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ORIGINAL RESEARCH

In situ production of cationic lipid coated magnetic nanoparticles in multiple emulsions for gene delivery

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ABSTRACT

Magnetic nanoparticles are effective delivery systems to target therapeutic genes by the attractive forces of magnetic fields. Curative effects depending on dose of nucleic acids or drugs increased, while cytotoxic effects minimized with these systems. In this study, a novel magnetic nanoparticle synthesis method was developed by combining advantages of microemulsion and multiple emulsion methods. Particle size, zeta potential, magnetization, complex formation with nucleic acids, DNase I protection ability, and cytotoxicity levels were examined. At last, magnetic nanoparticles were obtained with a promising synthesis method and it is determined that they are sufficiently small, non-toxic and have optimal surface properties for systemic delivery of nucleic acids.

Keywords: Iron oxide, superparamagnetic, cationic magnetic nanoparticle, multiple emulsion, gene delivery, magnetofection

INTRODUCTION

To discover and process the genetic factors of diseases; and to deliver the therapeutic genes or materials to the concerned sites are two main approaches for gene therapy. With the rise of the gene therapy in last decades, generating effective gene delivery systems became one of the most prominent research area in pharmaceutical biotechnology. Non-viral gene delivery systems are preferred for this aim because of their low-cost, low immunogenicity and easy productions (1–3). Genetic material can be delivered by nanoparticles either with passive or active transport techniques. One of the active transport technique, which shows great promise for both *in vitro* and *in vivo* transfection is magnetofection. Magnetofection is defined as the nucleic acids delivery to cells, site-specifically guided by the attractive forces of magnetic fields acting on nucleic acid vectors, which are associated with magnetic nanoparticles (MNPs) (4, 5).

MNPs should be coated with cationic materials to interact with DNA electrostatically. Most of the magnetic nanoparticle synthesis and coating methods include organic solvents or toxic agents (6–10) Another significant problem encountered in the synthesis of MNPs with iron compounds is the tendency of the reaction to synthesize non-magnetic iron compounds. This is mainly caused as a result of the effect of dissolved oxygen in the solutions. The dissolved oxygen must therefore be removed from the medium.

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This removal can be performed by creating a nitrogen gas environment (11–15). Maintaining of the medium pH in the acidic range is another method (6, 16) For the multiple emulsion method that we developed, solutions of Fe^{+2} and Fe^{+3} are in the form of droplets surrounded by lipid layer of water/oil microemulsion. In this situation, the rate of contact with the external environment or atmospheric oxygen is lower. Another point is that the lipids participating in the microemulsion composition exhibit a protective effect by reducing the pH, as they contain fatty acids.

In this study, we developed a novel method based on physics and chemistry techniques to obtain non-toxic, biocompatible and targetable MNPs with optimal size and surface properties for gene delivery. Afterwards obtained MNPs were characterized and then MNP:DNA complexes were formed. Protection efficiencies against DNase I enzyme and cytotoxicity levels were examined.

MATERIALS

Plasmid DNA which encoding green fluorescent protein (pEGFP-C1) was used as genetic material (Invitrogen, California, USA). Ethanol, Tween 80, $FeCl_2$, $FeCl_3$ and Sodium dodecyl sulfate (SDS), were provided from Merck-Co. (Hohenbrunn, Germany). Palmitic acid, Span 80, and dimethyldioctadecylammonium bromide (DDAB) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Materials for agarose gel electrophoresis and cell culture reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ultra-pure distilled water was used at all formulations. XTT cell proliferation assay kit was provided from Biological Industries (Beit-Haemek, Israel). Chinese Hamster Ovary (CHO) cell line was purchased from ATCC.

METHODS

MNP Synthesis

In order to develop MNPs, microemulsion and multiple emulsion techniques were combined and MNPs were synthesized in the core of multiple emulsion. To obtain triangle phase diagram of oil in water (o/w) microemulsion is the first step of this method. Microemulsions were formed with palmitic acid as oil phase, Span 80 as non-ionic surfactant, ethanol as co-surfactant and Fe^{+2} and Fe^{+3} solutions as water phase ($\mathbf{w_1}$/o) over the lipid melting point (73°C). Transparent regions belongs to w/o microemulsion area in phase diagrams were determined. DDAB was incorporated to the oil phase of the microemulsion for gaining cationic property to the MNPs. Then, obtained microemulsion were used as interior emulsion of multiple emulsion ($\mathbf{w_1/o}/w_2$). Tween 80 was used as an outer surfactant and ultra-pure water used as an outer water phase ($w_1/o/\mathbf{w_2}$). Afterwards 1 N NaOH was used to increase pH of the system. [OH^-] ions leak to the interior water phase of the multiple emulsion. Therefore, magnetic iron oxide particles were synthesized in the core of cationic lipids regarding to the equation given below (6,18–21).

