Template Oligomerization of DNA-Bound Cations Produces Calibrated Nanometric Particles

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Cationic polymers and cationic lipid mesophases are efficient gene delivery vectors for eucaryotic cells in vitro.[1,2] However they compact DNA into rather large polymorphic particles which severely limits their in vivo performances due to size-restricted diffusion. In contrast, oligocations, such as spermine[3] or cationic detergent micelles,[4,5] have low binding cooperativity and fast exchange rates and so are capable of collapsing DNA into more homogeneous populations of small particles. Under stoichiometric ionic ratio conditions, charge neutralization of the macromolecular polyanion is not complete[3] and the resulting anionic particles repell each other enough to ensure colloid stability. Unfortunately, ionic interactions of oligocations and detergents with DNA are too weak to withstand physiological salt concentrations. As a result, DNA is decondensed in biological fluids and such molecules are unable to transfect cells.[6,7]

We searched for a way of retaining the attractive features of both small and large cationic structures, i.e., for obtaining a monodisperse population of individually collapsed DNA molecules which would *in fine* be stable in a physiologic medium. A simple solution to this dilemma was to chemically convert the cationic molecule into a polymer (or the detergent into a lipid) once the particles were formed (Figure 1a). In such conditions, the rate of oligomerization of the condensing agent should benefit from a local concentration increase, while simultaneously "freezing" the particles.

According to these hypotheses, we first synthesized a polymerizable cation **C-sper-C** (Figure 1b) from natural cysteine and spermine precursor molecules.[8] Spermine binds to the minor groove of B-DNA,[9] which we expected to serve as a matrix for air-induced thiol/disulfide oligomerization of **C-sper-C** (a comparable protamine α-helix oligomerization reaction occurs in the major groove of genomic DNA during its final condensation stage into sperm heads[10]). Thiol oxidation rates of **C-sper-C** were compared in the presence and in the absence of plasmid DNA. DNA was indeed found to very much enhance the oxidation rate (Figure 2) while being condensed, as shown by a 15% turbidity increase at 260 nm. The mean size of the resulting particles was measured by laser light scattering to be 50 ± 15 nm. Particles were stable for at least 1 week (Figure 3a). Gel electrophoresis confirmed that the cooperative condensation process occurred for amine/phosphate ratios > 1 (Figure 3b, lanes 2 and 3) and led to polymerized **(C-sper-C)$_n$**/DNA complexes stable in electrophoresis conditions (lane 3), in contrast to spermine/DNA complexes (lane 4). Addition of excess reducing dithiothreitol (DTT) to the **(C-sper-C)$_n$**/DNA complexes converted the cationic polymer back to **C-sper-C** thus releasing the DNA (lane 5). Raising the ionic concentration to physiological levels (150 mM NaCl) after the oxidation step gave similar results.

The physical collapse/chemical stabilization process outlined in Figure 1a was also applied to the DNA-assisted dimerization of a cationic detergent **C_{10}-C^{G+}** (structure in Figure 1b) into a geminal-type lipid **(C_{10}-C^{G+})$_2$**.[11] The surface charge potential was found more negative for the **(C_{10}-C^{G+})$_2$**/DNA complexes (−40 mV, 15 mM Tris buffer pH 8.4, Zetamaster 3000) than for the **(C-sper-C)$_n$**/DNA complexes (−10 mV, 2 mM HEPES pH 7.0). This may explain why the latter complexes showed a tendency to slowly aggregate in 150 mM NaCl while the former did not. Yet the major difference between polymer and lipid-condensed DNA was revealed by the shape and size of the particles. Transmission electron microscopy of **(C-sper-C)$_n$**/DNA complexes (Figure 4a) showed polymorphic 50 nm tores eventually flattened out to ca. 100 nm rods. In contrast, the **(C_{10}-C^{G+})$_2$**/DNA complexes formed a very homogeneous population of 25 nm spheres (Figure 4b). These strikingly different morphologies may reflect an intrinsically higher DNA bending ability for a curved cationic amphiphile surface, leading to spheres, as compared to a cationic polymer that leads to toroïds. The volume of a 5.6 Kb plasmid is 9×10^3 nm^3 assuming a diameter of 2.5 nm for the hydrated double helix.[12] A rough estimate of the volumes of the **(C-sper-C)$_n$**/DNA tores led to values of $7-25 \times 10^3$ nm^3 hence containing 1−3 plasmid molecules. Lower DNA concentrations, which may favor formation of the smaller particles, would be unusable for gene delivery purposes. The **(C_{10}-C^{G+})$_2$**/DNA spheres had a volume of 8×10^3 nm^3, strongly suggesting monomolecular collapse.

