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Synthetic Viruslike Particles for Targeted Gene Delivery to $\alpha_v\beta_3$ Integrin-Presenting Endothelial Cells

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Abstract: Progress in the design of gene delivery systems is of utmost importance for cancer gene therapy since several physiological and intracellular barriers remain. We previously developed a technology for condensing a single gene into a single and stable globular nanometric system. In this manuscript, we have decorated the nanometric particles with cyclic RGD ligands in order to target endothelial cells. The potential of these artificial viruses as targeted gene delivery vehicles is demonstrated *in vitro* with $\alpha_v\beta_3$ integrin-expressing primary endothelial cells.

Keywords: Synthetic virus; DNA condensation; RGD peptide; integrin; gene delivery; transfection

Introduction

Gene therapy relies on the availability of gene delivery systems. Their efficacy depends on their ability to bind to cell surfaces and to divert internalization mechanisms to unload the gene into the nucleus of target cells. Most common synthetic systems are based on cationic lipids or polymers with endosomolytic activities and are simply assembled by electrostatic association between the DNA phosphates and excess of the cationic vector. This fast and kinetically driven process leads to colloidally unstable, polydisperse and large cationic complexes[1] that bind electrostatically to readily accessible anionic proteoglycans of adherent cells.[2,3] Subsequent endocytosis occurs spontaneously, and vectors with endosomolytic activities may then lead to gene escape into the cytosol and generally to robust transfection. *In vivo*, additional extracellular barriers decrease the efficiency of this process. Yet synthetic systems are still used for cancer gene therapy when large amounts of complexes can be physically administrated *in vivo* to reach directly the targeted cell surface.[4−6] Another potentially favorable situation can be encountered in cancer therapy when fast-growing tumor metastases induce formation of

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leaky blood vessels.[7] Blood-injected nanomaterials can thus passively accumulate within the tumor mass, directly targeting the tumor cells or the endothelial cells that are recruited for angiogenesis if they are equipped with appropriate ligands.[8,9] For this to occur, complexes must remain stable in the bloodstream and circulate for some time. Gene delivery systems (lipoplexes, polyplexes) based purely on cationic vectors are incompatible for intravenous injection because they aggregate with erythrocytes[10] or serum proteins.[11] The consequence is liver and lung damage at doses efficient for gene transfection.[12] Approaches to solve these specific circulation problems have been to use polymers for shielding cationic systems[13,14] and retargeting strategies.[15,16]

To address this crucial diffusion issue, we have developed convertible detergents for condensation of a single plasmid DNA into thermodynamically and chemically stabilized 30 nm diameter DNA complexes.[17,18] These delivery systems are prepared using cationic cysteine-based detergents that condense the wormlike DNA into complexes containing a single DNA molecule (monomolecular DNA condensation).[19] Reversion of the condensation process is then stopped by *in situ* chemical transformation of detergents into insoluble lipid-like molecules using mild oxidation of thiol to disulfide. This process leads to the production of stable DNA complexes of the smallest possible size that can display increased stability and extended circulation times in the bloodstream without the need of any polymeric coating protection.[17] These types of DNA complexes can also be easily decorated, and we showed that a polyethyleneglycol−folate envelope leads to selective particle engulfment into endocytic vesicles of cancer cells overexpressing a high affinity folic acid receptor.[20−22] However, these nanometric gene delivery particles were poor transfection agents, even in the presence of the endosomolytic agents DOPE and chloroquine. Three overlapping reasons may explain this shortcoming. The role of polyethyleneglycol (PEG) in shielding particles from interacting with extracellular fluids may become a hurdle in endosomal compartments where it may prevent DNA release.[23] Moreover, the size of the complexes, conjugated with restricted availability of ligand-specific cell receptors, may restrict entry of material into the cells at doses too low to ensure gene trafficking across stringent intracellular conditions into the nucleus. Finally, internalization of particles into vesicles, and hence subsequent escape, occurs by multiple and highly regulated mechanisms.[24] These mechanisms differ not only in the nature of the ligand and its receptor but also in the size of the complexes or the cell type.[25−27] Entry of complexes using the high affinity folic acid receptor may be unproductive since this endocytic

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pathway has been linked to transcytosis.[28] Together, these reasons prompted us to seek another cell targeting element, to remove the PEG envelope from the DNA nanoplexes and to equip the system with an endosome-escaping helper.

