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Gene delivery into human cancer cells by cationic lipid-mediated magnetofection

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ABSTRACT

In this study, a combination of magnetic nanoparticles (MNPs) together with cationic lipid *N*,*N*-di-*n*-hexadecyl-*N*,*N*-dihydroxyethylammonium chloride formulated with colipid cholesterol, upon magnetofection, enhanced DNA uptake into human glioblastoma-astrocytoma, epithelial-like cell line U-87 MG, hepatocellular carcinoma Hep G2, cervical cancer HeLa and breast cancer MDA-MB-231 cells. Having confirmed this, we monitored uptake of plasmid DNA mediated by ternary magnetoplexes by fluorescence microscopy, flow cytometry and reporter gene expression assays in the presence and absence of a magnetic field. Our observations clearly indicate enhanced transfection efficiency *in vitro*, upon magnetofection, in the presence of serum as seen from β-Gal reporter gene expression. The observed activity in serum suggests the suitability of MNPs for *in vivo* applications. Further, we measured the transverse relaxation time (T_2) and obtained T_2-weighted MRI images of treated U-87 MG cells. T_2 determined for MNP-VP-Me_{22} and MNP-VP-Et_{22} corresponds to 22.6 ± 0.8 ms and 36.0 ± 2.1 ms, respectively, as compared to 47 ± 1.7 ms for control, suggesting their applicability in molecular imaging. Our results collectively highlight the potential of lipid-based approach to augment magnetic-field guided-gene delivery using MNPs and additionally towards developing intracellular molecular probes for magnetic resonance imaging.

1. Introduction

It is a widely established fact that synthetic non viral vectors are relatively safer than their viral counterparts (Pedroso de Lima et al., 2001) to deliver therapeutic nucleic acids into cells. Cationic lipids as non viral vectors are known to enhance nucleic acid delivery into cells (Pedroso de Lima et al., 2001; Guo and Huang, 2012) to achieve efficient expression of transduced genes for therapeutic benefit. Limited delivery of DNA to the nucleus, due to entrapment in endosomes and compartmentalization of the genetic material, precludes delivered DNA from gaining access to the transcriptional machinery leading to reduced transfection efficiency and hence the prime motivation towards developing carriers that are stable, efficient and minimally toxic. Efforts to develop non-viral carriers that package and protect therapeutic nucleic acids, in addition to cationic lipids, has been demonstrated through use of cationic polymers (Mindemark et al., 2012), peptides (Rajagopalan et al., 2007) and proteins (Jeyarajan et al., 2010) for *in vitro* and *in vivo* applications.

Several physical methods mediated by lipoplexes have been developed to enhance transfection efficiency (Kamimura et al., 2011). Nucleic acid vectors associated with magnetic particles have been developed as a novel strategy where a physical parameter such as the magnetic force is utilized to increase molecular contact of vectors with target cells and thereby transduce DNA for biomedical applications. Mykhaylyk et al. (2007a,b) developed '*Magnetofection*' protocols for viral and non viral gene delivery. The procedure entails association of nucleic acids with superparamagnetic nanoparticles followed by the application of the magnetic field which engender concentration on the cell surface (Scherer et al., 2002; Plank et al., 2003) resulting in significant enhanced uptake of DNA (Schillinger et al., 2005). Magnetofection rapidly concentrates therapeutic agents (*e.g.*, drugs and genetic materials) coupled with iron oxide nanoparticles to target cells or tissues to provide site-specific targeting (Dobson, 2006; Huth et al., 2004). Since the inception of this technology, magnetofection procedures have been constantly modified through the use of cationic entities (Kievit et al., 2010) and cationic lipid (Lee et al., 2011), further promoting rapid and enhanced nucleic acid delivery.

Cationic amphiphiles have been shown to augment uptake of nucleic acids mediated by magnetic nanoparticles (Mykhaylyk et al., 2010) to significantly enhance gene transfection. Recently Liu et al. (2011) developed dendrimer-based magnetoplexes to

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target cells, representing an example that efficiently delivers DNA into cells upon magnetofection. Conventional transfection protocols to deliver DNA involve prolonged incubation to allow molecular contact with the cell membrane to facilitate maximal uptake. In contrast, a brief period of magnetofection which in principle minimizes contact time between DNA and nucleases (Mykhaylyk et al., 2007a,b) underscores usage of magnetic nanoparticles (MNPs) as reagents particularly for rapid and effective theranostic purposes that involves simultaneous imaging and therapy. The present study deals with liposomal magnetofection using a versatile monocationic amphiphile *N*,*N*-di-*n*-hexadecyl-*N*,*N*-dihydroxyethylammonium chloride (DHDEAC) formulated with cholesterol (Chol). A bromide variant[1] DHDEAB, was earlier reported to efficiently transfect cells *in vitro* (Banerjee et al., 1999). In this study, ternary complexes prepared with DNA, MNP-VP-Et_{22} or MNP-VP-Me_{22} and DHDEAC:Chol when evaluated *in vitro* in the presence of a magnetic field, resulted in notable enhancements in transfection when tested in human glioblastoma-astrocytoma, epithelial-like cell line U-87 MG, hepatocellular carcinoma Hep G2 cells, cervical cancer cells, HeLa and human breast cancer cell line MDA-MB-231. Additionally, T_2 relaxivity studies of the two MNPs indicated that MNP-VP-Me_{22} is a promising candidate for generating T_2-based contrast and magnetic field-guided applications.

