Viruslike DNA Particles

Targeted Gene Delivery to Cancer Cells: Directed Assembly of Nanometric DNA Particles Coated with Folic Acid**

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This work is dedicated to Marc Zuber and Marie-Hélène Fauré

Gene therapy is gaining credit as a means to fight cancer from an angle other than radio- and chemotherapies.[1] However, it has yet to enjoy any real success because viral vectors are immunogenic, thus excluding repetitive treatment, and because gene delivery is too complex to be achieved with a single carrier molecule.[2] Therefore, effective multicomponent delivery vectors need to be developed.[3–5] The core of synthetic vectors is invariably a polycation capable of inducing condensation of anionic DNA. Conversion of the filiform DNA macromolecule into a compact particle improves both its chemical stability and physical properties. However, DNA compaction by cationic liposomes or polymers is a quasi-irreversible process that leads to the formation of 0.2–1-μm-sized microprecipitates containing hundreds of DNA molecules per particle.[6] For the transfection of cells in culture dishes, large complexes are advantageous because they sediment onto the cells. However, as one would expect, in animal models their gene-delivery properties are weak as a result of size-restricted diffusion.[7] In light of these factors, we have designed a novel synthetic DNA supramolecular assembly with key viral properties, such as diffusion and cell recognition.

We have developed a general method for the monomolecular condensation of genes that leads to a monodisperse and stable population of particles 30 nm in diameter.[8] The technique takes advantage of the condensation of DNA by a cationic cysteine-based detergent. This is a reversible interaction that can be used to drive the system entropically toward the largest number of particles. Particle stability is obtained subsequently by template-assisted oxidative dimerization of the detergent into a gemini lipid (see Figure 1, first two steps). As our goal is for particles to reach distant tumor metastases after injection into the blood stream, particles had to be protected from capture by liver macrophages and had to

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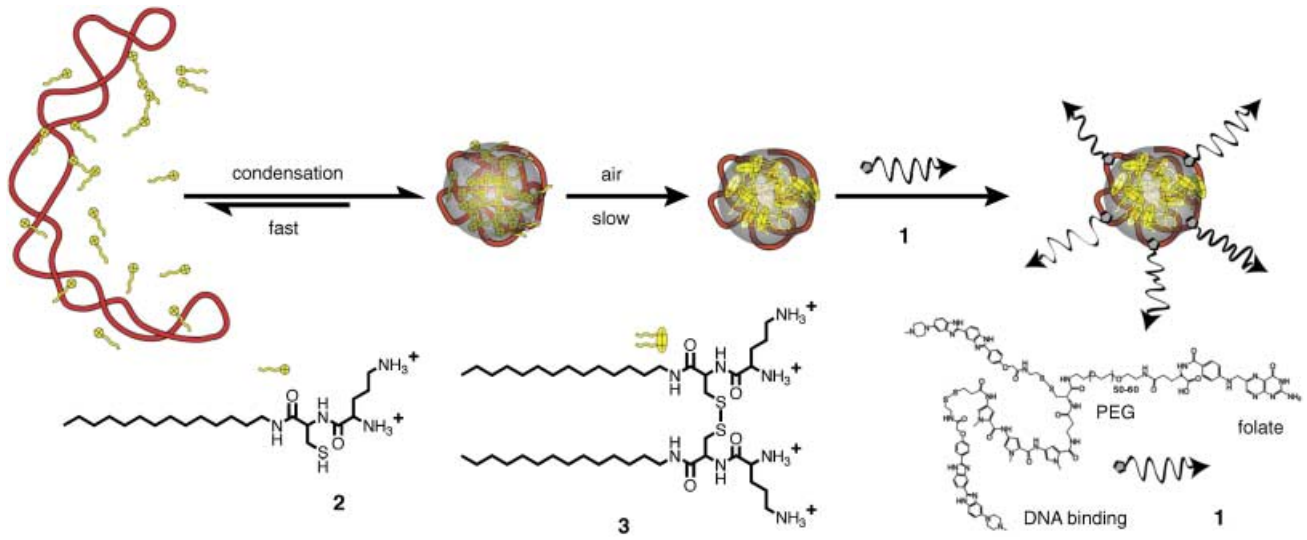
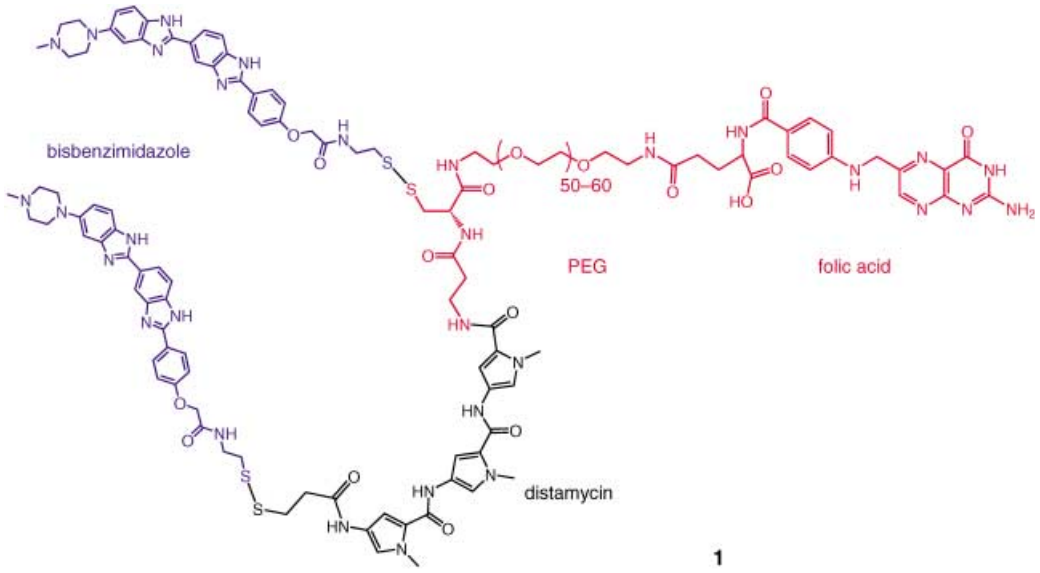