Tumor growth often triggers vascular remodeling and angiogenesis.[7] During these processes, endothelial cells show increased expression/presentation of several cell surface molecules, including the $\alpha_v\beta_3$ integrin. In addition to its role in angiogenesis and cell-matrix adhesion via recognition of RGD motifs, $\alpha_v\beta_3$ integrin was shown to be an efficient gateway for entry of the foot-and-mouth disease virus mostly via a clathrin-mediated endocytosis.[29] Moreover, evidence indicated that DNA lipoplexes use and divert at some point clathrin-coated vesicles for gene delivery.[30] The availability of high affinity and rather selective ligands for the $\alpha_v\beta_3$ integrin[31] prompted us to prepare a lipid−ligand conjugate capable of decorating the DNA nanometric complexes via anchorage to the lipid phase. We next evaluated the gene transfection activities of these nanometric systems for human primary endothelial cells in the presence of dioleoylphosphatidylethanolamine (DOPE), a hexagonal phase-forming lipid, that often improves the transfection efficacy of lipoplexes,[32] and chloroquine.[33]

Materials and Methods

Materials. N-α-NH_2-Fmoc protected amino acids and Fmoc-Gly-preloaded NovaSyn TGT resin were purchased from Novabiochem, Merck KgaA (Darmstadt, Germany). DOPE was from Fluka (St Quentin, France). All other chemicals were at least of analytical grade and were used as supplied. The 6367 bp pCMV-EGFP-Luc plasmid DNA (pDNA) was purchased from TebuBio (Le Perray-en-Yvelines, France). The linear peptide was prepared using standard automated solid phase Fmoc chemistry. HBTU was used as the condensing agent. Dioctadecylglycylamine and the tetradecylcysteinylornithine ($C_{14}CO$) were prepared as described elsewhere.[18] Synthesis of the cyclic peptide−lipid conjugates is described in the Supporting Information. Protein concentrations were determined using bicinchoninic acid assay (BCA, Uptima, Interchim, Montluçon, France). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed in the positive ion mode using α-cyano-4-hydroxycinnamic acid as matrix. UV/vis analysis was performed on a Uvikon 930 spectrometer (Kontron Instruments, Saint-Quentin, France). ^{1}H NMR spectra were performed on Bruker DPX 300 or 200 MHz spectrometers. Cell culture media were from Eurobio (Courtaboeuf, France), and fetal bovine serum (FBS) was from Perbio (Brebieres, France). Lysis and luciferin solutions for monitoring luciferase activity were purchased from Promega (Charbonnières, France) and used as described. The luminescence was measured using a luminometer (Mediator PhL, Wien, Austria).

Particle Formation. Typically and to decorate particles with 500 cRGD motifs: the plasmid (30 μM bp) in 10 mM HEPES-Na buffer, pH 7.4, containing 0.1 mM EDTA and 5% glucose (500 μL volume) was added at once to an ethanolic solution of $C_{14}CO$ (15 μL from a 1 mM EtOH stock concentration), DOPE (7.5 μL from a 0.5 mM EtOH stock concentration) and the RGD−lipid (1.25 μL from a 1 mM EtOH stock solution). The reaction was kept at room temperature for 48 h for a full thiol oxidation. Particles were then analyzed by 0.9% agarose gel electrophoresis (8 V/cm; 90 min) in 40 mM tris-acetate buffer, pH 7.0 (TAE). The gel was stained with ethidium bromide to reveal DNA. Particles without DOPE (w/o DOPE) were prepared similarly except with the omission of the 7.5 μL ethanolic solution of DOPE.

Cell Culture. KB cells (human nasopharyngeal cancer cell line) were cultured in Dubelcco's modified Eagle's medium (DMEM) (Sigma) containing 10% heat inactivated fetal calf serum, 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin. Human umbilical vein endothelial cells (HUVEC) were extracted from human navel cords (C.H.U., Strasbourg) by collagenase (Worthington, Biovalley, France). The primary cells were grown and maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 0.5 ng/mL epithelial growth factor (EGF), 2 ng/mL fibroblast growth factor (FGF), 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in an incubator at 37 °C and in a 5% CO_2 humidified atmosphere.

Transmission Electron Microscopy. Electron microscopy analyses were performed on a Philips EM operated at 80 kV. Samples were transferred onto ultrathin carbon film grids (Ted Pella, 1822-F, Formvar removed) by placing the grid on top of a 10 μL drop for 1 min. Grid with adherent particles was wicked from one side, placed on a 100 μL water drop for 30 s for washing, wicked, placed for 1 min on a 60 μL drop of freshly filtered 1.33% uranyle acetate, wicked again and air-dried before visualization.

Light Scattering Measurements. After 48 h oxidation, size and ζ-potential of complexes that were prepared as described above were determined by dynamic light scattering using a nanoZS (Malvern instruments, Paris, France) with

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the following specifications: initial plasmid concentration, 30 μM bp; medium viscosity, 1.054 cP; refractive index, 1.34; temperature, 25 °C. Data were analyzed using the automatic mode of the multimodal number distribution software included with the instrument. ζ-Potentials were measured with the following specifications: sampling time, 30 s; 16 to 30 measurements per sample; medium viscosity, 1.054 cP; medium dielectric constant, 80; temperature, 25 °C; beam mode $F(\mathrm{Ka}) = 1.5$ (Smoluchowsky).

