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Click assembly of magnetic nanovectors for gene delivery

Souvik Biswas[†], Laura E. Gordon[‡], Geoffrey J. Clark[‡], and Michael H. Nantz^{†,*}

[†]Department of Chemistry, Brown Cancer Center, University of Louisville, Louisville, Kentucky 40292

[‡]Department of Medicine, Brown Cancer Center, University of Louisville, Louisville, Kentucky 40292

Abstract

Functionalization of iron oxide nanoparticles with quaternary ammonium ion-based aminooxy and oxime ether substrates provides a flexible route for generating magnetic gene delivery vectors. Using the MCF-7 breast cancer cell line, our findings show that pDNA magnetoplexes derived from the lipid-coated nanoparticle formulation dMLP transfect in the presence of 10% serum with or without magnetic assistance at significantly higher levels than a commonly used cationic liposome formulation, based on luciferase assay. The present ionpairing, click chemistry approach furnishes Fe₃O₄ nanoparticles with lipid layers. The resultant magnetic nanovectors serve as transfection enhancers for otherwise transfection-inactive materials.

1. Introduction

Since the seminal report by Mah's group on the use of magnetic nanoparticles to enhance viral-mediated transduction [1], much research effort has been directed toward the development of nano-sized magnetic vectors for intracellular delivery of polynucleotides [2]. Indeed, the rising interest in 'magnetofection', the term coined by Scherer *et al.* [3] to denote magnet-assisted gene delivery [4, 5], has led to a plethora of new strategies for functionalizing magnetic particles to promote association with negatively charged oligonucleotides [6], DNA or siRNA [7]. The majority of approaches have relied on coating iron oxide nanoparticles or nanocomposites with cationic polymers, such as poly-L-lysine [8] or, in particular, polyethylenimine (PEI) [9–11]. The high positive charge [12] of PEI effectively promotes DNA transfer into cells. Additionally, the polyamine backbone of PEI enhances escape of DNA complexes entrapped within endosomes by means of a proton sponge effect that increases osmotic pressure and causes endosome rupture [13]. Magnetic PEI-coated nanoparticles generally are prepared by mixing suspensions of magnetite (Fe₃O₄) nanocrystals with solutions of linear or branched PEI [14]. More recently, an *in situ* preparation [15] involving precipitation of iron oxide in the presence of PEI and a method

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*To whom correspondence should be addressed. michael.nantz@louisville.edu.

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for covalent attachment [16] of PEI to chitosancoated iron oxide nanoparticles have been described. Elaboration of PEI-coated iron oxide nanoparticles, such as by addition of cationic peptide fragments [17] or single chain antibodies [18], is also an area under intense investigation. Irrespective of the mode of generation or subsequent functionalization, magnetic PEI-coated iron oxide nanovectors readily combine with polynucleotides on simple mixing to generate the corresponding magnetic, electrostatic charge-affinity complexes. The resultant ‘magnetoplexes’ (nomenclature derived by extrapolation of the established term magnetoliposome [19] and the terms lipoplex and polyplex [20]) have been used principally for gene delivery *in vitro*; however, successful magnetofection also has been demonstrated *in vivo* by externally applying a magnetic gradient to the treatment area, for example [21].

One concern that plagues applications involving PEI is the associated cellular toxicity. The high positive charge density of PEI disrupts cellular membranes [22]; thus, complications arising from vector toxicity afflict many PEI-based gene therapy approaches [23]. To overcome this limitation, a recent focus in the field of magnetofection has been to develop lipid-based magnetoplexes. This strategy has been realized in part by mixing preformed lipoplexes (cationic lipid : helper lipid : DNA) with magnetite [24] or by adding transfection-active cationic liposome formulations (cationic lipid : helper lipid) to either oleic acid-coated [25, 26] or dextran-coated [27] magnetite prior to complexation with nucleic acids to form magnetoplexes.

Given that the molecular structure of cationic lipids has a profound influence on gene delivery [28], it is surprising that few magnetofection studies have exploited structure-activity optimizations offered by a modular, lipid-based approach. Our program on lipid-based transfection vectors [29] and interest in click chemistry [30] led us to consider devising a flexible method for stepwise construction of a transfection-active lipid coating directly on the surface of iron oxide nanoparticles. Without the aid of established transfection lipids and their co-lipid formulations, the use of lipid-coated nanoparticle vectors for gene transfer remains largely unexplored. Inspired by the nanoparticle ‘click’ conjugate work of Miller [31] and Mirkin [32], we sought to harness click chemistry [33] to attach lipid sidechains onto functionalized iron oxide nanoparticles. We report herein such an approach toward assembling transfection-active, lipid-coated magnetic nanoparticles for gene transfer applications.