Figure 1. Stepwise assembly of stealth nanometric DNA particles. Plasmid DNA is condensed with a cationic cysteine detergent under aerobic conditions. The resulting gemini lipid–DNA particles are coated with a polyethyleneglycol–folate envelope that is anchored to the DNA by a minor-groove-binding moiety.

be equipped with a ligand that allows binding to and entry into cancer cells.

The biodistribution of different particle types, including liposomes, has been shown to benefit greatly from coating with a layer of polyethyleneglycol (PEG).[9] This effect is a result of the inertness of the polyether backbone, combined with brushtype polymer crowding, which prevents opsonization and capture by macrophages. Fast-dividing cancer cells overexpress the folic acid (vitamin B6) receptor,[10] which is capable of engulfing, besides folic acid, particles as large as the SV40[11] and Ebola viruses.[12] We therefore planned to cover the monomolecular DNA particles with a folate–PEG envelope. The envelope was anchored to the particle surface with a strong DNA-minor-groove-binding agent prepared from distamycin[13] and Hoechst 33258. Finally, as the PEG coat may also interfere with the release of DNA into the cell nucleus, linkages that can be broken by the cytoplasm were introduced into the molecule in the form of reducible disulfide bonds, to give compound **1**.



The synthesis of compound **1** was performed according to standard methods[14] by linking a distamycin central domain to folic acid by means of a commercially available heterobifunctional PEG. For the distamycin portion, the pyrrole amino acids were synthesized and then oligomerized stepwise. Bisbenzimidazole (Hoechst dye 33258) was *O*-alkylated and activated as a 2-pyridyl disulfide derivative. The final compound **1** was obtained by treating the distamycin–PEG–folate conjugate with 2 equivalents of the bisbenzimidazole fragment and purified by HPLC. It was characterized by UV and MALDI-TOF mass spectrometry ($M_w = 4479$ Da).