Interaction of RGD Particles to Endothelial Cells. To assay binding of cRGDfK-coated particles to integrins, 2.5 cm^2 chambered coverglass (Labteck 155383, Nunc, Rochester, NY) was treated for 1 h at room temperature with a 0.5 mL PBS solution containing 0.5 μg of vitronectin from human plasma (Sigma-Aldrich). PBS was then removed to seed the endothelial cells. After overnight incubation at 37 °C, DNA complexes (200 μL; 4 μg of plasmid) were added to cell medium without serum (200 μL) to reach a final concentration of 10 μg DNA/mL. Cells were observed after 2 h incubation at 37 °C.

Transfection Experiments. Transfection experiments were performed in 96 well plates or in solution. JetPEI (PolyPlus-transfection, Illkirch, France) and Transfectam were used as positive controls according to described protocols. For transfection of adhered cells, cells (either HUVEC or KB) were seeded in 96 well plates 24 h prior to the experiment. DNA complexes (40 μL; 0.8 μg of plasmid) were then added to the cells in serum-free medium (40 μL) and in the presence of chloroquine (100 μM final concentration). Medium (100 μL), serum (20 μL) and chloroquine (100 μM final concentration) were added 2 h later. Cells were lysed 24 h post-transfection, and luciferase gene expression was quantified. Results were expressed as relative light units (RLU) integrated over 10 s per milligram of cell protein lysate (RLU/mg of protein). The errors bars represent standard deviation derived from triplicate transfection experiments. For transfection of cells in suspension, DNA complexes (100 μL; 2 μg of plasmid) were added to HUVEC (10^6 cells freshly harvested by treatment with 0.56 mM EDTA) in suspension in serum-free media (100 μL). After 45 min incubation at 37 °C in the presence of chloroquine (100 μM), the cells were diluted with medium containing 15% (v/v) serum (3 mL) and seeded in 24 well plate in triplicates. Cells were lysed 24 h post-transfection, and luciferase gene expression was quantified as described earlier.

Results and Discussion

Synthesis. The tetradecylamide of ornithyl-cysteine **1** (C_{14}CO) was prepared as previously described.[18] Many peptides and analogues bind the $\alpha_v\beta_3$ integrins.[34,35] Among them, we chose to use the cyclic pentapeptide RGDfK (f being the D-phenylalanine)[36] because it binds with high affinity to $\alpha_v\beta_3$ integrin (K_d in the nM range) and is easily conjugated to many molecules or delivery system via the free amine of the lysine residue without apparent loss in binding properties.[37−40] Crystal structure of the $\alpha_v\beta_3$ integrin complexed with the cyclic RGDfN(Me)V confirmed indeed that the side chain of the fifth amino acid, corresponding to the valine or lysine residue, is excluded from the binding pocket and is pointing toward the aqueous phase.[41] The cyclic peptide was synthesized according to a previously described procedure[36] and conjugated to a lipid anchor (Scheme 1).

Briefly, the linear peptide was prepared with an automatic synthesizer using Fmoc-protected amino acids, glycine preloaded on trityl resin, and HBTU as the condensing agent. The peptide was then cleaved from the resin by a mild acidic treatment (acetic acid/trifluoroethanol/CH_2Cl_2) and cyclized in DMF using diphenylphosphorylazide. The benzyloxycarbonyl protecting group was removed by hydrogenolysis over Pd/C, and the amine reacted to dioctadecylaminoglycylglycolate succinimidyl ester **10**. The protecting groups were finally cleaved off with trifluoroacetic acid to afford the cRGDfK−lipid **3**.

It was shown that replacing the glycine for an alanine diminishes drastically the affinity of the cyclic peptide for integrins (at least 100-fold decrease in affinity).[42] We therefore prepared the cRADfK−lipid **4**, in a similar manner, but starting from an alanine-loaded resin.

