Programmed packaging of multicomponent envelope-type nanoparticle system for gene delivery

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A programmed packaging strategy to develop a multicomponent envelope-type nanoparticle system (MENS) is presented. To this end, we took specific advantage of using in-house tailored liposomes that have been recently shown to exhibit intrinsic endosomal rupture properties that allow plasmid DNA to escape from endosomes and to enter the nucleus with extremely high efficiency. Transfection efficiency experiments on NIH 3T3 mouse fibroblasts indicate that MENS is a promising transfection candidate. © *2010 American Institute of Physics*. [doi:10.1063/1.3427354]

Nonviral DNA delivery systems have been developed to facilitate gene entry into mammalian cells.[1] The cell presents multiple barriers to DNA/vector complexes en route to the nucleus.[2,3] 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 and transfection may fail. An efficient nanocarrier should therefore incorporate different functional devices, but it is difficult to integrate them into a single system and to have each function exerted at the appropriate time and correct place. Recently, a programmed packaging concept in which various tools that control intracellular trafficking are packed into single nanoparticles has been proposed.[4] Here we apply such a strategy to develop a multicomponent envelope-type nanoparticle system (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.[5] Such a core is coated with highly-fusogenic multicomponent (M) anionic liposomes that have intrinsic endosomal rupture properties.[6–8] Finally MENS was then surface-functionalized with poly-L-arginine. transfection efficiency (TE) experiments on NIH 3T3 mouse fibroblasts cells indicate that MENS is a promising transfection candidate.

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 routinary procedures (total lipid concentration 1 mg/ml).[9] Double-stranded calf-thymus (CT) DNA, purchased from Sigma (St. Louis, MO) and used without further purification, was dissolved in water (1 mg/ml). Plasmid DNA (pGL3 which codifies for firefly luciferase) was purchased from Promega (Madison, WI) and dissolved in bidistilled water (from Carlo Erba Reagenti, Milan, Italy) (final concentration 1 mg/ml). 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 at several weight ratios r_W=0.25, 0.5, 1, 1.5, 2, 3, 5, and 10. Positive P/DNA particles were mixed with negative multicomponent SUV dispersions at several volume ratios, r_V=0.25, 0.5, 1, 1.25, 1.75, 2, 2.25, 2.5, 5, and 10. After 24 h equilibration, finally, the MENS was prepared by mixing p/DNA multicomponent lipoparticles with a poly-L-arginine containing dispersion (1 mg/ml) [r_A=poly-L-arginine/DNA (Vol/Vol) =0.1]. A processing overview is given in Fig. 1. All sizing and ζ-potential measurements were made on a Zetasizer Nano ZS90 (Malvern, U.K.) at 25 °C with a scattering angle of 90°. For luminescence analysis, mouse fibroblast NIH 3T3

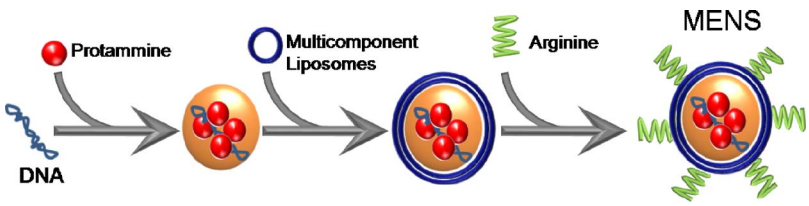

FIG. 1. (Color online) Schematic diagram illustrating preparation of MENS. DNA is first condensed with protamine sulfate via electrostatic interaction. The condensed particle is coated by the assembly of negatively charged multicomponent SUVs around the positive core. The lipid DNA-loaded nanoparticle is then modified by poly-L-arginine to reverse the surface charge.

