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High performance mRNA transfection through carbonate apatite–cationic liposome conjugates

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ABSTRACT

mRNA instead of DNA provides a new and attractive approach for gene therapy and genetic vaccination. Delivery of mRNA can bypass nuclear localization step enabling protein expression directly in cytoplasm through transcription. Current technologies for mRNA delivery are predominantly based on cationic liposomes with low activity for transfection. We, previously reported that applying inorganic nano-particles of carbonate apatite onto cationic liposome of DOTAP {*N*-[1-(2,3-dioleoloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium chloride} resulted in high transfection potency for luciferase mRNA both in mitotic and non-mitotic cells. In this paper, we expanded the previous work and performed in detail study especially on two important parts, evaluating the image of the complex and analyzing the steps of gene delivery to detect the determinant factor for enhanced transfection potency. Transmission electron microscopic (TEM) observation clearly indicated the presence of inorganic carbonate apatite particles on mRNA–liposome complex and demonstrated the structure of the new hybrid carrier material. Due to apparently higher gravitational force of absorbed inorganic nano-particles, cellular contact and internalization of hybrid-particle-associated mRNA were significantly enhanced compared to DOTAP. This analysis indicates rather than downstream steps, initial steps of cell membrane binding and subsequent way of internalization could be the determinant factor for final protein expression. Moreover, we compared transfection efficiency of mRNA and pDNA in Human Umbilical Vein Endothelial cell (HUVEC) to demonstrate advantages of mRNA delivery.

1. Introduction

mRNA transfection is a very rare occurrence in the field of gene delivery or genetic vaccination. It is known that mRNA has some therapeutic potential over DNA if we consider the real state of the majority of cells *in vivo* which are mostly non-dividing or slowly dividing. The employment of mRNA as a gene delivery tool is a very attractive way to treat those cells where DNA based gene expression is low due to the absence of cell cycle-dependent breakdown of the nuclear envelope [1,2]. mRNA-based expression of certain proteins sustains for a limited time but such transient expression is desirable in some clinical disorders [3,4]. mRNA transfection strategy is also applicable in the field of cancer vaccination or immunotherapy as the preclinical study on DC transfected with autologous tumor mRNA has shown feasibility for vaccination [5,6]. Moreover, mRNA-based gene transfer is now recognized as a versatile non-viral transfection tool for *ex vivo* modification of target cells (cell therapy). To date, this technology has already found its way to the clinic in the field of cancer immunotherapy, but there is no doubt that RNA transfer will find more clinical applications, such as in the stem cell area, as a way to change the cellular behavior of stem cells for the purpose of tissue engineering or regenerative medicine. Thus, some clues to develop more efficient mRNA delivery system are highly expected for diversified applications. So far most published reports on mRNA delivery are based on cationic lipids with DOTAP showing the highest activity among them [7–9]. We previously showed inorganic carbonate apatite accelerated DOTAP mediated delivered mRNA expression [10]. We, here report on the characterization of the component particles of the cationic liposome–apatite complex evaluating the existence of its hybrid nature. Additionally, analysis of the gene delivery steps has been performed to show the reason of high mRNA transfection potency. Our work represents two strategies for the development of an improved gene transfection methodology, first one is understanding of the importance for hybrid carrier in gene delivery system i.e., surface modification of the carrier, another is to improve initial gene delivery steps to get final high expression profile. Moreover, transfection efficiency of mRNA and pDNA in

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Human Umbilical Vein Endothelial cell (HUVEC) was investigated to demonstrate advantages of mRNA delivery over DNA.

2. Materials and methods

2.1. Cell culture

HeLa cell line was cultured in 25-cm^2 flasks in DMEM supplemented with 10% fetal bovine serum (FBS), 50 μg penicillin/ml, 50 μg streptomycin/ml and 100 g neomycin/ml at 37 °C in a humidified 5% CO_2-containing atmosphere. HUVEC line (Lonza, USA) was cultured in the recommended EGM®-2 bulletkit® endothelial growth medium (Lonza, USA) in a humidified 5% CO_2-containing atmosphere and subcultured using reagent pack™ subculture reagent (Lonza, USA). Cells were seeded in a 24-well tissue culture plate on the day before transfection.