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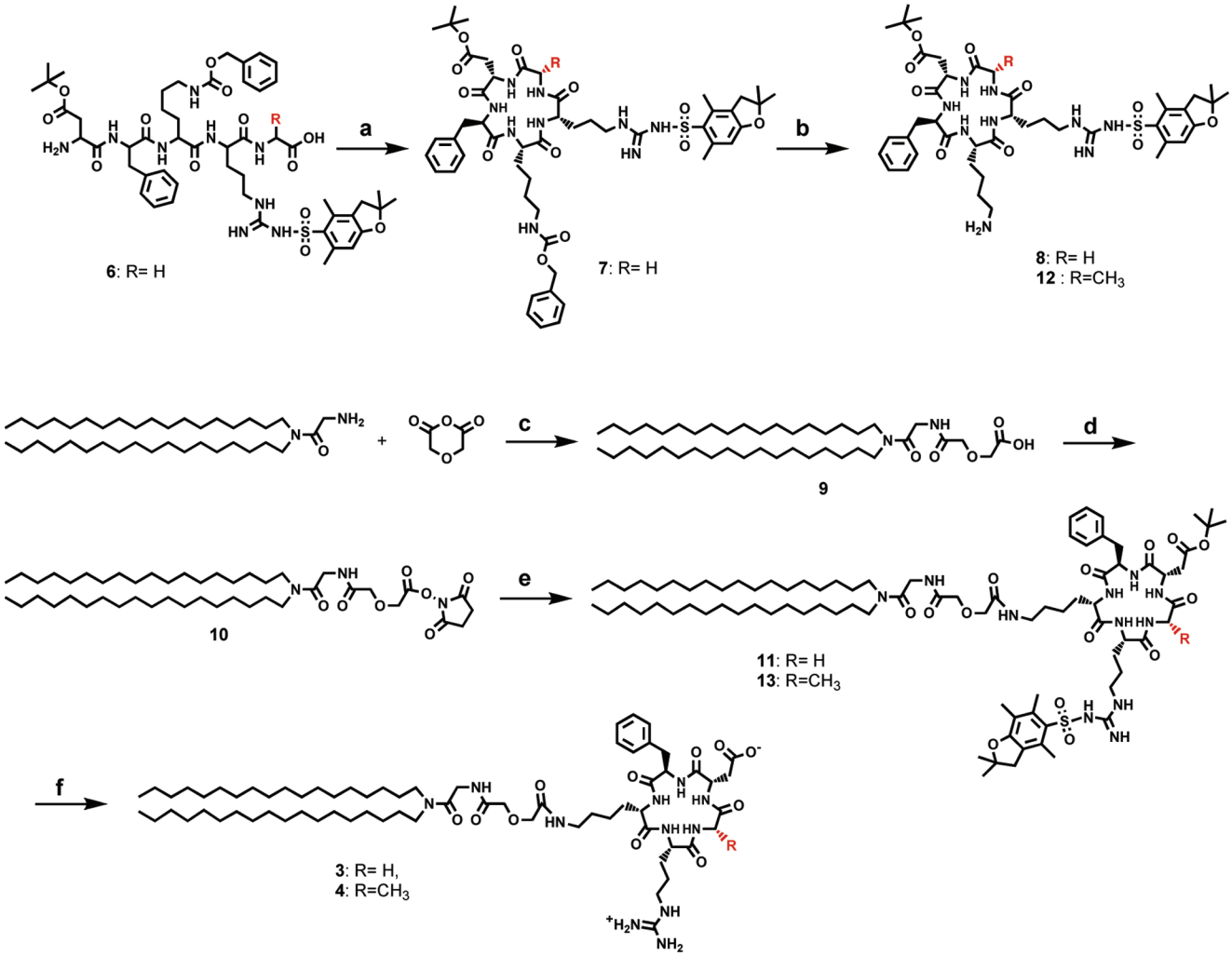
Scheme 1[a]



[a] (a) $(C_6H_5O)_2P(O)N_3$, $NaHCO_3$, DMF; 75%; (b) H_2, Pd/C, CH_3OH; (c) CH_2Cl_2, 80%; (d) DCC, NHS, CH_2Cl_2, 80%; (e) DMF/CH_2Cl_2, 96%; (f) TFA/H_2O 95/5, 79%.

Formation of DNA Particles. Monomolecular condensation of DNA into stable nanometric particles was achieved according to the two-step procedure depicted in Figure 1. At first, anionic plasmid molecules (30 μM in bp, 4.68 nM in pEGFPLuc plasmid) were individually and quantitatively condensed with the cationic detergent **1** at an amine detergent to DNA phosphate (N/P) ratio of 1. The particles were then stabilized by oxidation of every couple of detergents **1** into one single water-insoluble lipid-like molecule **2**. To ensure full disulfide bond formation, the reaction was allowed to proceed for 48 h. Previous experiments suggested that the weak transfection efficacy of these nanometric systems might originate from a lack of efficient endosomolytic element.[21] We therefore loaded the DNA particles with the transfection-helper DOPE at an input detergent to DOPE molecular ratio of 4 to 1. To do so, we took advantage of the detergence properties of **1**, which allowed aqueous solubilization of DOPE and integrin-targeting lipids, as well as initial monomolecular DNA plasmid condensation. Homogeneity of mixed micelles was nonetheless favored by premixing the amphiphiles in ethanol at the desired ratio. After 48 h, effective formation of disulfide bonds was monitored by quantifying absence of free thiols in the reaction mixtures according to Riddles et al.,[46] and formation of nanometric particles was monitored by agarose gel electrophoresis (Figure 2).

Indeed, reversible electrostatic interactions, the ones that instigate monomolecular plasmid condensation, would easily be destroyed in an electrical field[19] and, on the other hand, large polymolecular DNA aggregates would remain in the wells of the gel.[18] After ethidium bromide staining, condensed [pEGFPLuc/**2**] complexes (lane 2) appear as a single band with an increased electrophoretic mobility with respect to the naked and unfolded plasmid (lane 1). This gel accelerating behavior agrees with the fact that electrostatic condensation with cationic detergents leaves, on the complex surface, phosphate residues that are not neutralized. These compact and globular particles move through an agarose network more readily than semirigid and unfolded linear polymers.