$$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O$$

1 mL of hot multiple emulsion with magnetic core was taken in an injector and dropped into the ice-cold distilled water (0-2°C). MNPs were formed when multiple emulsion droplets met with cold water (MNP-Pa) (22–24). A neodium magnet was used for separation of MNP-Pa. MNP-Pa washed with distilled water for two times and the characterization studies were carried on (6,19,25).

To understand the effects of formulation on magnetization, one formulation (NP-Ø) was prepared as same as described above but ultra-pure water used instead of (1M Fe^{+2}) and (2M Fe^{+3}) solutions as the interior water phase. MNPs were also synthesized by a well-known co-precipitation method (MNP-CoP) (26).

Preparation of MNP:DNA Complexes

MNP:DNA complexes were formed by mixing different ratios of pEGFP-C1 (0.1 µg/mL) and cationic MNP-Pa suspension for 30 minutes at 25°C. The blocking effect of MNP-Pa on the electrophoretic mobility of pEGFP-C1 was examined on agarose gel electrophoresis to determine optimal complex ratio (27,28).

Size and Zeta Potential Measurements

The particle size and zeta potential of MNPs and MNP:DNA complexes were determined with dynamic light scattering (DLS) method by using Zetasizer Nano ZS (Malvern Instruments, Worcestershire, U.K.). Experiments were carried out at least in triplicate and standard deviations were calculated.

Magnetization measurements

Magnetic properties of nanoparticles were evaluated by using Lakeshare Vibrating Sample Magnetometer (VSM).

DNase I Protection Study

DNase I was added to MNP:DNA systems to a final concentration of 1 IU DNase I/2.5 µg pDNA, and the mixtures were incubated at 37°C for 30 min. Afterwards 10% (w/v) SDS solution was added to the samples to a final concentration of 1% (w/v) to release pEGFP-C1 from the complex (1,29,30). Samples were then analyzed by electrophoresis on %1 agarose gel (containing %0.5 ethidium bromide) for 1 hour at 100 volts. The bands were visualized with a transilluminator (Biocapture, Vilber Lourmat).

Cytotoxicity

CHO cells were seeded in a 96 well plate ($6x10^3$ cells per well) 24 h before the cytotoxicity test. Various volume of MNP-Pa and MNP:DNA complexes prepared within 100 μL DMEM media without serum. Culture media was aspirated, and the prepared samples were added to the wells. After 24 h incubation at 37 °C and 5% CO_2, XTT cell proliferation test was examined. Cell viability was calculated with specific absorbance corresponding to control group. Experiments were carried out at least in triplicate and standard deviations were calculated.

RESULTS AND DISCUSSION

In the method that we developed, the iron compounds are in the form of droplets in limited volume reaction pools, within the inner water phase of multiple emulsions. As the droplet size is limited by the reaction volume for production of MNPs, the dimensions also become more controllable. For this purpose, firstly w/o microemulsions were prepared. The w/o microemulsion area was determined by the aid of triangle phase diagram and transparent water in oil systems were formed (Figure 1). Formulation which has the largest volume of inner water phase was selected in the region of transparent systems. Micelles, liquid crystals and reaction derivate were removed and MNPs were collected by using a neodium magnet (Figure 2). Characterization studies were performed as described in the methods.