2.2. Synthesis of mRNA transcript

mRNA encoding luciferase was prepared by in vitro transcription of the luciferase SP6 control DNA having a poly (A) tail (A30) (Promega) using an SP6 RiboMAX ™ kit as described by the manufacturer (Promega) with or without m7G(5′)pppG(5′) cap analog (Ambion) or anti-reverse cap analog, ARCA (Ambion) at a ratio of 5:1 cap/GTP. When the cap analog was omitted, the GTP concentration was raised accordingly to 5 mM. In vitro transcribed mRNAs (with or without cap) were characterized by gel electrophoresis and were all ~1800 bp in length.

Plasmids, pGL3 (Promega) containing a luciferase gene under SV40 promoter was propagated in the bacterial strain XL-1 Blue and purified by QIAGEN plasmid kits.

2.3. Liposome formation

For the preparation of liposome, solid DOTAP {*N*-[1-(2,3-dioleoloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium chloride; Sigma} was initially dried from chloroform, subsequently dispersed in dH_2O and shaken at a temperature above the gel-to-liquid-crystalline transition temperature of the lipid (~0 °C) for 15 min. followed by sonication of the milky solution for 10 min.

2.4. Complex formation for transfection

Cells from the exponential growth phase were seeded at 100,000 cells per well into 24 well plates to attain 80% confluency on the day before transfection. For mRNA transfection, 2 types of complexes were prepared. mRNA–DOTAP and mRNA–DOTAP–apatite. For mRNA–DOTAP complex formation, 3 μg of heat-denatured mRNA (10 min at 65 °C) and 6–10 μg of DOTAP were diluted separately in 100 μl DMEM media (pH 7.5). After 15 min, DOTAP was added to the RNA solution, followed by incubation for 40 min at room temperature as recommended by the manufacturer. Later, final volume was adjusted to 1 ml by DMEM (pH 7.5). mRNA was complexed with an optimized concentration (5 mg/ml) of lipofectamine 2000 and transfection was carried out according to the manufacturer's protocol. pDNA–DOTAP and pDNA–DOTAP–apatite complexes were formed in the same manner using 3 μg pDNA and 6 μg of DOTAP.

In case of co-transfection with polyadenylic acid {poly (A)} [Sigma], mRNA was mixed with or without poly (A) at a concentration of 20 μg/ml. After 15 min, DOTAP was added to the RNA solution containing poly (A) solution, followed by incubation for 40 min at room temperature as recommended by the manufacturer. Later, final volume was adjusted to 1 ml by DMEM (pH 7.5).

For preparation of mRNA–DOTAP–apatite complex, at first mRNA–DOTAP solution was prepared like before and incubated for 15 min at room temperature. After adjusting final volume with DMEM to 1 ml, 4 μl of 1 M $CaCl_2$ was added to reach 4 mM $CaCl_2$ in 1 ml solution and then incubated for 30 min at 37 °C. For fluorescein-labeled mRNA same procedure was followed to form all of the complexes as described above. pDNA–DOTAP and pDNA–DOTAP–apatite complexes were formed in the same manner using 3 μg pDNA and 6 μg of DOTAP. The solution containing each type of transfection complexes was added with 10% FBS to the rinsed cells. Treated cells were incubated at 37 °C in a 5% CO_2 humidified environment for 2–4 h. After incubation, the transfection mix was discarded and 1 ml of fresh serum medium was added to the cells. Cells were cultured for 6–12 h for mRNA and 24 h for DNA before analysis of reporter gene expression. The transfected cells were lysed by a lysis buffer (Promega) and the luciferase activity was measured using the luciferase assay system by a luminometer (TD-20/20 Luminometer, USA). Transfection efficiency was measured as mean light units per milligram of cell protein.

2.5. Dynamic light scattering studies

The size and surface charge (zeta potential) of all the complexes were measured by using a Light Scattering Apparatus, ELS 200TI (Otsuka Electronics, Japan).

