

Surface Functionalized Multicomponent Envelope Type Nanosystems for Improved Gene Delivery

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ABSTRACT

Multicomponent envelope-type lipid nanosystem (MENS) is an emerging nanoparticle technology. These nanoparticles were assembled according to a recently proposed ‘programmed packaging concept’ in which various devices that control intracellular trafficking are packed into a single multicomponent envelope-type nanoparticle system. The uptake ability and final intracellular fate of MENS formulations was evaluated by means of confocal laser scanning microscopy. Our results revealed that MENS show a high cellular internalization and that are not specifically targeted to metabolic degradation. These studies will contribute to rationally design novel delivery systems with superior transfection efficiency.

Keywords: lipid nanoparticles, multicomponent, anionic liposomes, confocal laser scanning microscopy, colocalization.

1 INTRODUCTION

Non-viral delivery systems have been developed to facilitate gene entry into mammalian cells [1]. Very early steps in the transfection process involve binding of the vector to the cell surface and its internalization via multiple mechanisms. Once in the cell, plasmid DNA must be able to escape endosomal trafficking. If DNA is not released from endosomes, it is shuttled to the lysosomes, where it is degraded by the abundant nucleases leading to poor transfection. An effective nanocarrier should therefore integrate functional tools with specific function to be exerted at the correct time and place. Due to electrostatic issues, lipid gene vectors are usually made of cationic lipids. However, cationic lipids have often been shown to stimulate significant cytokine production, leading to reduced reporter gene expression and systemic toxicity. It is possible that by avoiding the use of cationic lipids, the potential toxicity of formulations can be avoided.

Recently, a new programmed packaging strategy in which various “soft devices” that control intracellular trafficking are incorporated into single nanoparticles has been proposed [2]. Here we apply such a novel concept to develop multicomponent envelope-type nanosystems (MENS) for overcoming intracellular membrane barriers. MENS was made of a plasmid DNA core condensed with protamine sulfate. The compacted nature of protamine sulfate/DNA complex (P/DNA) is supposed to confer some resistance to nucleases [3]. Such a core is coated with highly-fusogenic multicomponent (M) anionic liposomes that have intrinsic endosomal rupture properties [4-6]. Finally MENS was then surface-functionalized with poly-L-arginine. Inside the cell, the carrier escapes from the endosome into the cytosol in a process mediated by the “proton sponge” effect. pH-sensitive polymer envelope as well as the outer endosome-fusogenic lipid membrane resulted in efficient endosomal escape. The encapsulated DNA cargo is delivered to the interior of the nucleus through successive fusions of the lipid inner nucleus-fusogenic membrane with the two membranes of the nucleus. During stepwise fusion, the DNA/polycation core is released from the lipid envelope, enabling efficient transcription.

Confocal laser scanning microscopy (CLSM) experiments show that MENS formulations present high cellular internalization and that are not specifically targeted to metabolic degradation indicating that MENS are promising non-viral delivery candidate.

2 MATERIALS AND METHODS

2.1 MENS Preparation

Anionic dioleoylphosphatidylglycerol (DOPG) and dioleoylphosphatidic acid (DOPA), and neutral dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Multicomponent (M) small unilamellar vesicles (SUVs) incorporating DOPG, DOPA, DOPE, and DOPC within the

lipid bilayer were prepared according to routine procedures (total lipid concentration 1 mg/ml) [7]. Double-stranded calf-thymus (CT) DNA, purchased from Sigma (St. Louis, MO) and used without further purification, was dissolved in water (1 mg/ml). For confocal fluorescence microscopy experiments, Cy3-labeled 2.7-kbp plasmid DNA (Mirus Bio Corporation, Madison, WI) was used. Protamine sulfate salt (P) from salmon (MW = 5.1 kDa) and poly-L-arginine (A) (MW = 5–15 kDa) were both purchased from Sigma. Positively charged P/DNA microspheres were prepared. Positive P/DNA particles were mixed with negative multicomponent SUV dispersions. After 24 h equilibration, finally, MENS were prepared by mixing P/DNA multicomponent lipoparticles with a poly-L-arginine containing dispersion (1 mg/ml). Preparation of surface functionalized MENS is illustrated in Figure 1.

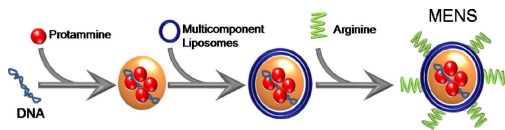


Figure 1. Preparation of MENS (adapted from ref. [8]).

