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A general strategy to achieve ultra-high gene transfection efficiency using lipid-nanoparticle composites

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ABSTRACT

Gene therapy provides a new hope for previously "incurable" diseases. Low gene transfection efficiency, however, is the bottle-neck to the success of gene therapy. It is very challenging to develop non-viral nanocarriers to achieve ultra-high gene transfection efficiencies. Herein, we report a novel design of "tight binding-but-detachable" lipid-nanoparticle composite to achieve ultrahigh gene transfection efficiencies of 60~82%, approaching the best value (~90%) obtained using viral vectors. We show that Fe@CNPs nanoparticles coated with LP-2000 lipid molecules can be used as gene carriers to achieve ultra-high (60–80%) gene transfection efficiencies in HeLa, U-87MG, and TRAMP-C1 cells. In contrast, Fe@CNPs having surface-covalently bound N,N,N-trimethyl-N-2-methacryloxyethyl ammonium chloride (TMAEA) oligomers can only achieve low (23–28%) gene transfection efficiencies. Similarly ultrahigh gene transfection/expression was also observed in zebrafish model using lipid-coated Fe@CNPs as gene carriers. Evidences for tight binding and detachability of DNA from lipid-nanoparticle nanocarriers will be presented.

1. Introduction

Among various therapeutic approaches, gene therapy is a very promising method for the treatment of many diseases that are previously considered incurable, such as, Parkinson disease, cystic fibrosis, as well as various kinds of cancers [1–4]. Most of the biomedical investigations and clinical treatments involving gene therapy are of limited success owing to their poor cellular uptake and limited gene transfection efficiencies. Hence, an effective delivery system is vital to successful gene delivery/therapy [5–7]. To this end, viral (e.g., adenovirus and retrovirus) and non-viral (e.g., polymers, nanoparticles and liposomes) vectors have been developed. Viral vectors provide very efficient gene delivery and transfection efficiencies (~90%). However, viral vectors have many serious drawbacks, including, immunogenicity, carcinogenicity, inflammation, limited DNA carrying capacity, difficult to prepare in large scale, and high cost [7,41]. Non-viral vectors are much safer than viral vectors, and have the advantages of simple/easy preparation, absence or negligible specific immune-response. However, the gene transfection efficiencies of non-viral vectors are far lower than viral vectors in general. Among non-viral vectors, liposomes, cationic polymers, such as poly (ethyleneimine) (PEI), poly (amidoamine) (PAMAM) based dendrimers were widely used as gene delivery vectors. For example, the PAMAM based polypeptide dendrimers can achieve ~33% GFP gene transfection efficiency in HeLa cells [8]. In the case of bone marrow cells, the gene transfection efficiencies are 20% and 35% for PEI and PAMAM nanocarriers, respectively [9]. The causes responsible for low gene transfection efficiencies (20~35%) in non-viral vector systems are still not clear, albeit, most of non-viral vectors are able to bind with DNAs very effectively. Recently, many new nanomaterials such as, carbon nanotubes [10–12], graphene [13], silica nanoparticles [14], quantum dots [15], gold nanoparticles [16], nanorods [17], etc. were reported to act as DNA cargoes for gene therapy applications. However, these nanomaterial-based non-viral vectors suffer from many problems,