2. Materials and methods

2.1. ao-NP synthesis

Iron oxide nanoparticles (**NP**) (5.0 mg), prepared according to a literature procedure [34], were suspended in ultrapure water (5 mL) by brief sonication (ca. 10–15 min, bath sonicator). To the suspension was added a solution of *N, N*-bis-(2-aminooxyethyl)-*N, N*-dimethylammonium iodide (17.0 mg, 0.058 mmol) in water (5 mL). The reaction suspension was stirred at room temperature. After 12 h, the coated particles were isolated by magnet-assisted sedimentation followed by removal of the supernatant. The isolated particles then were rinsed with water, and the sedimentation procedure was repeated. After rinsing a total

of three times, the resultant **ao-NP** slurry was freeze dried to afford **ao-NP** (6.9 mg) as a dry powder.

2.2. MLP synthesis

To **ao-NP** (6.9 mg) suspended in methanol (5 mL) was added myrist-aldehyde (20.0 mg, 0.94 mmol). The suspension was stirred at room temperature. After 12h, the lipid-coated nanoparticles were isolated by magnetic sedimentation, washed with methanol (2X), and then dried under vacuum 5h to yield the magnetic lipid particles (**MLP**; 7.8 mg).

2.3. dMLP synthesis

To **NP** (5.0 mg) suspended in methanol (5 mL) was added *N, N*-dimethyl-*bis*(2-tetradecylideneaminoxy-ethyl)ammonium iodide (**4**; 11.0 mg, 0.016 mmol). The suspension was stirred 12h at room temperature. The resultant particles then were isolated by magnet-assisted sedimentation, washed with methanol (2X), and dried under vacuum 5h to give **dMLP** (9.25 mg).

2.4. Nanoparticle characterization

Methanol suspensions of **NP** or **dMLP** samples were deposited on transmission electron microscopy (TEM) Cu grids coated with a carbon film. TEM microstructures and energy dispersive X-ray (EDX) spectra then were measured using a JEOL JEM 3200FS at 300 kV equipped with an EDX detector. X-Ray diffraction (XRD) patterns were obtained using a BRUKER D8 diffractometer with a CuK α radiation source ($\lambda = 0.15418$ nm). The phase identification was performed using JCPDS-ICDD 2000 software (The International Centre for Diffraction Data; ICDD).

2.5. Zeta potential measurements

The zeta potentials of **NP**, **ao-NP**, **MLP**, **dMLP** and derived magnetoplexes were measured in water using a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corporation; Model 90 Plus). The concentration of iron oxide in the aqueous particle samples was 0.1 mg/mL. Magnetoplexes were formulated at a concentration of 0.5 μ g pDNA.

2.6. Cell culture

Human breast cancer cells (MCF-7) were purchased from American Type Culture Collection VA, USA. Cells were grown to 50–60% confluency in DMEM and 1% Pennstrep (Mediatech, Inc, VA) with 10% FBS (Valley Biomedical, Winchester, VA).

2.7. Luciferase gene expression

Luciferase transfections were performed in triplicate using 0.025 μ g of pDNA (pCMV Luc)/well in MCF-7 cells. MCF-7 cells were seeded up to 1×10^5 cells/well in a 24-well plate to give 50–60% confluence. Magnetoplexes (MLP•pDNA, dMLP•pDNA) were prepared at nanoparticle:pDNA ratios of 30, 60, 90, 120, 180, 360 and 540 by adding the required volume of aqueous suspension of MLP or dMLP to a pDNA solution (3 μ L, 0.025 μ g DNA/ μ L). 200 μ L of serum free DMEM then was added to each magnetoplex solution followed

by incubation for 30 min at room temperature. The magnetoplex solutions were diluted to 600 μ L with serum free DMEM, and then 200 μ L of the final magnetoplex formulation were added directly to each well. For magnetofection, the cell plate was placed on top of a magnetic plate (Oz Biosciences) for 1 h at 37.5 $^{\circ}$ C. After 18h incubation at 37.5 $^{\circ}$ C, the cells were lysed and luciferase gene expression was quantified using a commercial kit (Promega) and luminometer according to the vendor's protocol. Lipofectamine 2000 (Invitrogen) was used as a positive control. Transfections were also performed in similar manner without the assistance of a magnet.

2.8. Cytotoxicity measurements

Cell cytotoxicity and proliferation of cells treated with **dMLP** formulations were assessed by an alamarBlue assay. After transfection of MCF-7 cells using dMLP-derived magnetoplexes (0.025 μ g of DNA/well) and incubation for 18 h, cell viability was measured according to the vendor's (Invitrogen) protocol. Fluorescence intensities were measured using a spectrofluorometer (Molecular Devices: Gemini EM) by excitation at 540 nm and emission at 600 nm.