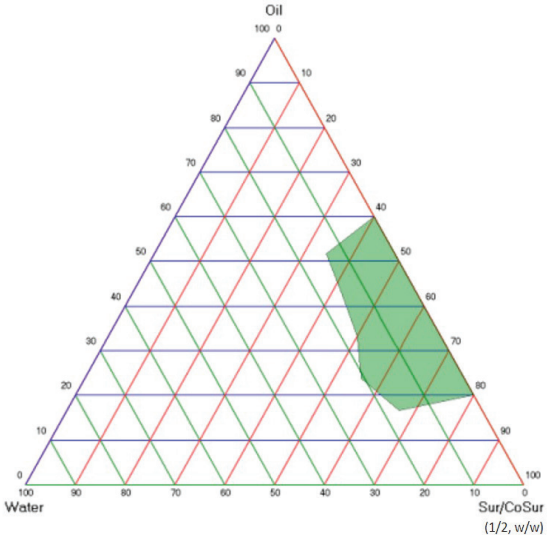

Figure 1. Triangular phase diagram of w/o microemulsion formed with palmitic acid as oil phase, Span 80 as non-ionic surfactant, ethanol as co-surfactant and Fe^{+2} and Fe^{+3} solutions as water phase. Green area shows transparent w/o microemulsion region.

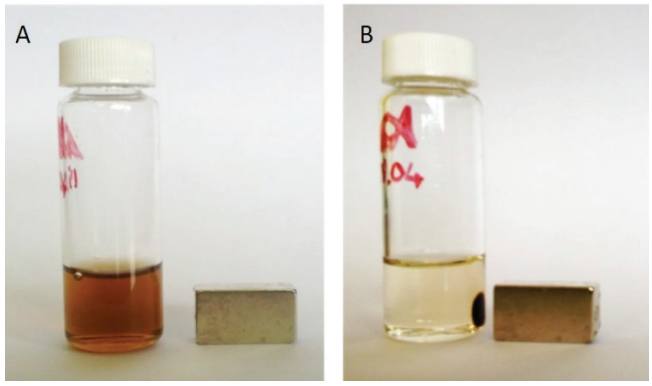

Figure 2. Optic photographs of obtained MNPs. MNP-Pa suspension (**A**) and magnetic separation of MNPs by using a neodium magnet (**B**).

Magnetization of MNPs were analyzed by VSM. The magnetic properties such as saturation magnetization (Ms), remanent magnetization (Mr) and coercivity (Hc) were evaluated from the magnetization hysteresis of MNPs. As seen in Figure 3, MNPs are in paramagnetic behavior and Mr and Hc values are approximately zero. Although saturation magnetization of MNP-CoP is higher than the coated samples that we prepared, MNP-Pa has reasonably sufficient Ms values for magnetic targeting of nucleic acids (2, 7, 24, 31, 32). Ms value was measured 20 emu g^{-1} for lipid coated MNP-Pa while 35.2 emu g^{-1} for MNP-CoP.

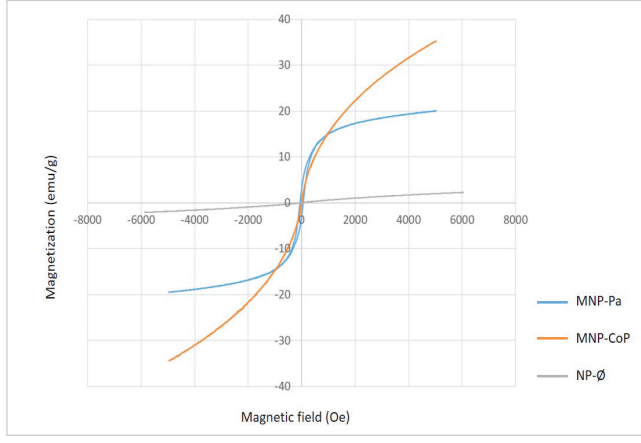

Figure 3. Magnetization hysteresis loops for MNP-Pa, MNP-CoP, and NP-Ø.

Particle size, polydispersity index and zeta potential values of MNPs were measured for characterization studies (Table 1). MNP-CoP and MNP:DNA complex (3:1, v/v) were also prepared for characterization and comparison.

Table 1. Characterization of MNP-Pa, MNP-CoP and MNP:DNA Complex

	Formulation	Particle Size (nm) (±SD)	PDI (±SD)	Zeta Potantial - ζ (mV) (±SD)
1	**MNP-Pa**	21.75 (0.153)	0.234 (0.008)	19.24 (1.5)
2	**MNP-CoP**	9.52 (0.204)	0.338 (0.045)	-25.9 (2.15)
3	**MNP:DNA Complex (3:1, v/v)**	35.56 (0.461)	0.486 (0.004)	4.71 (0.656)

MNPs were produced in nano-scale size. Particle size was measured smaller than 30 nm and it is found appropriate to superparamagnetic character (15, 31, 33). The increase of particle size was determined for in situ coated MNP-Pa samples comparing to the MNP-CoP while both MNP-Pa and MNP:DNA complex were found sufficiently small to traverse the microvascular system. Therefore obtained MNPs are suitable for systemic delivery (31, 34, 35). PDI values showed that more homogeneous particle size distribution was obtained for the MNP-Pa compared to MNP-CoP and MNP:DNA complex.