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such as, poor cellular uptake, inefficient gene release, high cytotoxicity and susceptible to intracellular degradation of foreign genes which results in poor transfection efficiencies. Although a variety of nanomaterials have been reported in the literature as nanocarriers for gene delivery and gene therapy with a wide range of gene transfection efficiencies, it remains very challenging to design a nanocarrier system to achieve ultra-high gene transfection efficiencies, similar to that obtained by viral vectors. The gene transfection efficiencies achieved upon using various non-viral vectors reported in the literature were summarized in Table 1. In general, many factors could possibly affect the gene transfection efficiencies, including, (a) high binding affinity between the nano-cargo and DNA polyplex, (b) high intracellular uptake of the DNA-nanocargoes [18], (c) efficient endosomal/lysosomal degradation of foreign DNA [19], and (d) poor DNA release from nanocarriers into the nucleus [18]. The factors (a) and (b) are favorable towards high gene transfection efficiency, but (c) and (d) are negative factors. Very high binding affinity between cationic nanocarriers and polyanionic DNA will favor carrying large quantity of genes, but it is un-favorable for efficient release of the gene being delivered [18]. Most of non-viral vectors reported in the literature to date emphasize efficient gene delivery via increasing the positive charges on nanocarriers), but still suffer from poor transfection efficiencies, presumably due to very poor gene release. If a nanocarrier can achieve both high gene binding affinity and high gene release efficiency simultaneously, high gene transfection efficiencies will be possible. To this end, we report a generalized approach in this paper to easily achieve ultra-high transfection efficiencies via a design of a nanocarrier system having tight binding affinity towards poly-anionic DNAs, but the neutral "DNA-lipid" complex is still detachable from the surface of nanocarriers.

Overall, we develop to prepare "tight binding-but-detachable" lipid-nanoparticle composites to achieve ultra-high gene transfection efficiencies in three different cell lines and as well as *in vivo* zebrafish model. Using our strategy, designing nanocarriers for efficient gene delivery/release for gene therapy treatments of various kinds of diseases can be easily explored in the clinical biomedicine.

2. Materials and methods

2.1. Synthesis of core/shell iron/carbon nanoparticles using solid state microwave arcing:

The magnetic core/shell iron encapsulated carbon nanoparticles were prepared by following the literature procedure [20–23]. In brief, a $C_{60/70}$ and ferrocene (1:1 wt ratio) powder mixture together with small pieces of silicon ($1 \times 1 \times 1 \sim 2 \times 2 \times 1$ mm^3 from a broken silicon wafer) was irradiated with microwave inside a focused microwave oven (2.45 GHz, Discover system, CEM Corporation, USA) under an argon atmosphere (1 atm) for 15 s. The microwave irradiation process was repeated twice to have more completely carbonization of the carbon-containing powder. Finally, the magnetic products were collected using an external magnet, and structure characterization was performed by using a transmission electron microscope (TEM, JEOL, JEM-2100F, 200 kV).

Table 1
Comparison of gene transfection efficiencies achieved using various non-viral vectors reported in the literature to that of "tight binding but detachable" lipid-Fe@CNPs.

Non-viral vector	Cell line	% Gene transfection efficiency	Reference
Dendrimer	Bone marrow cells	20	[8]
Poly (amidoamine)	Bone marrow cells	35	[9]
LP-2000	HeLa	27	[12]
Carbon nanotubes	HeLa	38	[12]
GO-PEI	HeLa	13	[13]
PEI-Silica nanoparticles	COS-7	44	[14]
Quantum dots	A549	40	[15]
Gold nanoparticles	Eukaryotic cells	25	[16]
Gold nanorods	HeLa cells	51	[17]
Gemini cationic lipids	HeLa cells	60	[45]
Lipid-Fe@CNPs	HeLa cells	78	Present study
Lipid-Fe@CNPs	U87-MG cells	80	Present study
Lipid-Fe@CNPs	TRAMP-C1	68	Present study

2.2. Surface functionalization of iron-encapsulated carbon nanoparticle (Fe@CNPs) [24,25]

To introduce fluorescent properties to Fe@CNPs, we modified the surface of the magnetic carbon soot. To the toluene solution (7 mL) containing magnetic carbon soot (50 mg) was added to the styrene monomer (1 mL, Aldrich) and benzoyl peroxide (BPO, 0.25 mL, 0.4 M) followed by ultrasonication (20 min) to make the carbon soot become well dispersed. The solution was transferred immediately to a domestic microwave oven (2.45 GHz, 600 W) and exposed to microwave irradiation for 10 s. During microwave irradiation the solution temperature rises rapidly, causing decomposition of the BPO radical initiator and initiation of polymerization. This addition of styrene-BPO-microwave irradiation process was repeated twice. The third time an additional component, namely, methacryloxy thiocarbomyl Rhodamine-B (MATCR, 10 mg in 1 mL THF), was added and the solution was sonicated and irradiated with microwaves under the same conditions. The fluorescent moiety, i.e., MATCR, was added to the Fe@CNPs-containing solution at a subsequent stage of surface grafting to avoid close contact with the graphene shells as photo-excited fluorescent moiety might be electronically quenched by the graphene shells on the Fe@CNPs surface. Finally, the surface-functionalized Fe@CNPs (designated as Fe@CNPs-PS-PMATCR) were collected and separated from free unbound polymers on repeated washing with THF and toluene and centrifugation at 12,000 rpm.