Finally, the diameters of particles resulting from the detergent-mediated aerobic collapse of DNA of various sizes and topologies were measured by laser light scattering. The supercoiled pCMVluc plasmid (5.6 Kb), as well as linear phage λ (48 Kb) and phage T4 (166 Kb) DNAs were successfully condensed into monodisperse population spheres of increasing sizes. The particle diameter was plotted against the cubic root of the number of DNA base pairs (Figure 5). The data fitted with a straight line crossing the origin which is again evidence for monomolecular collapse (the volume of a sphere varies as the cube of its size). We speculate that still larger DNAs can be condensed by this method, bringing mammalian artificial chromosomes into the scope of nonviral gene transfer technology.

In summary, we have developed a general approach to the monomolecular condensation of DNA into stable nanometric particles. This approach can also be extended to the design of

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(8) 1,4-Diaminobutane was bis-cyanoethylated with acrylonitrile (2 equiv in CH_3CN, 18 h in the dark) and protected with BOC-ON (2.2 equiv plus 2.2 equiv of NEt_3, 3 days); overall yield 44% after recrystallization from ether. Reduction with Raney nickel/H_2 (7 days in CH_3OH/THF/2 M aqueous NaOH 3:3:1) yielded the diamine (88%) which was coupled with *N*,*S*-bis-*tert*-butoxycarbonyl-L-cysteine (2 equiv plus 2 equiv of DCC in CH_2Cl_2, 24 h); 66% yield after silica gel chromatography. Final deprotection of an aliquot was performed in TFA/CH_2Cl_2 1:1 (2 h, quantitative yield as judged by free thiol titration). A 100 mM **C-sper-C** stock solution in deuterated ethanol was kept under argon at −80 °C and checked by NMR for degradation/oxidation prior to each series of measurements. 300-MHz ^{1}H NMR, δ (CD_3CD_2OD): 1.80−1.91 (m, 4H, CH_2-C**H$_2$**-C**H$_2$**-CH_2); 1.91−2.04 (m, 4H, 2 X C**H$_2$**-CH_2-NH-CO); 3.01−3.17 (m, 12H, 6 × C**H$_2$**-NH); 3.31−3.48 (m, 4H, 2 × C**H$_2$**-SH); 4.08 (t, J = 6 Hz, 2 × C**H**-NH_2). MS (FAB$^+$), *m*/*z*: 410.1 (MH^+).
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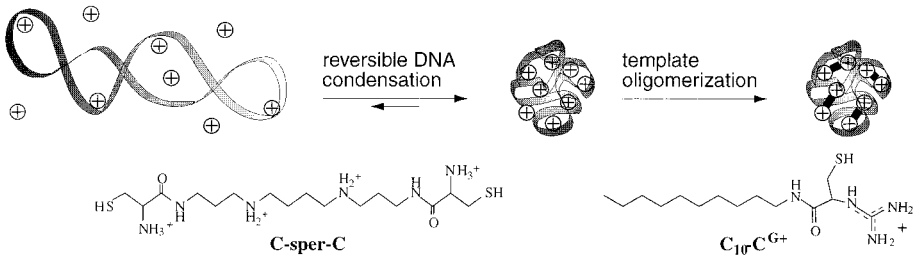

Figure 1. (a) Reversible condensation of DNA is induced by cations with low binding cooperativity. The resulting particles are stabilized by template oligomerization of the monomeric cationic species. (b) Structures of the cationic spermine bis-cysteine (**C-sper-C**) and cysteine detergent ($\mathbf{C_{10}\text{-}C^{G+}}$) monomers.

