conjugation degree in amphiphilic structure simultaneously leads to increase in polymer hydrophobic properties, closer polymeric interactions in aqueous medium and assembly of smaller particles. Indeed, decreasing the size will subsequently increase the surface/volume ratio of particles. These phenomena lead more particles to communicate and destabilized the aqueous medium [22].

Conclusions

Through higher association of stearic acid with dextran in DSASP-pDNA/Fe₃O₄ complexes, the surface/volume ratio increases alongside decreasing complex size which consequent more hydrophobic interaction with plasma membrane. Considering DSA₆SP with more amine content and also the efficient role of positive charges, the lower transfection efficacy was represented for this carrier compared to DSA₈SP. Finally, the importance of hydrophilic content in transfection assay is explained based on the efficient effects on total size of complexes and enhancement of their cellular attachment due to plasma cell membranes.

Abbreviations

DSASP
dextran-stearic acid-spermine
pDNA
plasmid DNA
MNPs
magnetic nanoparticles
PEI
polyethylenimine
VSM
vibrating sample magnetometer
DLS
dynamic light scattering

Declarations

Ethical Approval and Consent to participate

Not applicable.
Consent for publication
Not applicable.

Availability of data and materials
All data analyzed in this study are included in this published article.

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
M.K.A. performed the experiments and analyzed the data and also wrote the original draft. B.H.V. and M.S. contributed in conceptualizing, planning and supervising the methodological approaches. P.A. as corresponding author conducted research, sponsored the study and made all the arrangements. S.H. and H.Sh. conceived the study and methodology. All authors contributed to the study design and reviewed the manuscript.

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References
1. Gozuacik D, Yagci-Acar H, Akkoç Y, Kosar A, Dogan-Ekici Al, Ekici S. Anticancer use of nanoparticles as nucleic acid carriers. J Biomed Nanotechnol. 2014;10(9):1751–83.
2. Sudhakar A. History of cancer, ancient and modern treatment methods. Journal of cancer science therapy. 2009;1(2):1.
3. Eliyahu H, Makovitzki A, Azzam T, Zlotkin A, Joseph A, Gazit D, Barenholz Y, Domb A. Novel dextran–spermine conjugates as transfecting agents: comparing water-soluble and micellar polymers. Gene
therapy. 2005;12(6):494–503.

4. Schaffer DV, Lauffenburger DA. Optimization of cell surface binding enhances efficiency and specificity of molecular conjugate gene delivery. J Biol Chem. 1998;273(43):28004–9.

5. Teixeira HF, Bruxel F, Fraga M, Schuh RS, Zorzi GK, Matte U, Fattal E. Cationic nanoemulsions as nucleic acids delivery systems. International journal of pharmaceutics. 2017;534(1–2):356–67.

6. Wong SY, Pelet JM, Putnam D. Polymer systems for gene delivery—past, present, and future. Prog Polym Sci. 2007;32(8–9):799–837.

7. Cho K, Wang X, Nie S, Shin DM. Therapeutic nanoparticles for drug delivery in cancer. Clinical cancer research. 2008;14(5):1310–6.

8. Mykhaylyk O, Antequera YS, Vlaskou D, Plank C. Generation of magnetic nonviral gene transfer agents and magnetofection in vitro. Nature protocols. 2007;2(10):2391.

9. Yin H, Kanasty RL, Eltoukhy AA, Vegas AJ, Dorkin JR, Anderson DG. Non-viral vectors for gene-based therapy. Nat Rev Genet. 2014;15(8):541–55.

10. Ye S, Cole-Strauss A, Frank B, Kmiec EB. Targeted gene correction: a new strategy for molecular medicine. Molecular medicine today. 1998;4(10):431–7.

11. Nóbrega C, Mendonça L, Matos CA. A Handbook of Gene and Cell Therapy, Springer2020.

12. Sohrabijam Z, Saeidifar M, Zamanian A. Enhancement of magnetofection efficiency using chitosan coated superparamagnetic iron oxide nanoparticles and calf thymus DNA. Colloids Surf B. 2017;152:169–75.

13. Putnam D, Gentry CA, Pack DW, Langer R, Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini, Proceedings of the National Academy of Sciences 98(3) (2001) 1200–1205.

14. Azzam T, Eliyahu H, Makovitzki A, Linial M, Domb AJ. Hydrophobized dextran-spermine conjugate as potential vector for in vitro gene transfection. J Controlled Release. 2004;96(2):309–23.

15. Li S, Yi J, Li W, Wang L, Wang Z. Synthesis and characterization of three novel amphiphilic dextran self-assembled micelles as potential drug delivery system. Journal of Materials Science. 2017;52(21):12593–607.

16. Hosseinkhani H, He W-J, Chiang C-H, Hong P-D, Yu D-S, Domb AJ, Ou K-L. Biodegradable nanoparticles for gene therapy technology. Journal of nanoparticle research. 2013;15(7):1–15.