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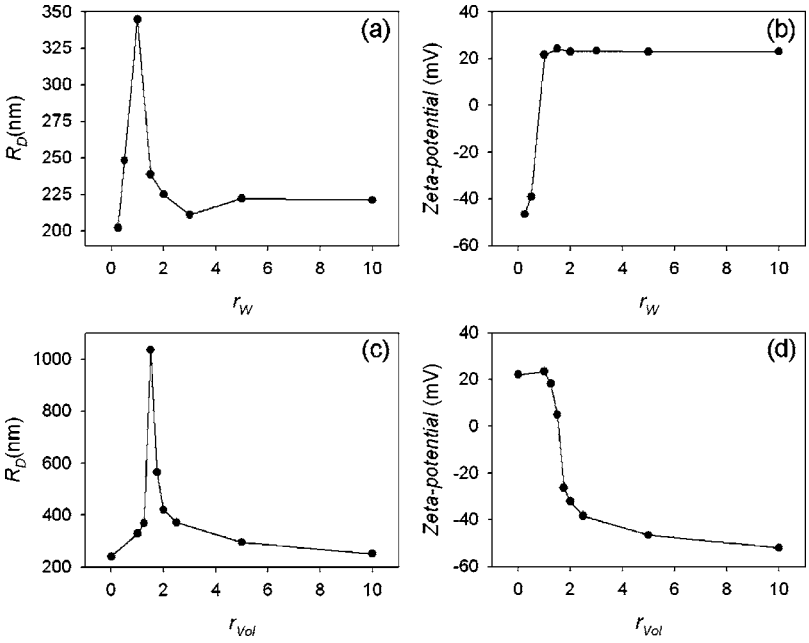

FIG. 2. (a) Radius of P/DNA complexes as a function of the P/DNA weight ratio, r_W. This behavior is typical of the reentrant condensation effect. (b) Zeta-Potential of P/DNA complexes as a function of r_W. The charge inversion effect occurring for $0.5<r_W<1$ changes the overall charge of the aggregates from negative (DNA excess) to positive (protamine excess). (c) Radius of P/DNA multicomponent lipoparticles as a function of the volume ratio, r_V (P/DNA dispersion volume per multicomponent SUV dispersion volume). (d) Zeta-Potential of P/DNA multicomponent lipoparticles as a function of r_V. The charge inversion effect occurring for $1.5<r_V<1.75$ changes the overall charge of the aggregates from positive to negative.

cells were transfected with pGL3 control plasmid. MENS were diluted in buffer and left for 20 min at room temperature before adding them to the cells.

To protect DNA by enzymatic degradation, it is advantageous to condense DNA into a compact core prior to inclusion into a lipid envelope.[10] To this end, CT-DNA was packaged into a condensed core via electrostatic interactions with cationic protamine sulfate (step 1 in Fig. 1). Precise knowledge of this process is important for controlling the size and charge of the condensed DNA. P/DNA complex formation was investigated by measuring the average hydrodynamic radius, R_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, namely, the reentrant condensation and the charge inversion effect.[11] These two typical effects are shown in Fig. 2 where the average dimensions and the zeta-potential of P/DNA particles are plotted against the P/DNA weight ratios, r_W, [panels (a) and (b), respectively]. As can be seen, with the increase in r_W, complexation begins and the size of the complexes gradually increases until a maximum is reached (at $r_W \sim 1$). Our results are in good agreement with previous studies showing that P/DNA particles show a neutral charge at $r_W \sim 0.9$.[5] Further increase in the P content determines the formation of decreasing-size complexes until the size of the original P/DNA core is approximately reached again (reentrant condensation). Aggregates also undergo the charge inversion effect, documented by the zeta-potential values whose sign changes for $0.5<r_W<1$, differentiating negatively and positively charged aggregates. DNA-protection ability is a central requisite of lipid nanocarriers. Electrophoresis experiments (not reported for space consideration) showed that, starting from $r_W \sim 0.75$, plasmid DNA was completely protected by protamine. On the basis of these results, P/DNA complex at $r_W=1.5$ was therefore chosen because it guaranteed complete DNA protection, exhibited positive charge (20 mV) and appropriate dimensions (~240 nm) with the minimum protamine content.