3. Results and discussion

Oximation (Scheme 1), the coupling between aminooxy and aldehyde or ketone carbonyl groups, is a robust, chemoselective ligation reaction that has been used effectively in aqueous systems [35]. We envisioned that oximation could be applied to conjugate hydrophobic chains onto magnetic nanoparticles if aminooxy moieties were affixed at the surface of the particles. To actualize this concept, we prepared iron oxide nanoparticles [34] by modification of a literature procedure so that the particles retained an overall net negative charge (ζ -potential, -32 mV). XRD and EDX measurements confirmed Fe_3O_4 magnetite, and TEM analysis indicated particles with an average 5–10 nm diameter. The nanoparticles then were treated with excess (*ca.* 3.3 wt. equivalents) cationic aminooxy reagent **1** [36] (Scheme 1) to furnish the surface with an aminooxy layer. The aminooxy-coated nanoparticles (**ao-NP**) were readily isolated by magnet-assisted sedimentation and, using this method, were washed multiple times to remove any unassociated aminooxy compound. Dry weight measurements consistently indicated a deposition of **1** on the surface of **ao-NP** in the range of 1.27 – 1.30 μ mol/mg. The close association of **1** with the nanoparticles presumably is driven by a combination of hydrogen bonding and electrostatic interactions [37]. The relative extent of these associative interactions is unclear. While the observed reduction in zeta potential for **ao-NP** to -16 mV reflected a partial neutralization of surface charge by the ammonium ion of **1**, the availability of unbound aminooxy groups for condensation with carbonyl substrates was undetermined.

We were gratified to find that reaction of **ao-NP** with excess myristaldehyde (**2**, Scheme 1) resulted in smooth attachment of hydrophobic chains via oximation to give the corresponding magnetic lipid particles (**MLP**, Scheme 1). Multiple washings were performed to remove any uncondensed aldehyde, and this process delivered the **MLP** formulation having a significantly more positive zeta potential. Weight analyses indicate roughly one equivalent of myristaldehyde is captured during the oximation step, suggesting

that lipid **3**, or likely a mixture of lipids **3** and **4** (Figure 1), is bound at the **MLP** surface, possibly as a surrounding bilayer, as suggested by the shift in zeta potential.

With a route to positively charged, lipid-coated nanoparticles in hand, we next evaluated the transfection efficiency of **MLP** in MCF-7 breast cancer cells using a luciferase reporter plasmid (Figure 2). Cells were plated at 1×10^5 /well in a 24 well plate 24 hours prior to transfection in DMEM/10% FBS. MLP-magnetoplexes were prepared at various weight ratios by direct mixing with pDNA followed by application to cells in the presence of a static magnetic field. The data (Figure 2) indicate that **MLP** transfection efficiency is roughly equivalent or slightly higher than that of the transfection agent Lipofectamine 2000 (positive control) at magnetoplex weight ratios exceeding 120, while activity drops off rapidly at higher ratios. Uncomplexed pDNA (negative control, not depicted) did not show significant transfection (*ca.* 400–600 RLUs). Not unexpectedly, given the negative zeta potential of *ao*-NP, various combinations of *ao*-NP:pDNA also did not transfect (data not shown). Unfortunately, as with most magnetic nanoparticle delivery systems, removal of the magnetic field resulted in substantial loss of transfection activity for the **MLP**-magnetoplexes.

Encouraged by this first demonstration that a lipid coating elicits activity under magnetofection conditions, we sought to further enhance performance by increasing the hydrophobic component of **MLP**. Since additional myristaldehyde could not be attached by further reaction with **MLP**, we probed whether direct attachment of the dual-chain lipid **4** (Figure 1) to iron oxide nanoparticles (NP) could be accomplished. Reaction of excess **4** [37] with NP followed by our washing protocol delivered **dMLP**, magnetic lipid particles formed on direct exposure to the cationic oxime ether lipid. Lipid loading was measured at 1.25 – 1.35 $\mu\text{mol/mg}$. The TEM micrograph of **dMLP** (Figure 3) shows spherical shaped nanocrystalline particles around 5–10 nm in diameter

Evaluation of **dMLP** in the transfection of MCF-7 cells (Figure 4) revealed notable differences in comparison to transfections using **MLP**. Under magnetofection conditions (Figure 4A) and in the presence of serum, **dMLP**-magnetoplexes are considerably more active than **MLP**-magnetoplexes at all magnetoplex ratios and substantially more efficacious. The optimal **dMLP**-magnetoplex-120 exhibits more than three orders of magnitude higher transfection activity (*ca.* 1425-fold) than the transfection standard Lipofectamine 2000. More remarkable are the activities of **dMLP**-magnetoplexes in the absence of a magnet (Figure 4B). The **dMLP**-derived magnetoplexes are not dependent on magnet-assisted sedimentation for their activity, unlike **MLP**-magnetoplexes. At magnetoplex ratios between 90 and 540, the data indicate **dMLP** significantly promotes transfection relative to the positive control (e.g., magnetoplex-180 exhibits >40-fold higher activity than Lipofectamine 2000).