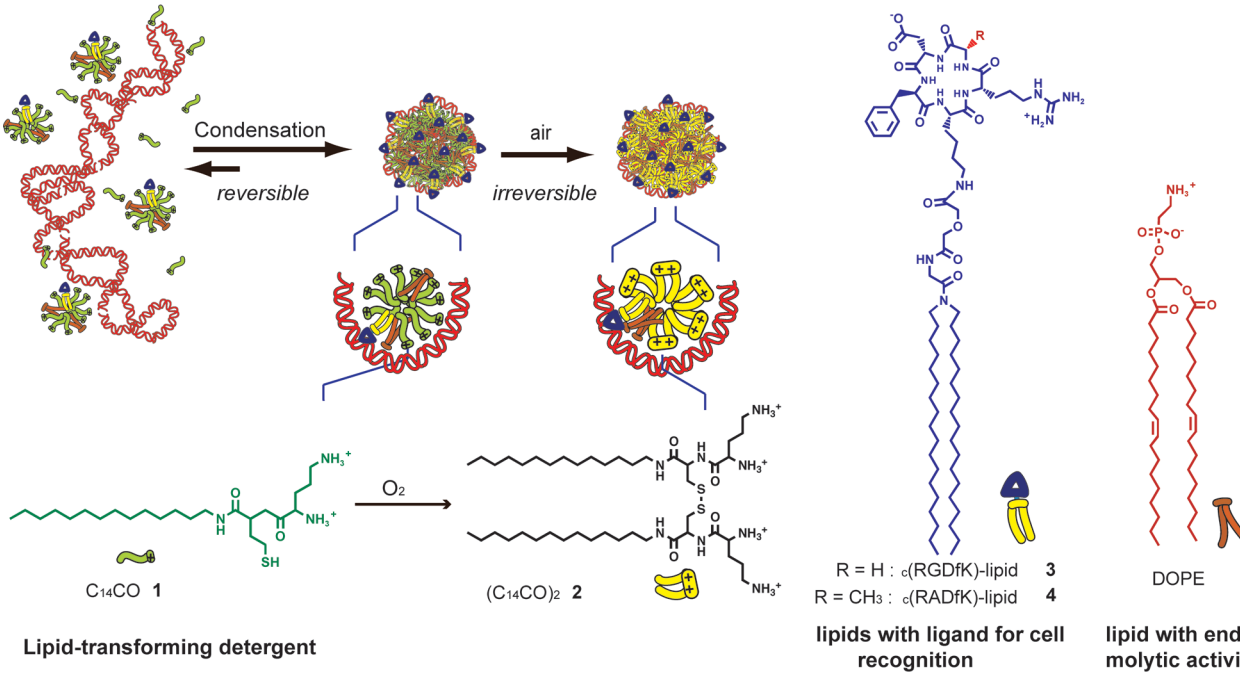


Figure 1. Mechanism and partners that are involved in the formation of stable, nanometric and integrin-targeting gene delivery systems. A plasmid DNA molecule is monomolecularly condensed using a thiol-containing cationic detergent **1** at an equimolar charge stoichiometry, in the presence of DOPE and integrin-targeting lipid (cRGD−lipid **3**). Reversible micellar domains are then stabilized by oxidation of the detergent into a lipid-like molecule **2**, leading to stable and cRGD-decorated DNA complexes. Untargeted control lipid is the cRAD−lipid **4**.

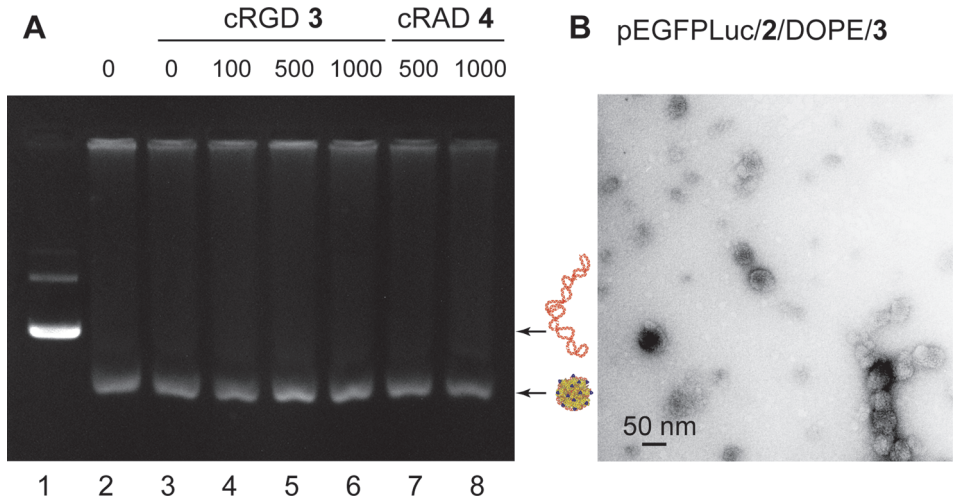


Figure 2. Characterization of decorated DNA complexes by agarose gel electrophoresis and transmission electron microscopy. The gel shows a 6.4 kbp pEGFPLuc plasmid (lane 1) and its complex with **2** (lane 2). The other lanes show particles incorporating DOPE and increasing input amounts of cRGDfK−lipid **3** (lanes 3 to 6) or cRADfK−lipid (lanes 7 and 8). The indicated values represent the number of ligands per particle based on the assumption that all lipid molecules are incorporated within the particles. For EM visualization, particles prepared as in lane 5 were deposited onto a carbon support and stained with uranyl acetate.