Then, the preassembled P/DNA core was coated by a lipid envelope through membrane fusion of negatively charged multicomponent SUVs (−55.2 mV, 122.4 nm), triggered by the electrostatic attraction around the positively charged core (step 2 in Fig. 1).[4] According to recent findings,[6–8] special multicomponent liposomes were chosen because they have intrinsic endosomal rupture properties. The main results are summarized in Fig. 2 where we show the average size [panel (c)] and the zeta-potential [panel (d)] as a function of P/DNA multicomponent lipoparticles volume ratio, r_V Both the reentrant condensation and the charge inversion are clearly recognizable. The complex at $r_V=10$ 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 index (pdi=0.22). The difference in diameters between the condensed p/DNA core and the final P/DNA multicomponent lipoparticles product is about 12 nm, which extends the difference in thickness to 2–3 lamellar layers (4–6 nm each).

However, it is also possible that a water phase exists between the core and the outer lipid envelope. Since plasma membrane (−30 to −60 mV negative inside) and mitochondrial membrane (−150 to −170 mV negative inside) possess large membrane potential, a positive charge owing to MENS modification may be a driving force for attraction and membrane interaction. Thus, we functionalized the lipid surface by poly-L-arginine a cell-permeable peptide that is expected to induce the cellular uptake of the particle by macropinocytosis (step 3 in Fig. 1).[4] An advantage of macropinocytosis-dependent uptake is that it permits endosomal escape of the carrier before it can be degraded in the lysosome. Mixing P/DNA multicomponent lipoparticles with a poly-L-arginine containing dispersions ($r_A=0.1$) resulted in the formation of stable positively charged MENS aggregates (around 50 mV). The very same phenomenology was observed when plasmid DNA was employed. The encapsulation efficiency of the DNA, as determined by electrophoresis on agarose gels, was about 100%.

Among the barriers to transfection, endosomal escape is considered to be rate-limiting. A now emerging viewpoint

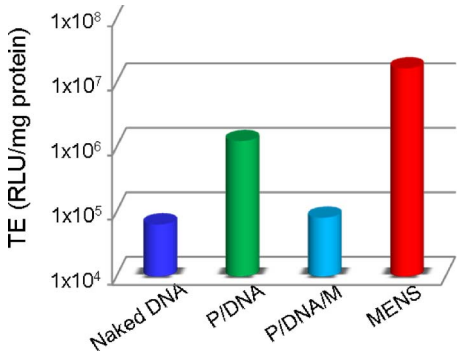

FIG. 3. (Color online) Transfection activity of MENS compared to those of P/DNA complexes, P/DNA multicomponent lipoparticles (P/DNA/M) and naked DNA.

states that lipid mixing between the outer envelope of lipid nanocarriers and endosomal compartments resulting in cytosolic DNA release can be achieved in a lipid composition-dependent manner.[4] In the case of MENS, encapsulated cargo can be released to the cytosol by decoating the double layered envelope structure. Thus, when endosome-fusogenic lipid formulations will be identified properly, the very same programmed packaging strategy will be used to cover the P/DNA core with the proper lipid envelopes. To evaluate the ability of MENS to deliver plasmid DNA, transfection experiments were performed in NIH 3T3 cells (Fig. 3). MENS showed high activity when compared with that of P/DNA. Indeed, surface functionalization increased the transfection activity by more than one order of magnitude compared to P/DNA nucleus. This increased transfection activity is most likely to result from enhanced cellular internalization by the presence of poly-L-arginine, which is known to enhance cellular binding and uptake. We also observed that DNA core coated with few multicomponent lipid bilayers alone exhibited low TE comparable to the level achieved with naked DNA. Such a reduction in TE is most likely to depend on the negative charge of P/DNA multicomponent lipoparticles [Fig. 2, panel (d)] that unfavorably interact with the plasma membrane containing cell surface proteoglycans with negatively charged sulfate groups on the extracellular side.

In conclusion, we have used a programmed packaging strategy to develop a MENS that is composed of condensed DNA as a core, which is covered with lipid bilayers mimicking envelope type viruses. MENS was positively charged and exhibited complete DNA-protection ability. TE measurements reported in Fig. 3 demonstrate that MENS is a promising transfection candidate.

In the near future, 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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