Preliminary binding studies of **1** to 5.5 Kbp pCMVLuc plasmid DNA were performed by agarose gel electrophoresis (Figure 2, lanes 1–4). Compound **1** induced concentration-dependent DNA retardation and reduced ethidium bromide staining of DNA, both of which are indicative of strong binding. The bulk reducing power inside cells is known to arise from millimolar concentrations of glutathione.[15] As an intracellular reversibility test, **1**–DNA complexes were incubated with 1 mM glutathione. A 15-min incubation period was sufficient to restore ethidium bromide staining and plasmid mobility (Figure 2, lane 4; a slight amount of plasmid retardation and blue fluorescence were observed as a result of some residual bisbenzimidazole binding to the plasmid).

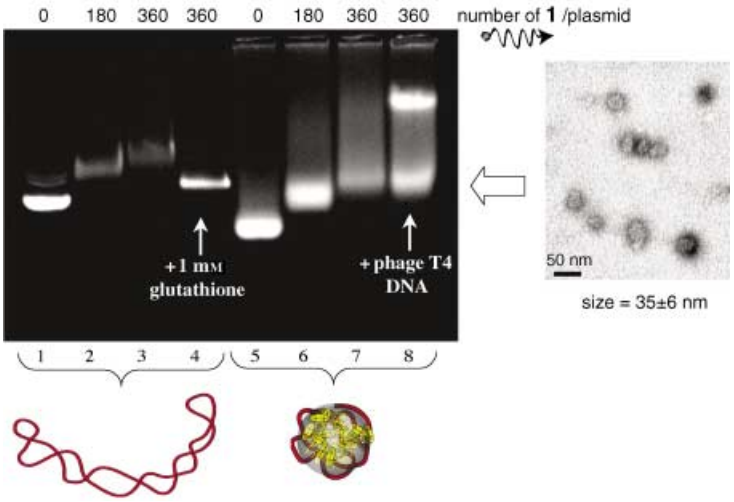


Figure 2. Agarose gel electrophoresis shows complex formation between DNA, the cationic detergent, and compound **1**. Plasmid DNA (30 μM-base pairs (bp), lane 1) was mixed with increasing amounts of **1** (1 μM, lane 2 and 2 μM, lanes 3 and 4). Lane 4: DNA–**1** complexes were incubated for 15 min with glutathione (1 mM). Lanes 5–8: nanometric **3**–DNA particles were prepared as in Figure 1. After overnight incubation, compound **1** (1 μM, lane 6 and 2 μM, lanes 7 and 8) was added to the particles. Lane 8: 1 h incubation of the complexes with phage T4 DNA. Image: Transmission-electron-microscopy picture of the final **3**–DNA–**1** complexes shows monomolecular DNA condensation into compact particles.

The preparation of the monomolecular DNA particles was carried out as described previously.[8] Thus, anionic DNA was condensed with an equimolar charge amount of the cationic detergent ornithyl-cysteine tetradecylamide (**2**), which was allowed to dimerize overnight under aerobic conditions (Figure 1). The electrophoretic mobility of the condensed DNA species through the agarose gel (Figure 2, lane 5) was found to be faster than reptation of the elongated DNA molecule (Figure 2, lane 1). Addition of the bulky conjugate **1** to the particles somewhat decreased their mobility (Figure 2, lanes 6 and 7). **3**–pDNA–**1** particles were stable in the presence of excess DNA (Figure 2, lane 8), thus indicating a quasi-irreversible binding of **1** to the particle surface.

The morphology of the PEG–folate-enveloped particles was observed by transmission electron microscopy[16] (Figure 2). Complexes appeared as a rather homogeneous population ($n = 53$) of compact particles with an average size of 35 ± 6 nm, indicative of monomolecular plasmid-DNA condensation.[17] If it is assumed that every molecule of **1** present binds to the DNA, the enveloped particles prepared as in Figure 2, lane 7 were each coated with 360 molecules of PEG–folate, which corresponds to approximately one PEG polymer per 10 nm^2. Such polymer density is within the range of the so-called "weakly overlapping mushroom regime",[9] and therefore should both provide stealth properties and allow folate-receptor-binding to the particles.