2. Materials and methods

2.1. Materials

DHDEAC was synthesized as described previously for the bromide variant (Banerjee et al., 1999). Cholesterol and 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified eagle's medium (DMEM) was from Invitrogen. All solvents used were of analytical grade. Plasmids $pEGFPN_3$ and pCMVβ-Gal obtained from laboratory stocks were amplified in *Escherichia coli* and purified using Nucleobond AX plasmid purification kits by Machery Nagel, Germany. Nucleic acid labelling kit (Oregon green) was obtained from Invitrogen. Lab-Tek chambered glass was from Nalge Nunc International (Rochester, NY, USA). PolyMag and Super Magnetic Plate MF-10000, 96-well format, were purchased from OZ Biosciences, France. DMSO, chlorpromazine, filipin, cytochalasin D, nystatin, MβCD, nocadazole, wortmannin and bafilomycin A_1 were obtained from Sigma–Aldrich. Alexa488 conjugated transferrin was obtained from Molecular Probes (Invitrogen, Carlsbad, USA), Rhodamine DHPE was obtained from (Avanti Polar Lipids, Inc., Alabaster, Alabama). Cholera toxin B (CT-B) Alexa Fluor® 555 conjugate was obtained from Molecular Probes, Invitrogen, Carlsbad, USA.

2.2. Preparation of magnetic nanoparticles

Magnetic nanoparticles (MNPs) were prepared according to a method previously reported by Hyeon et al. (2001). Briefly, 2.0 ml of Fe $(CO)_5$ was added to a mixture containing 100 ml of *n*-octyl ether and 12.8 g of oleic acid at 100 °C. The mixture was heated to reflux temperature and was stirred for 1 h. The resulting black solution was cooled to room temperature and 3.4 g of dehydrated $(CH_3)_3NO$ was added. The mixture was heated to 130 °C under a nitrogen atmosphere and stirred for 2 h. The reaction temperature was slowly increased to reflux temperature and continued for 1 h. The solution was cooled to room temperature, and ethanol was added to yield a black precipitate, which was then separated by centrifugation. The precipitate was washed with ethanol and collected by centrifugation. Elemental analysis indicated that the obtained MNPs slurry included 62 wt% of oleic acid. The obtained black powder was dried in vacuum. On the other hand, poly(4-vinyl pyridine) with reactive group at the one end was prepared by the modified telomerization method with mercaptopropyl trimethoxy silane as a telogen (Takafuji et al., 2005). The degree of polymerization of the obtained polymer determined by ^1H NMR was found to be 22 (VP_{22}). The obtained polymer was mixed with MNPs in toluene/methanol and refluxed for 96 h, following which nanoparticles were separated by centrifugation and washed with diethyl ether. The resultant particles (MNP-VP_{22}) were characterized by Fourier transform infrared (FT-IR) spectroscopy and elemental analysis. The pyridyl groups on MNP-VP_{22} were quaternized by alkyl halides with different alkyl chain length. Briefly, methyl iodide was added to the MNP-VP_{22} dispersion in methanol and stirred at reflux temperature for 48 h. The resultant particles (MNP-VP-R_{22}, R = Me (methyl) and Et (ethyl)), were centrifuged and washed with diethyl ether three times.

2.3. Fourier transform infrared spectroscopy (FT-IR), size and zeta potential

MNP-VP_{22} and MNP-VPR_{22} were characterized by diffuse reflectance FT-IR spectroscopy (FT/IR-4100, JASCO Co., Japan). The size and the surface charge (zeta-potential) of MNP-VPR_{22} in deionized water, binary and ternary complexes containing MNP-VP-Me_{22} and MNP-VP-Et_{22} were evaluated by using a Zetasizer Nano-ZS (MALVERN Instruments Ltd., USA). The mixtures were diluted in HEPES buffered saline, pH 7.6.

2.4. Cell culture, uptake of plasmid DNA

Adherent human glioblastoma-astrocytoma, epithelial-like cell line U-87 MG, hepatocellular carcinoma Hep G2 and human breast cancer cell line MDA-MB-231were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% foetal bovine serum (Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, and kanamycin (sigma). Chinese hamster ovary (CHO), cervical carcinoma cell line HeLa and human ovarian cancer cell line SK-OV-3 was also grown similarly and maintained. All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

The cellular uptake of plasmid pCMVβ-Gal DNA was monitored using fluorescently labelled DNA (Oregon Green) and assessed by fluorescence confocal microscopy and flow cytometry. Cells were seeded in two-chambered coverglass/6 well plate and incubated overnight. The following day, binary or ternary complexes were prepared and added to cells and further incubated for 30 min under the magnetic field at 37 °C at 5% CO_2. The cells that were not magnetized served as controls. Culture medium was washed with 1× PBS and visualized using LSM510 META NLO confocal microscopy and analyzed using LSM 5 Image Examiner.

2.5. Flow cytometry

Quantitative cell uptake was examined by transfecting U-87 MG and Hep G2 cells with ternary complexes composed of DNA, MNP-VP-Et_{22} or MNP-VP-Me_{22} and cationic lipid DHDEAC:Chol at 1:1:1 charge ratios. Cells were incubated with the prepared magnetoplexes for 30 min at 37 °C and 5% CO_2 in the presence of magnetic field. Cells were then trypsinized, washed and resuspended in 1× PBS and subjected to flow cytometry and 30,000 events were counted using FACS Calibur. Cell populations represented by histograms, of treated and untreated cells were analyzed using BD CellQuest Pro software. Populations of cells represented by

[1] US Patent # 6,333,433B1.