2.2 Size and Z-Potential

Size distribution of MENS were measured at 25 °C by a Malvern NanoZetaSizer spectrometer equipped with a 5 mW HeNe laser (wavelength $\lambda = 632.8$ nm) and a digital logarithmic correlator. The normalized intensity autocorrelation functions were detected at 90° and analyzed by using the CONTIN method, which analyzes the autocorrelation function through an inverse Laplace transform [9,10] in order to obtain the distribution of the diffusion coefficient D of the particles. This coefficient is converted into an effective hydrodynamic radius R_H by using the Stokes-Einstein relationship:

$$R_H = \frac{K_B T}{6\pi\eta D} \quad (1)$$

where $K_B T$ is the thermal energy and η the solvent viscosity. Our clusters invariably show a size distribution, and the values of the radii reported here correspond to the so-called “intensity weighted” average [11]. The electrophoretic mobility measurements were carried out by means of the laser Doppler electrophoresis technique, the same apparatus used for size measurements. The mobility u was converted into the Z-potential using the Smoluchowski relation:

$$Z = \frac{u\eta}{\varepsilon} \quad (2)$$

where η and ε are the viscosity and the permittivity of the solvent phase, respectively.

2.3 Cell Culture, Transfection and Colocalization Assays

CHO-K1 cells were purchased from American Type Culture Collection (CCL-61 ATCC) and were grown in Ham’s F12K medium supplemented with 10% of Fetal Bovine Serum at 37°C and in 5% CO₂. For transfection experiments, MENS were prepared in PBS (Invitrogen). The complexes were left for 20 min at room temperature before adding them to the cells. We performed colocalization assays in living cells. To evaluate the ultimate intracellular fate of MENS, CHO-K1 cells were cocultured with MENS and 50 mM Lysosensor for 30 min to label lysosomes. Colocalization of green and red signals was analyzed by means of the Manders coefficients using the *Intensity Correlation Analysis* plugin of the ImageJ software (NIH Image; <http://rsbweb.nih.gov/ij/>) [12].

2.4 CLSM Experiments

CLSM experiments were performed with the Olympus Fluoview 1000 (Olympus, Melville, NY) confocal microscope interfaced with a 405 nm diode laser and 543 nm Helium-Neon laser. Glass bottom Petri dishes containing transfected cells were mounted in a temperature-controlled chamber at 37 °C and 5% CO₂ and viewed with a 60X 1.25 numerical aperture water immersion objective. The following collection ranges were adopted: 555-655 nm (Cy3) and 460-530 (Lysosensor). Images were collected in sequential mode to eliminate emission cross talk between the various dyes.

3 RESULTS AND DISCUSSION

3.1 Size and Z-potential

P/DNA complex formation was investigated by measuring the average size, D , and the electrophoretic mobility of the diffusing complexes in the solution. The combined use of these two techniques allowed us to study both of the two typical phenomena occurring in these systems, that is, the *reentrant condensation* and the *charge inversion* effect [13]. P/DNA complex at weight ratio $R_w=1.5$ was chosen because it exhibited positive charge (20 mV) and appropriate dimensions (~240 nm) with the minimum P content. The pre-assembled P/DNA core was coated by a lipid envelope through membrane fusion of M (-55.2 mV, 122.4 nm) that have intrinsic endosomal rupture. The complex at a volume ratio $R_{vol}=10$ was finally chosen because it exhibited negative charge (-52.1 mV) as well as the lowest colloidal dimensions (251.8 nm) and the lowest polydispersity (pdi=0.22). Finally P/DNA/M complexes at $R_{vol}=10$, were surface-functionalized with poly-L-arginine, (Figure 2). Complexes at a ratio $R_{vol}=0.5$ resulted in the formation of stable positively charged MENS aggregates whose size (~180 nm) and Z-potential (~50 mV) remained stable over 24 h.

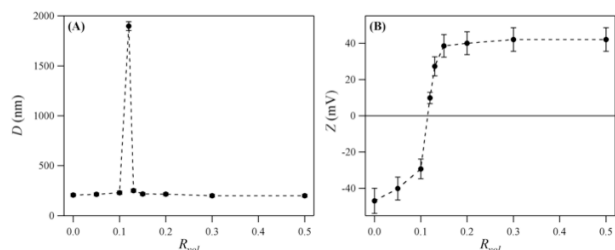


Figure 2. (A) Diameter and (B) Zeta-potential of MENS as function of the volume ratio, R_{vol} (P/DNA/M dispersion volume per poly-L-arginine dispersion volume).

3.2 Cellular Uptake and Intracellular Final Fate

Figure 3 shows CHO-K1 cells after 3 h (panel A), 6 h (panel B) and 24 h (panel C) of treatment with MENS. It is clearly revealed that 3 h is enough to observe the fluorescent signal of MENS inside the cell. After 3 h of treatment, MENS were mainly distributed at the cell periphery, while at 24 h complexes largely overrun the cytoplasm.