The cellular fate of the particles was tracked by using fluorescence-activated cell sorting (FACS) and confocal microscopy. KB cells derived from a human nasopharyngeal cancer line were chosen as a target because they conditionally express a large number of folic acid receptors upon folic acid starvation. Particles were made fluorescent with the DNA-intercalating dye YOYO (36 molecules/plasmid) and were incubated for 3 h with folate-receptor-expressing KB cells.[18] FACS analysis showed about a tenfold increase over background of the average cellular fluorescence (Figure 3). KB cells that did not overexpress the folate receptor did not show enhanced fluorescence, thus confirming specific and homogeneous folate-receptor-mediated cell binding. The combination of faster diffusion as a result of monomolecular DNA compaction,[19] stability, and cell specificity highlight the potential of these complexes for in vivo gene delivery.

To examine whether binding was followed by endocytosis, 1-μm optical sections were taken through the median plane of the cells by confocal fluorescence microscopy.[20] As shown in Figure 4, complexes ended up in large perinuclear endosomal compartments. A similar picture was observed previously for classical transfection agents such as cationic lipids.[21] However, in contrast to the latter compounds, folate complexes were poor transfection agents (ca. 0.5 pg luciferase/mg cell protein with the pCMVluc plasmid).

In an effort to understand this discrepancy, we studied the cell-binding thermodynamics by FACS. Half saturation of the receptor was obtained for a

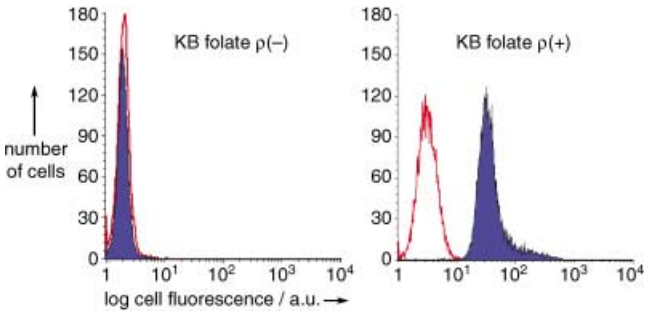


Figure 3. **3**–DNA–**1** complexes specifically bind to folate-receptor-expressing KB cells (KB folate ρ(+)). Cells were incubated for 3 h with fluorescent DNA complexes and sorted by cytometry according to their fluorescence. Only cells overexpressing the folic acid receptor (right graph) show an increased fluorescence (filled trace) over untreated cells (hollow trace).

particle concentration of about 5 nM (Figure 4). This value is close to the affinity measured for folic acid itself,[22] thus ruling out the cooperative binding of each particle to multiple folic acid receptors. The total number of plasmid molecules internalized at saturation (10^5) is within the range that was estimated for cationic lipid-mediated transfection.[21,23] It is therefore unlikely that the observed low transfection level is a result of insufficient cell entry through the folate receptor. However, if the PEG envelope is not removed in endosomes, it may prevent DNA release.

In summary, we have demonstrated that a synthetic DNA supramolecular assembly with diffusion and cell-recognition properties can be built. However, these do not yet exhibit

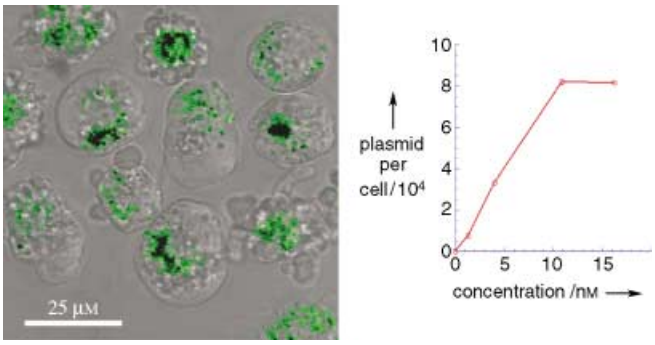


Figure 4. Intracellular fate of the fluorescent **3**–DNA–**1** particles in KB folate ρ(+) cells as shown by confocal microscopy after 3 h incubation. The particle binding isotherm shows an affinity of 5 nM for the receptor and up to 8×10^4 particles internalized per cell.

molecular functions such as endosome release and intracellular trafficking to the nucleus. Classical transfection has been shown to benefit greatly from "proton sponges" able to buffer the acidity of endo- or lysosomes[24] as well as from nuclear-localization-signal peptides conjugated to the DNA.[25,26] Work is currently in progress toward the incorporation of such additional features into ligand-bearing nanometric DNA particles.