Monomolecular DNA condensation is best achieved at a cationic detergent concentration just below its critical micellar concentration because interaction of the DNA polyanionic backbone with a single cation ensures a fast equilibrium toward a thermodynamically stable DNA/detergent complex. Aqueous solubilization of lipids by cationic detergents may lead to cationic micelles that might impact dramatically the monomolecular plasmid condensation step and the chemical transformation of **1** to **2**. Fortunately, incorporation of the overall neutral lipid DOPE, **3** or **4** still allows the detection of bands (lanes 3 to 8), that migrate in the agarose gel, suggesting also an effective monomolecular DNA condensation.

Sizes and ζ-potential of the DNA nanoparticles incorporating the various lipids were then evaluated from dynamic light scattering data using a multimodal analysis (Table 1). All complexes were found to form a monodisperse population.

Table 1. Sizes and ζ-Potential of the Particles[a]

composition of the particle	mean diameter (nm) [PDI][b]	ζ-potential[c] (mV)
[pEGFPLuc/**2**]	37 [0.164]	−27.9 ± 6.4
[pEGFPLuc/**2**/DOPE]	37 [0.183]	−32.1 ± 6.5
[pEGFPLuc/**2**/DOPE/$\mathbf{3}_{500}$]	54 [0.115]	−32.3 ± 6.4
[pEGFPLuc/**2**/DOPE/$\mathbf{4}_{500}$]	47 [0.117]	−31.1 ± 7.3
[pEGFPLuc/PEI] N/P 5	159 [0.349]	+31.5 ± 7.4

[a] Determined by light scattering measurements in 10 mM Hepes buffer, pH 7.4 containing 0.1 mM EDTA. [b] PDI: polydispersity index. [c] Average and standard deviation.

The presence of DOPE did not modify the average diameter of the [pEGFPLuc/**2**] complexes (37 nm). The lipid−RGD **3** or lipid−RAD **4** led however to a slight but significant increase of the average diameter of the particles to about 50 nm. Finally, a similar and negative ζ-potential value (−30 mV) was measured for each DNA nanoparticle, regardless of its composition in either of the zwitterionic lipid DOPE, **3** or **4**.

The morphology of the [pEGFPLuc/**2**/DOPE/$\mathbf{3}_{500}$] complexes was then evaluated by electron microscopy (Figure 2). Complexes as prepared in lane 5 and in Table 1 were transferred onto a carbon-coated grid and stained with uranyl acetate. Complexes appeared to form two populations of globular particles with average diameters of 30 and 50 nm, suggesting the presence of DOPE and **3** is making the monomolecular DNA condensation process diverge slightly from its optimum.

Gene Delivery Activities. Although physicochemical measurements provided evidence of DNA particle formation in the presence of DOPE and cRGD−lipids, effective decoration needed to be confirmed. To do so, we chose human umbilical vein endothelial cells (HUVEC) for their property to express high levels of $\alpha_v\beta_3$ integrins[43] (the $\alpha_v\beta_3$ level was evaluated to be 10^6 receptors/cell by flow cytometry analysis, see Figure S1 in the Supporting Information). The cells were then seeded onto vitronectin-coated wells to favor cell attachment to extracellular matrix proteins via the $\alpha_v\beta_3$ integrins.[44] Particles ([pEGFPLuc/**2**/DOPE/**3**]) (2.36 nM in DNA particles; 500 cRGD−lipid/particle) were incubated for 2 h with HUVEC, and the cells were observed by microscopy (Figure 3).

While bare particles did not remodel the normal morphology of the cells, particles incorporating the cRGD−lipid conjugate caused cells to detach from the support and to adopt a rounded appearance. Strikingly, the concentration of the DNA particles (2.36 nM) is close to the IC_{50} value of 4.2 nM found for inhibition of vitronectin binding to the isolated $\alpha_v\beta_3$ by c(RGDfK) alone, even if the initial concentration of the lipid−cRGD is 500 times higher (1.18 μM). A similar degenerate situation[21] was observed previously and is consistent with the thermodynamic behavior of macromolecules that adopt a Brownian motion in solution, for whom the limiting step remains the initial interaction of a single cRGD motif with $\alpha_v\beta_3$ integrin. These results demonstrated that the ([pEGFPLuc/**2**/DOPE/**3**]) particles compete with vitronectin RGD motifs for binding to integrins and confirmed an effective presentation of the cyclic RGD ligands on the surface of the DNA nanoparticles.