17. Su H, Liu Y, Wang D, Wu C, Xia C, Gong Q, Song B, Ai H. Amphiphilic starlike dextran wrapped superparamagnetic iron oxide nanoparticle clusters as effective magnetic resonance imaging probes. Biomaterials. 2013;34(4):1193–203.

18. Ghadiri M, Vasheghani-Farahani E, Atyabi F, Kobarfard F, Hosseinkhani H. In-vitro assessment of magnetic dextran-spermine nanoparticles for capecitabine delivery to cancerous cells. Iranian journal of pharmaceutical research: IJPR. 2017;16(4):1320.

19. Meng T, Wu J, Yi H, Liu J, Lu B, Yuan M, Huang X, Yuan H, Hu F. A spermine conjugated stearic acid-g-chitosan oligosaccharide polymer with different types of amino groups for efficient p53 gene
therapy. Colloids Surf B. 2016;145:695–705.

20. Azzam T, Eliyahu H, Makovitzki A, Domb A. Dextran-spermine conjugate: an efficient vector for gene delivery, Macromolecular Symposia. Wiley Online Library; 2003. pp. 247–62.

21. Liu Z, Zhang Z, Zhou C, Jiao Y. Hydrophobic modifications of cationic polymers for gene delivery. Prog Polym Sci. 2010;35(9):1144–62.

22. Shaki H, Ganji F, Kempen PJ, Dolatshahi-Pirooz A, Vasheghani-Farahani E. Self-assembled amphiphilic-dextran nanomicelles for delivery of rapamycin. Journal of Drug Delivery Science Technology. 2018;44:333–41.

23. Alvizo-Baez CA, Luna-Cruz IE, Vilches-Cisneros N, Rodríguez-Padilla C, Alcocer-González JM. Systemic delivery and activation of the TRAIL gene in lungs, with magnetic nanoparticles of chitosan controlled by an external magnetic field. Int J Nanomed. 2016;11:6449.

24. Dobson J. Gene therapy progress and prospects: magnetic nanoparticle-based gene delivery. Gene therapy. 2006;13(4):283–7.

25. Mohammad-Taheri M, Vasheghani-Farahani E, Hosseinkhani H, Shojaosadati SA, Soleimani M. Fabrication and characterization of a new MRI contrast agent based on a magnetic dextran–spermine nanoparticle system. Iran Polym J. 2012;21(4):239–51.

26. Qu J, Liu G, Wang Y, Hong R. Preparation of Fe3O4–chitosan nanoparticles used for hyperthermia. Adv Powder Technol. 2010;21(4):461–7.

27. Wu W, He Q, Jiang C. Magnetic iron oxide nanoparticles: synthesis and surface functionalization strategies. Nanoscale Res Lett. 2008;3(11):397–415.

28. Rasouli E, Basirun WJ, Rezayi M, Shameli K, Nourmohammadi E, Khandanlou R, Izadiyan Z, Sarkarizi HK. Ultrasmall superparamagnetic Fe3O4 nanoparticles: honey-based green and facile synthesis and in vitro viability assay. Int J Nanomed. 2018;13:6903.

29. Scott V, Clark AR, Docherty K, The gel retardation assay, Protocols for gene analysis (1994) 339–347.

30. Magdeldin S. Gel electrophoresis: Principles and basics, BoD–Books on Demand2012.

31. Schnurr B, Ahrens T, Regenass U. Optical assays in drug discovery, (2007).

32. Namgung R, Singha K, Yu MK, Jon S, Kim YS, Ahn Y, Park I-K, Kim WJ. Hybrid superparamagnetic iron oxide nanoparticle-branched polyethylenimine magnetoplexes for gene transfection of vascular endothelial cells. Biomaterials. 2010;31(14):4204–13.

33. Yu Y, Hu Y, Li X, Liu Y, Li M, Yang J, Sheng W. Spermine-modified Antheraea pernyi silk fibroin as a gene delivery carrier. Int J Nanomed. 2016;11:1013.

34. Tauxe L, Mullender T, Pick T. Potbellies, wasp-waists, and superparamagnetism in magnetic hysteresis. Journal of Geophysical Research: Solid Earth. 1996;101(B1):571–83.

35. Ghandoor HE, Zidan H, Khalil MM, Ismail M. Synthesis and some physical properties of magnetite (Fe3O4) nanoparticles. Int J Electrochem Sci. 2012;7(6):5734–45.

36. Mohamed MA, Jaafar J, Ismail A, Othman M, Rahman M. Fourier transform infrared (FTIR) spectroscopy, Membrane Characterization, Elsevier2017, pp. 3–29.
37. ur Rahman O, Mohapatra SC, Ahmad S. Fe3O4 inverse spinal super paramagnetic nanoparticles. Mater Chem Phys. 2012;132(1):196–202.

38. Cao H, Li J, Shen Y, Li S, Huang F, Xie A. Green synthesis and surface properties of Fe3O4@ SA core–shell nanocomposites. Applied surface science. 2014;301:244–9.