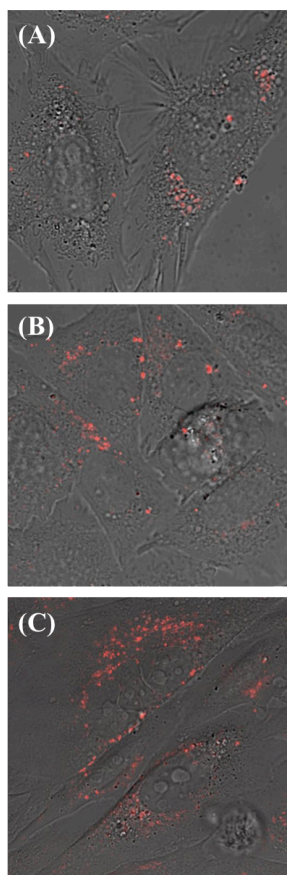


Figure 3. Confocal microscopy of CHO-K1 cells 3 h (panel A), 6 h (panel B) and 24 h (panel C) after treatment with MENS that contained Cy3-labeled plasmid DNA (red).

The ability of MENS to penetrate cells make them ideal candidates for nanoparticle delivery. However, once in the cell, DNA must be able to escape endosomal trafficking. If DNA is not released from endosomes, it is shuttled to the lysosomes, where it is degraded by the abundant nucleases leading to poor transfection.

The ultimate intracellular fate of MENS was investigated by CLSM. To this end, we used Lysosensor, which accumulates in acidic cell organelles, and is primarily a lysosome marker (Figure 4). Thus, the DNA signal colocalized with Lysosensor signal (yellow clusters) was interpreted as DNA in lysosomes. After 24 h of incubation with MENS, minor colocalization of red and green signals was observed, together with detectable MENS escape from lysosomes, as testified by the large uncolocalized red patches (Figure 4). The colocalization of green and red signals was analyzed by means of the Manders coefficients [12], calculated as average of 30 cells, by means of the *Intensity Correlation Analysis* plugin of the ImageJ software.

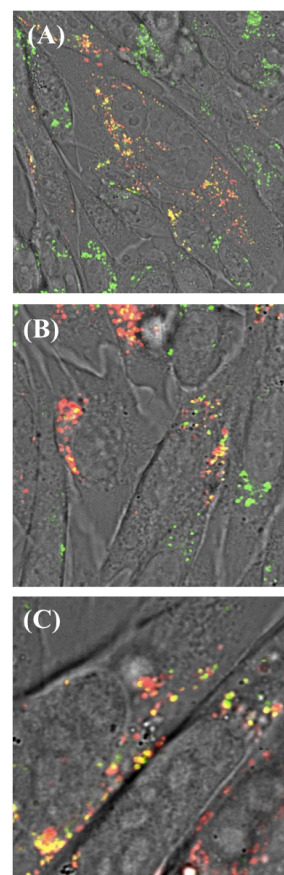


Figure 4. Representative colocalization of MENS signals (red) with Lysosensor (lysosome marker, green), after 24 h of MENS treatment. The presence of the two fluorescent labels in the same pixels (i.e. colocalization) gives yellow hotspots. Large uncolocalized red patches show that MENS avoid lysosomal entrapment.

We obtained $M_1=0.32\pm 0.18$ and $M_2=0.26\pm 0.12$, which indicate conditions of low colocalization, as clearly shown in Figure 4 (low overlapping of red and green signal).

The absence of colocalization of MENS with Lysosensor demonstrates that this formulation is not specifically targeted to metabolic degradation.

4 CONCLUSIONS

We have used a programmed packaging strategy to develop a multicomponent envelope-type nanosystems (MENS) composed of plasmid DNA condensed with protamine sulfate as a core, covered with anionic lipid bilayers and then surface-functionalized with poly-L-arginine, mimicking envelope type viruses. MENS was positively charged and exhibited complete DNA-protection ability.

We performed a detailed preliminary characterization concerning the size and the Z-potential of MENS (Figure 2). The proper MENS formulations were chosen according to the following general considerations: (i) MENS must be positively charged to associate electrostatically with mammalian cells, which contain surface proteoglycans with negatively charged sulphated groups; (ii) homogeneous small-size complexes are better internalized and processed by cells. As a result, complexes at a ratio $R_{\text{vir}}=0.5$ (P/DNA/M dispersion volume per poly-L-arginine dispersion volume) were chosen because resulted in the formation of stable positively charged MENS aggregates whose size (~180 nm) and Z-potential (~50 mV) remained stable over 24 h.

CLSM experiments have unambiguously revealed that MENS formulations abundantly penetrate CHO-K1 cells. The high cellular internalization is due to the presence of poly-L-arginine, which is known to enhance cellular binding and uptake. CLSM have also shown that MENS formulation do not colocalize with lysosomes, avoiding metabolic degradation.

Our results seem to indicate that MENS formulation are optimal candidate for non-viral gene delivery. Transfection efficiency experiments aimed at evaluating the ability of MENS to release the encapsulated DNA are currently in progress.

Moreover, the very same programmed packaging strategy will be applied to develop a proper MENS able to compete with the efficiency of viral vectors. It will be further rationally equipped with functional devices to control intracellular fate and intranuclear DNA release.

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