2.3. Synthesis of lipid-folate conjugates [25]:

In a typical experiment, 1:1 equivalents of 4-phenyl butyl amine and folic acid (Aldrich) were mixed with 1.1 equivalents of N,N'-Dicyclohexylcarbodiimide (DCC, Fluka) and dissolved in 15 mL dichloromethane (DCM) and stirred at room temperature for 24 h under nitrogen atmosphere. The resulting mixture was poured into a 5 wt% brine solution and undergone layer separation. The organic layer was washed and extracted for several times and final product was obtained by rotary evaporation of the residual solvent.

2.4. Preparation of lipid-Fe@CNPs

In empty round bottom flask, 50 μL of LP-2000 (Invitrogen, USA) and 50 μL of 5 wt% lipid-folate were spread over the bottom surface area and diluted with 300 μL of distilled water. To this solution, 1 mg of Fe@CNPs-PS-PMATCR was added and ultrasonicated for 5–10 min to get a homogenous dispersion. Then the mixture was further diluted with 600 μL distilled water to make up a volume until 1 mL and ultrasonicated again for another 5 min. Finally the lipid-to-nanoparticle weight ratio was maintained as 1:10. For the sake of clarity, the lipid-Fe@CNPs-PS-PMATCR will be designated as lipid-Fe@CNPs.

2.5. Preparation of DNA-lipid-Fe@CNPs and DNA-Fe@CNPs-TMAEA complexes

Different concentrations of DNA plasmids were diluted with PBS buffer and 10 μg of lipid-Fe@CNPs or Fe@CNPs-TMAEA were added and vortexed for 10 s and then incubated at 37 °C for 30 min to get stabilized. Then these complexes were further subjected to the cell line for the transfection assay experiments.

2.6. Surface modification of Fe@CNPs with N, N, N-trimethyl–N-(2-methacryloxyethyl) ammonium chloride

In a typical experiment, Fe@CNPs (50 mg) was suspended in an aqueous solution (8 mL) containing N,N,N-trimethyl–N-(2-methacryloxyethyl) ammonium chloride (TMAEA) (1 mL) monomer. The solution was then ultrasonicated in a bath-type ultrasonicator for 2 min to help disperse the core/shell iron/carbon nanoparticles. A tetrahydrofuran (THF) solution (0.25 mL) containing benzoyl peroxide (22.5 mg) was added to the solution, followed by ultrasonication for an additional 10 min. This process was repeated for 4 or 5 times with a total amount of 90–120 mg benzoyl peroxide added. The final solution was then diluted with deionized water, filtered through a nylon 66 (0.45 μm) membrane, and washed with deionized water several times to thoroughly remove free, unbound polymers [22].

2.7. In vitro magnetic resonance imaging (MRI) assays

MR images used in this study were acquired in a 7T MR imager (BioSpec 70/30 USR; Bruker). For T_2 measurements, lipid-Fe@CNPs and commercial Resovist agent (MagQu, Taiwan) with various concentrations of iron were dispersed in 0.5% agarose gel solution. The acquired MRIs and T_2 values were obtained using multislice multiecho sequence (MSME) with the following parameters, TR = 6000 ms; TE = 11~110 ms; matrix size = 256 × 256; field of view (FOV) = 60 × 60 mm^2; slice thickness = 6 mm. The total volume of each MRI phantom was 0.2 mL. Only 0.5% agarose gel solution was used as a control.