39. Mistry B, A handbook of spectroscopic data, Chemistry (2009).

40. Tassa C, Shaw SY, Weissleder R. Dextran-coated iron oxide nanoparticles: a versatile platform for targeted molecular imaging, molecular diagnostics, and therapy. Acc Chem Res. 2011;44(10):842–52.

41. Cen C, Wu J, Zhang Y, Luo C, Xie L, Zhang X, Yang X, Li M, Bi Y, Li T. Improving magnetofection of magnetic polyethylenimine nanoparticles into MG-63 osteoblasts using a novel uniform magnetic field. Nanoscale Res Lett. 2019;14(1):1–14.

42. Estelrich J, Escribano E, Queralt J, Busquets MA. Iron oxide nanoparticles for magnetically-guided and magnetically-responsive drug delivery. Int J Mol Sci. 2015;16(4):8070–101.

43. Lee H, Nguyen Y, Muthiah M, Vu-Quang H, Namgung R, Kim W, Yu M, Jon S, Lee I, Jeong Y. MR traceable delivery of p53 tumor suppressor gene by PEI-functionalized superparamagnetic iron oxide nanoparticles. J Biomed Nanotechnol. 2012;8(3):361–71.

44. Predescu AM, Matei E, Berbecaru AC, Pantilimon C, Drăgan C, Vidu R, Predescu C, Kuncser V. Synthesis and characterization of dextran-coated iron oxide nanoparticles. Royal Society open science. 2018;5(3):171525.

45. Amini R, Jalilian FA, Abdullah S, Veerakumarasivam A, Hosseinkhani H, Abdulamir A, Domb A, Ickowicz D, Rosli R. Dynamics of PEGylated–dextran–spermine nanoparticles for gene delivery to leukemic cells. Appl Biochem Biotechnol. 2013;170(4):841–53.

46. Holst CM, Oredsson SM. Comparison of three cytotoxicity tests in the evaluation of the cytotoxicity of a spermine analogue on human breast cancer cell lines. Toxicology in vitro. 2005;19(3):379–87.

47. Hosseinkhani H, Azzam T, Tabata Y, Domb A. Dextran–spermine polycation: an efficient nonviral vector for in vitro and in vivo gene transfection. Gene therapy. 2004;11(2):194–203.

Figures
Figure 1

The overview of amphiphilic polymer of dextran-stearic acid-spermine (DSASP) used to transfer nucleic acid via Fe3O4 encapsulation in the magnetofection process.
Figure 2

The (a, b) size and (c, d) zeta potential of Fe3O4 nanoparticle (MNPs) and dextran-stearic acid-spermine (DSASP)-pDNA/MNPs complexes were evaluated at different mass ratios.
Figure 3

Characterization of synthesized MNPs. The SEM images of (a) Fe3O4 MNPs and (b) dextran-stearic acid-spermine (DSASP)@Fe3O4. The vibrating sample magnetometer (VSM) shows superparamagnetic property of MNPs based on no hysteresis loop, $H_c = 0$ Oe (c) Fe3O4 nanoparticles and (d) DSASP-Fe3O4 nanocomplexes. (e) FTIR spectra of Fe3O4 nanoparticles (red peak) DSASP polymer (blue peak) and the magnetic nanocomplex of DSASP–Fe3O4 (green peak).
Figure 4

Gel retardation analysis of dextran-stearic acid-spermine (DSASP) on pDNA by electrostatic interaction efficacy on 1% agarose gel at different mass ratios 1, 2.5, 5, 10, 20, 25, 50, 100 w/w of (a) DSA8SP-pDNA/MNPs and (b) DSA6SP-pDNA/MNPs. Serum stability assay with DNaseI in mass ratio of 50 w/w indicates for (c) DSA8SP-pDNA/MNPs and (d) DSA6SP-pDNA/MNPs.
Figure 5

The effect of different mass ratios (w/w) of triplex complexes of (a) pDNA-dextran-stearic acid 8-spermine (DS8ASP)-MNPs and (b) dextran–stearic acid 6–spermine (DS6ASP) pDNA/MNP and (c) combination treatments with MNPs based on the mass ratio of 10 w/w in presence and absence of SMF 20 mT on viability of HEK 293T cells. Data are shown as the mean ± SD based on independent tests (n = 4). **p < 0.01; ***p < 0.001 show significant differences relative to control (CTRL), which were analyzed by one-way factorial ANOVA followed with post-hoc Newman–Keuls multiple comparison tests.
Figure 6

Evaluation the transfection efficiency of (a) dextran-stearic acid 8-spermine (DS8ASP) and (b) dextran-stearic acid 6-spermine (DS6ASP) polyamines at different mass ratios in triplex complexes based on magnetofection processes. Data are shown as the mean ± SD based on independent tests (n = 4). *p < 0.05; **p < 0.01; ***p < 0.001 show significant differences relative to cells in absence of SMF, which were analyzed by one-way factorial ANOVA followed with post-hoc Newman–Keuls multiple comparison tests.