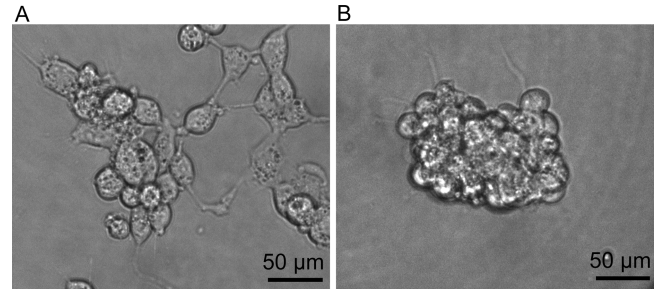


Figure 3. Evaluation of decorated DNA particle binding to $\alpha_v\beta_3$-presenting cells. cRGD enveloped DNA particles (image B) led to detachment of endothelial cells from vitronectin-coated glass support whereas uncoated DNA nanoplexes (image A) did not. Final DNA concentrations were 10 μg/mL (2.36 nM in particles). Images were taken after 2 h incubation at 37 °C.

HUVECs, which present large amounts of $\alpha_v\beta_3$ integrins on their external surface membranes,[43] were seeded into untreated plates to favor cell adhesion by other means than integrins. Cells were then treated with 2.36 nM cRGD-decorated [pEGFPLuc/**2**/DOPE] or nontargeting cRAD, and luciferase gene expression was evaluated 24 h later (Figure 4). Endothelial cells that were treated with DNA nanoparticles showed some luciferase expression with a 10-fold difference in favor of the $\alpha_v\beta_3$ targeting system. However, the gene expression level was poor compared to PEI/DNA complexes that have high intrinsic endosomolytic activities and internalize into adherent cells in much larger amounts via electrostatic binding to sulfated proteoglycans. Nonetheless, addition of chloroquine, a molecule known to confer gene delivery to polymers that are devoid of endosome buffering ability, was favorable and led to a further increase (10-fold) of luciferase gene expression. In this condition, the $\alpha_v\beta_3$-targeting cRGD decorated DNA particles (but not the untargetting cRAD ones) yielded significant transfection activity. The benefit of chloroquine was additive to that of DOPE since omission of the latter was counterproductive.

This first transfection experiment showed that the presence of DOPE and chloroquine helps overcome the poor endosomolytic activities of nanometric DNA nanoparticles. Moreover, equipment of particles with cRGD motifs clearly enhances transfection of endothelial cells, suggesting that selective recognition of $\alpha_v\beta_3$ and subsequent internalizing pathways could be advantageously used for nucleic acid delivery. The presence of $\alpha_v\beta_3$ on the cell surface, by itself,

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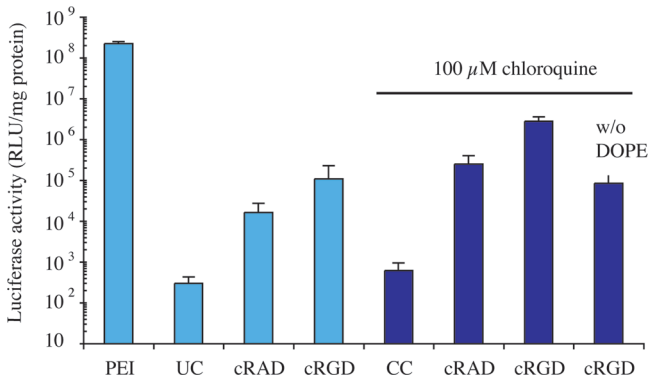

Figure 4. Transfection efficacy of the DNA delivery systems equipped with cRAD or c(RGD) motifs in $\alpha_v\beta_3$-presenting endothelial cells in the absence (light blue) or presence of chloroquine (dark blue). DNA particles contained DOPE (except when indicated), and 500 ligands/particle. Final DNA concentrations were 10 μg/mL (2.36 nM in particles). Expression of the luciferase reporter gene was monitored after 24 h by measuring the luciferase activity expressed as relative light unit/mg of protein cell lysate. PEI: cells transfected with PEI. UC: untreated cells. CC: chloroquine-treated cells. w/o DOPE: particles incorporating no DOPE.

does not guarantee transfection activity. Indeed, cell surface titration with a specific monoclonal antibody showed that KB cells also express $\alpha_v\beta_3$ integrins, though about 5-fold less than HUVECs (Figure S1 in the Supporting Information). Treatment of KB cells with decorated particles showed no significant transfection activity difference between uncoated and cRGD-coated particles (Figure S2 in the Supporting Information), indicating that the amount of integrin per cell and therefore a certain amount of material is required to bypass intracellular barriers and to yield transgene expression.