When MNPs used without coating, they are cleared from body in a short time by the immune system. While their biocompatibility is a positive trait, they need to be coated with polymers or lipids in their applications as a delivery systems for drug or genes (2, 6, 7, 31, 36). In addition to the coating process, it requires a second purification step (37). MNPs synthesized in multiple emulsions appear in the form of solid lipid nanoparticles. Since the synthesis of MNPs spontaneously ensures the production in lipid coated form there is no need an extra coating process as well as following purification steps. In the system that we developed, cationic lipid coating of the MNPs was ensured by the incorporation of DDAB in the oil phase of the microemulsion. Cationic zeta potential (19.24 mV) value was evaluated as a proof of cationic lipid coating after washing and magnetic separation processes (13, 38, 39).

Cationic lipid coating allows MNP-Pa to form complexes with oppositely charged molecules via electrostatic interactions. Nucleic acids are negatively charged molecules because of their phosphate groups (28, 29, 40, 41). MNP-Pa were formed complexes with pEGFP-C1 as genetic material. Optimal complex ratio which had protective effect on pEGFP-C1 against DNase I enzyme was determined by agarose gel electrophoresis (Figure 4).

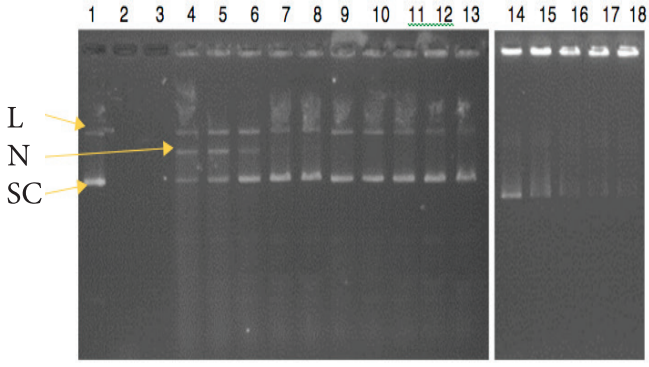

Figure 4. Agarose gel electrophoresis of MNP:DNA complexes. 1. pEGFP-C1, 2. pEGFP-C1+ DNaz I, 3.Ø, 4,5,6,7,8, respectively MNP:DNA (1:1, 2:1, 3:1, 4:1, 5:1) (v/v) + DNaz I+ %10 SDS, 9,10,11,12,13, respectively MNP:DNA (1:1, 2:1, 3:1, 4:1, 5:1) (v/v) + %10 SDS, 14,15,16,17,18, respectively MNP:DNA (1:1, 2:1, 3:1, 4:1, 5:1) (v/v), L: Linear, N: Nick, SC: Supercoiled.

Formation of compact complex is important issue for gene delivery systems as well as release of the nucleic acids from the complex. Release of the nucleic acids is required for gene expression in the cell (3, 28, 42). For this purpose SDS which is a negatively charged detergent molecule was used. Affinity of SDS to the cationic nanoparticles is higher than nucleic acids and final concentration of 1% SDS is sufficient to release nucleic acids from the complex (43).

Figure 4 features the agarose gel electrophoresis of resistant pEGFP-C1 in the complex with the increasing amounts of MNP-Pa against the degradation of DNase I enzyme. Complex formation demonstrated on band lanes 14-18 . It was observed that, the blocking effect of MNP-Pa on the electrophoretic mobility of pEGFP-C1 was started from the well 16 (MNP:DNA complex ratio of 3:1, v/v). The band on lane 6 demonstrated that, MNP:DNA complexes prepared at 3:1 (v/v) ratio was able to protect pEGFP-C1 against DNase I enzyme degradation when compared with pEGFP-C1 on lane 1. Release of pEGFP-C1 with the same complex ratio was shown on lane 11 and it was found sufficient.