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[16] Electron microscopy was performed on a Philips EM410 apparatus operated at 80 kV. Samples were transferred onto ultrathin-carbon-film grids (Ted Pella, CA, USA, 1822-F, formvar removed) by placing the grid on top of a 10 μL drop of the sample for 1 min. From one side of the grid the excess fluid was wicked away, then the grid was placed on a 100-μL water drop for 30 s to wash, excess fluid was again removed, the grid was placed for 1 min on a 60-μL drop of freshly filtered uranyl acetate (1.33 %), the excess fluid was again wicked away, and the grid was air dried.
[17] The volume of the 5500-bp DNA is 11800 nm^3 if hexagonal packing is assumed, in analogy with: J. A. Schellman, N. Parthasarathy, *J. Mol. Biol.* **1984**, *175*, 313. The DNA was neutralized with 2700 lipid molecules. If hydrated-lipid molecular dimensions of 0.8 $nm^2\times4$ nm are assumed, this gives a total volume of 20440 nm^3 for the complex, that is, a sphere of 34-nm diameter.
[18] KB cells were cultured in Dubelcco's modified Eagles medium (DMEM; Sigma, St Quentin, France) containing heat-inactivated fetal calf serum (10 %), glutamine (2 mM), penicillin (100 units mL^{-1}), and streptomycin (100 μg mL^{-1}) at 37 °C in a 5 %-humidified CO_2 atmosphere. Overexpression of the folic acid receptor was induced by growing the cells for three passages in folic acid depleted DMEM (FDMEM). For flow cytometry, particles were labeled with YOYO-1 (Molecular Probes, OR, USA; 1 YOYO/150 bp) by mixing the plasmid with YOYO before addition of the detergent. Cells were harvested by trypsination, washed with FDMEM, collected by centrifugation, and suspended in serum-free FDMEM. The cells (final concentration of 10^5 cells mL^{-1}) were then mixed with labeled particles in a total volume of 1 mL of serum-free DMEM and were incubated for 3 h at 37 °C in a 5 %-humidified atmosphere. DMEM (3 mL) containing calf-thymus DNA (200 μg mL^{-1}) was then added to account for nonspecific binding and the cells were collected by centrifugation and suspended in phosphate-buffered saline (PBS) with bovine serum albumin (BSA; 1 %). Flow cytometric analysis was performed on a FAC-StarPlus apparatus (Becton Dickinson, San Jose, USA) with an argon laser (excitation at 488 nm). Granulation, size, and fluorescence intensity (emission 515 nm) were recorded at a rate of 300 cells s^{-1}. Data were analyzed using cellQuest software. Calibration of the fluorescence intensity was performed by using 6-μm calibrated beads ($\lambda_{ex}=488$ nm, $\lambda_{em}=515$ nm). Quantum yields of FITC-labeled BSA, DNA-bound YOYO, and calibration beads were measured on a spectrofluorophotometer (Shimadzu RF-5301PC); FITC = fluorescein isothiocyanate.
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[20] KB folate ρ(+) cells were incubated for 3 h with fluorescent complexes and recovered as described[18] for cytometric analysis. Cells resuspended in FDMEM were deposited on chambered cover slides (Nalge Nunc, USA, Lab-Tek 155383). Images of living cells were collected on an AMRC-1024ES confocal laser scanning system equipped with an Eclipse TE300 microscope (Nikon) by using a 60× water-immersion objective. Series of 512×512 pixel images were acquired at different focal planes (z series) with a displacement of 1 mm between images. Green fluorescence, obtained from the 488-nm excitation line of an argon/krypton laser, and phase-contrast micrographs were loaded in the green and blue channels of the digital color image, respectively. Image contrast was corrected by using Adobe Photoshop software.
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