2.6. High voltage transmission electron microscopy

4 μl of 1 M $CaCl_2$ was mixed with just 1 ml of fresh serum-free HCO_3^- buffered [pH 7.4] medium (DMEM) and incubated for 30 min at 37 °C for complete generation carbonate apatite particles. DOTAP liposomes, mRNA–DOTAP, and mRNA–DOTAP–apatite complexes were prepared as described earlier. 5 μl of each of the samples was placed on a copper micro-grid (Nissin EM, Japan), the grid was dried and transmission electron microscopic (TEM) observation (Hitachi H-1250 S) was performed using the dried micro-grid using 1000 kV acceleration voltage. Images were recorded directly in electron microscopic films (Fuji Photo Film Co., Ltd., Japan). Films were then developed and analyzed.

2.7. Labeling of mRNA

Luciferase mRNA has been labeled using fluorescein RNA labeling mix (Roche, Germany) which contains fliuorescein-12-UTP through in vitro transcription of luciferase control DNA. Labeling was performed according to the manufacturer's protocol.

2.8. Inhibition study

To examine the mechanism of Luc-mRNA internalization through DOTAP–apatite carrier, cells were incubated in the absence or presence of different types of endocytosis inhibitors, cytochalasin B (25 mg/ml concentration) for 30 min, amiloride (5 mM) for 10 min, or filipin (1 μg/ml) for 1 h. Cells were washed in serum-free medium and incubated with the complexes for 3 h. The uptake of fluorescein-labeled mRNA through different carriers in HeLa cell was examined by fluorescence microscope. Luc-mRNA expression was measured at 6 h post-transfection. Luciferase activity was measured as RLU/mg of protein.

2.9. Confocal microscopy

The cells were grown on presterilized coverslips inside a petridish filled with the appropriate culture medium. After reaching the desired confluency the cells were transfected with fluorescein-lebeled mRNA. After certain periods (1 h), acidic compartments like endosomes were labeled with Lysotracker red DND-99 (diluted to 50–75 nM in PBS). For labeling, the medium containing transfection mix was removed and the pre-warmed (37 °C) probe containing medium was added and incubated for 30 min at 37 °C. After removing the probe solution, fresh serum-free DMEM was added and incubated again at 37 °C for distaining. Then after fixing the labeled cell using formalin, the cells were observed under confocal microscope.

2.10. Flow cytometry

Flow cytometry analysis of labeled mRNA transfected HeLa cell has been performed. Cells (both transfected and untransfected) were treated by trypsin–EDTA and after detachment of the adherent cells, centrifugation has been performed. Discarding supernatant, the cell pellet was resuspended in 1 ml serum-free DMEM media and used for flow cytometry. The intensity of fluorescein-labeled mRNA in HeLa cell was determined by gating cells at an excitation wavelength of 488 nm using an argon ion laser. The presence of the labeled mRNA was detected by emission at a wavelength of 525 nm.

3. Results and discussion

3.1. Physico-chemical characterization of the particles

Since lipoplex structure, size and the surface charge are the vital determinants of the carrier abilities to bind and deliver mRNA into the cells, we separately analyzed these parameters for all the components of the carrier complex using TEM which is a High Voltage Electron Microscope (HVEM) and Dynamic Light Scattering (DLS) apparatus.

Cationic liposome particles of DOTAP were detected by TEM, maintained the size range of 200–400 nm (Fig. 1A and B). When mRNA was complexed with DOTAP and analyzed under TEM, a bit aggregated particle was observed (Fig. 1C). It is already known that with the addition of negatively charged nucleic acid into liposome solution large chain like structures emerge and we might imagine the same from Fig. 1C. Carbonate apatite particles (50–200 nm in size), characterized before at our laboratory [11] were also observed under TEM and maintained the same size range (Fig. 1D). The inorganic nature of the carbonate apatite particles was confirmed from the diffraction coming from them (diffraction pattern not shown). Carbonate apatite particles were applied to the already