Increasing volume of MNP-Pa and MNP:DNA (3:1, v/v) complexes were evaluated for cytotoxicity test. After 24 h incubation at 37°C and 5% CO_2, XTT cell proliferation test was examined and % cell viability was calculated corresponding

to control with specific absorbance. Than results for all formulation groups were analyzed with graphical data as shown in Figure 5.

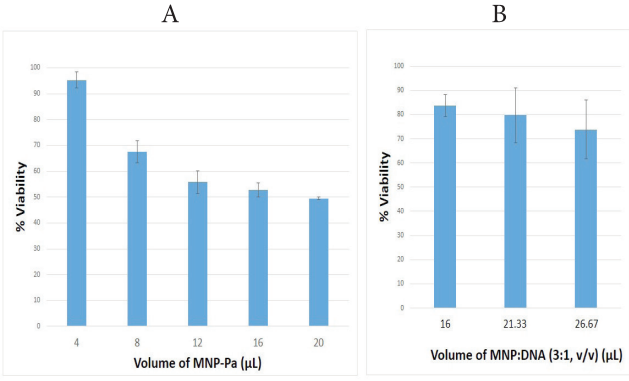

Figure 5. % Viability of CHO cells at increasing doses (µl/well) of MNP-Pa (**A**) and MNP:DNA complex (3:1, v/v) (**B**).

Cytotoxicity of MNP-Pa and MNP:DNA complexes were determined on CHO cells. No significant cytotoxicity was observed on CHO cells in the concentration range of 4-8 µl/well for MNP-Pa and all the applied doses for MNP:DNA complexes. Cell viability drops lower than 60% after 8 µL for the MNP-Pa while it sustains around 80% for MNP:DNA complexes. Zeta potential of MNP-Pa measured as +19.24 mV and it decreased to 4.71 mV for MNP:DNA complexes because of being in a complex with negatively charged nucleic acids. However, zeta potential reduction is not associated with transfection efficiency of MNPs (26, 44).

CONCLUSION

Obtained results showed that, MNPs have similar magnetization hysteresis compared with co-precipitation method and have superparamagnetic characteristics. Average particle size of MNPs-Pa and MNP:DNA (1:3, v/v) complex were measured 21.75 nm and 35.56 nm, respectively. No additional process for surface adjustments is needed with developed method. MNP-Pa are positively charged and are able to form complexes with pEGFP-C1 electrostatically. Zeta potential of MNP-Pa measured as +19.24 mV. Agarose gel electrophoresis showed that DNA:MNPs complexes are able to release pEGFP-C1 from complex and also protect it from DNase I (1 IU/ 2.5 µg pDNA) enzyme degradation. Cytotoxicity of MNP-Pa and MNP:DNA were examined and it was found that cell viability sustains around 80% for MNP:DNA complexes.

Consequently, a novel magnetic nanoparticle synthesis method which have appropriate particle size, zeta potential, and sufficient magnetic property for nucleic acid delivery was developed by combining advantages of microemulsion and multiple emulsion methods. Synthesized MNP-Pa are considered promising vehicles for non-viral gene delivery system for the treatment of genetic-based diseases.

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Gen aktarımı için katyonik lipid kaplı manyetik nanoparçacıkların çoklu emulsiyonlar kullanılarak yerinde üretimi

ÖZ

Manyetik nanopartiküller manyetik alanın çekim kuvvetini kullanarak terapötik genleri etkili bir şekilde aktaran sistemlerdir. Bu sistemlerde, ilaç ve nükleik asit dozuna bağlı tedavi edici etki artırılırken, sitotoksik etkiler en aza indirgenir. Bu çalışmada mikroemulsiyon ve çoklu emülsiyon metotlarının avantajları bir araya getirilerek yeni bir manyetik nanopartikül üretim yöntemi geliştirilmiştir. Partikül boyutu, zeta potansiyeli, manyetizasyon değeri, nükleik asitler ile kompleks oluşumu, DNaz I'e karşı koruma yeteneği ve sitotoksisite derecesi araştırılmıştır. Son olarak, manyetik nanopartiküller gelecek vaat eden bir sentez yöntemi ile elde edilmiş ve nükleik asitlerin sistemik taşınması için yeterli derecede küçük boyutta, toksik olmayan ve ideal yüzey özelliklerine sahip oldukları belirlenmiştir.

Anahtar kelimeler: demir oksit, süperparamanyetik, katyonik manyetik nanopartikül, çoklu emülsiyon, gen aktarımı, manyetofeksiyon.

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