Finally, we evaluated the influence of the nanoparticle's coating density on the gene transfection efficacy (Figure 5). The previous experiment suggested that the amount of integrin plays an important role in the overall transfection process. Since large amounts of integrin may be recruited on the part of the cell surface that is attached to the plastic plate, we modified our experimental setting by incubation of DNA particles with HUVEC cells in suspension. This condition enables full exposure of the cell surface integrins to the aqueous solution containing integrin-targeting DNA particles and chloroquine. After 45 min incubation at 37 °C, cells were allowed to spread on plates. Resulting gene expression was monitored after 24 h. As previously observed, decoration of the DNA particles with cRGD motifs led to significant luciferase expression with an optimal level of 500 cRGD motifs per particle. Decoration of particles with the control cRAD motif diminished considerably the transfection activity with values even lower than the one found for naked DNA particles, suggesting that the cyclic peptide shields [pEGFPLuc/**2**] complexes from unspecific binding to cell surfaces.[47] Full exposure of the HUVEC surfaces to DNA particles was useful and yielded a 4-fold increase in transfection activity compared to the experiment that was performed on adherent cells. This enhancement substantiates cell entry of cRGD-enveloped particles via selective interaction with integrins. Finally, even with increased accessibility of particles to integrins on the cell surfaces, gene expression was reduced for DNA particles prepared without DOPE.

Conclusion

In conclusion, we have shown that a DNA supramolecular assembly whose size and physicochemical properties are tailored for *in vivo* administration can lead to significant transgene expression upon implementing the appropriate functionalities. Equipment of monomolecular DNA particles with high affinity ligands for integrins enabled targeted internalization in primary endothelial cells. Subsequent routing of the gene to the nucleus was implemented using conventional endosomolytic agents. Our experiments altogether indicate that internalization mechanism, specific

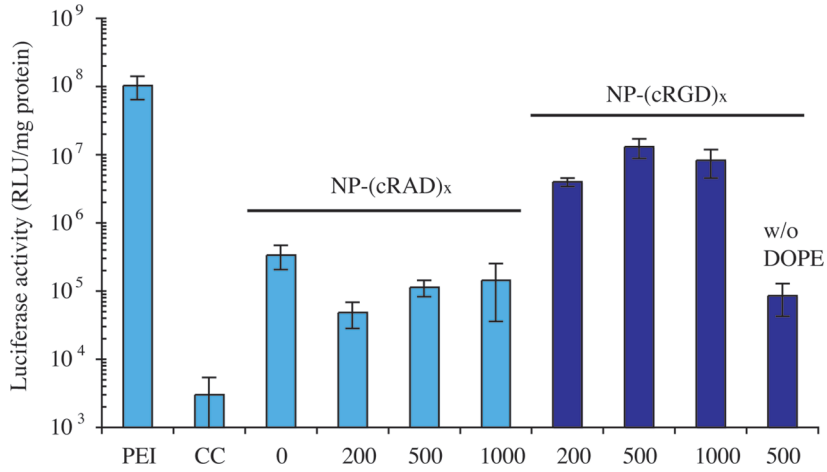

Figure 5. Transfection efficacy of the DNA delivery systems equipped with various amounts of cRAD or cRGD motifs in HUVEC. Particles (final DNA concentrations of 10 μg/mL) were incubated at 37 °C with a suspension of cells in the presence of 100 μM chloroquine. After 45 min, the cells were diluted and allowed to spread on 24 well plates. The luciferase gene expression was monitored after 24 h incubation. CC: chloroquine-treated cells. w/o DOPE: particles without DOPE.

activity and amount of material used are closely related. The requirement for high integrin amount per cell constitutes a definite advantage for discrimination between endothelial cells in neo-angiogenesis from resting ones. The requirement for chloroquine, which has systemic toxicity at doses efficient for endosomolysis,[45] clearly limits their *in vivo* use as such for cancer gene therapy applications and opens a challenging quest for conceiving agents with high endosomolytic activities. These results represent, however, a further step toward the conception of synthetic gene delivery systems with discrete and countable elements.

Abbreviations Used

TFE, trifluoroethanol; AcOH, acetic acid; Pbf, 2,2,4,5,7-pentamethyl-3-hydrobenzofuran-6-sulfonyl; Z, benzyloxycarbonyl; f, D-phenylalanine; FMoc, fluorenylmethoxycarbonyl; TFA, trifluoroacetic acid; DMF, *N*,*N*-dimethylformamide; DCM, dichloromethane; NHS, *N*-hydroxysuccinimide; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; DCC, dicyclohexylcarbodiimide; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; FBS, fetal bovine serum; pDNA, plasmid DNA; bp, base pair.

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Supporting Information Available: Procedures for the synthesis of peptide−lipid conjugates and for flow cytometry experiments. Figure S1 shows titration of integrin on cell surfaces. Figure S2 shows the absence of transfection with cRGD enveloped DNA particles on KB cells. Figure S3 shows cell binding properties of fluorescently labeled DNA nanoparticles using flow cytometric analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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(47) Figure S3 in the Supporting Information confirms that cRAD motifs protect the particles from unspecific binding to HUVEC.