To ascertain if the observed activities of **dMLP** in Figure 4 are the consequence of any disassociated lipid **4** functioning independently as a transfection agent, we examined **4** at various lipid:DNA ratios and found it to be essentially inactive (highest activity observed ~1500 RLU at a 3:1 lipid:DNA charge ratio $\approx$ activity of uncomplexed DNA, negative control). Comparison of the measured zeta potentials for **MLP**- and **dMLP**-magnetoplexes

(Figure 5) indicates different plasmid-binding properties. In agreement with the observed transfection activities, formulation using **dMLP** achieves net positive magnetoplexes at lower particle concentrations. Furthermore, the use of **dMLP** results in a fairly uniform magnetoplex charge over a wide range of formulation ratios as reflected by the consistent transfection activities observed for the **dMLP**-magnetoplexes, particularly in the absence of a magnet.

A cell viability study using an alamar blue kit (Invitrogen, Carlsbad CA) was used to examine the effects of **dMLP**-magnetoplexes on cells. Figure 6 shows that the nano-particle: DNA formulations have little associated cytotoxicity, particularly in the lower formulation ratios (1:20), and only a modest effect on viability at higher ratios. The oxime ether-based **dMLP** magnetoplexes are less toxic to cells than is Lipofectamine 2000. Cell viability data demonstrate a significant advantage of the **dMLP** system in comparison to the magnetic PEI-based transfection systems. Oxime ether moieties, previously unreported as linkages for tethering polar and hydrophobic domains of transfection lipids, are commonly used in pharmaceutical agents [38].

4. Conclusion

We have developed a flexible oximation approach for attaching lipid sidechains onto iron oxide nanoparticles. Elaboration of the aminooxy-coated particles **ao-NP** described herein into a magnetofection-active vector avoids the use of PEI and requires only simple mixing with a hydrophobic aldehyde. Using an even more direct approach, we demonstrated that reacting an ammonium ion-based oxime ether lipid with iron oxide nanoparticles generated a highly active, non-cytotoxic, magnetic transfection vector. Notably, the resultant lipid-coated nanovector **dMLP** functioned in the presence of serum to transfect a representative cancer cell line without the assistance of a magnet. Given the emerging *in vivo* applications that aim to exploit magnetic properties of gene delivery vectors post transfection, the development of a magnetic vector that does not require a magnet for the transfection stage may enable applications previously thwarted by ineffective magnetofection. Under magnetofection conditions, **dMLP** was orders of magnitude more active in MCF-7 cells based on luciferase assay than an industry-standard transfection agent. The results of our study demonstrate that Fe_3O_4 nanoparticles can function as transfection enhancers for an otherwise transfection-inactive material.

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Fig. 1.
Cationic oxime ether lipids formed on reaction with myristaldehyde.

Fig. 2.

Magnetofection of MCF-7 cells using MLP-magnetoplexes in the presence of 10% FBS. Results are expressed as total relative light units (RLU). Transfections were performed on 24-well plates using 0.025 μ g luciferase reporter construct (pCMVLuc) per well with an 18h transfection time. Each data point reflects the mean value of three separate transfections and the standard deviation from the mean. LFT = Lipofectamine 2000.

Fig. 3. Transmission electron microscopy. Micrograph of dMLP particles; inset of a single particle shows its polycrystalline core nature.

Fig. 4. Magnetofection (**A**) and transfection (**B**, no magnet) of MCF-7 cells using dMLP-derived magnetoplexes in presence of 10% FBS. The results are expressed as total relative light units (RLU). Transfections were performed in 24-well tissue culture plates using 0.025 mg luciferase reporter construct (pCMVLuc) per well with 18 h transfection time. Each data point reflects the mean value of three separate transfections. Error bars show the standard deviation from the mean. LFT = Lipofectamine 2000.

Fig. 5.
Zeta potential measurements (H_2O) of **MLP**- (lt. bars) and **dMLP**- magnetoplexes (dk. bars) at various nanoparticle to pDNA ratios.

Fig. 6. Cell viability study of MCF-7 cells transfected with DNA alone (DNA), Lipofectamine 2000 (LFT) and dMPL-magnetoplexes at various dMPL:pDNA ratios. At 18h post transfection, alamarBlue® was added to cells and the fluorescence intensity (arbitrary fluorescent units) was measured at the onset (lt. bar) and then measured again at 4h post addition (dk. bar, n = 3).

Preparation of magnetic lipid-coated particles using an oximation approach. Surface charge (ζ -potential) measured in H₂O.