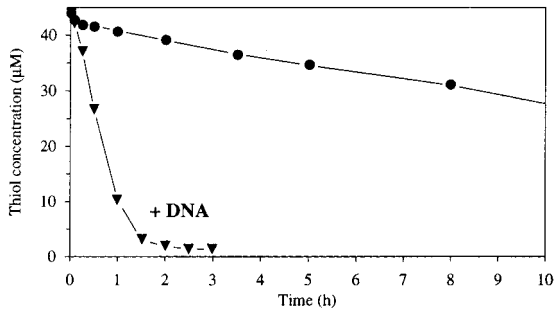

Figure 2. Oxidation rate of spermine bis-cysteine **C-sper-C** is enhanced in the presence of template DNA. **C-sper-C** was 22.5 μM in 10 mL of 2 mM HEPES pH 7.0 (pCMVluc is plasmid DNA containing the cytomegalovirus promoter inserted upstream of the coding sequence of the firefly luciferase and was 60 μM phosphate when present). For each time point, a 0.5 mL aliquot was removed and mixed with 0.5 mL 2× Ellman's reagent. The remaining free thiol was quantitated spectrophotometrically according to Riddles et al.[13]

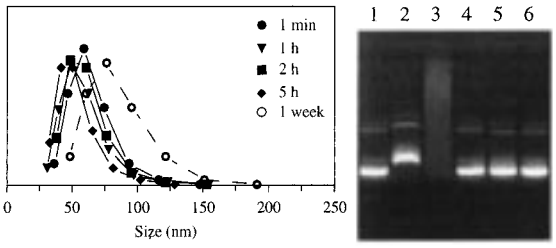

Figure 3. Stability of the $\mathbf{(C\text{-}sper\text{-}C)}_n$/DNA complexes. (a) Particle size does not significantly vary with time. Dynamic laser light scattering was performed with a Zetasizer 3000 (Malvern, Paris) on a 1 mL sample prepared as in Figure 2. (b) $\mathbf{(C\text{-}sper\text{-}C)}_n$/DNA complexes are stable during agarose gel electrophoresis whereas spermine/DNA complexes are not. Each 20 μL sample contained 0.4 μg of pCMVluc in 2 mM HEPES buffer pH 7.0 (control lanes 1 and 6), condensed with 15 μM **C-sper-C** (lane 2), 22.5 μM **C-sper-C** (lane 3), or 22.5 μM spermine (lane 4) and loaded after 5 h in air. The sample in lane 5 was as for lane 3 but adjusted to 6 mM dithiothreitol before loading. The 1% agarose gel was run for 90 min at 8 V/cm in 40 mM Tris-acetate buffer pH 8 and then stained with ethidium bromide.

any kind of calibrated nanometric particles required for material sciences. The process takes advantage of the low cooperativity of binding small monomeric counterions to a macromolecular polyion, followed by a zipper-oligomerization reaction which "freezes" the resulting condensed particles. The DNA particles

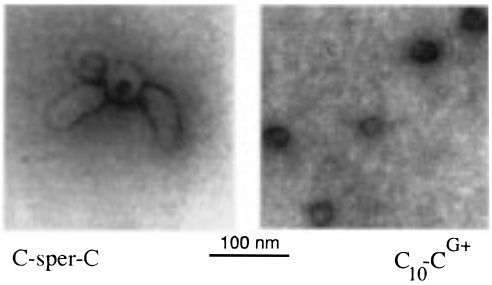

Figure 4. Representative transmission electron microscopy images of $\mathbf{(C\text{-}sper\text{-}C)}_n$/DNA and $\mathbf{(C_{10}\text{-}C^{G+})_2}$/DNA complexes. The polycation complex was prepared as for Figure 2 and left 5 h in aerobic condition. The lipid complex was prepared by mixing the detergent (40 μM) with the plasmid (30 μM phosphate) in 15 mM HEPES pH 7.4 and left in the air overnight. Carbon grids were glow-discharged (110 mV, 25 s), and a drop (5 μL) of sample solution was left on the grid for 1 min. Complexes were negatively stained with 30 μL aqueous uranyle acetate (1% w/w) for 20 s, and excess liquid was removed with blotting paper. Observations were performed at 80 kV with a Philips EM 410 transmission electron microscope.

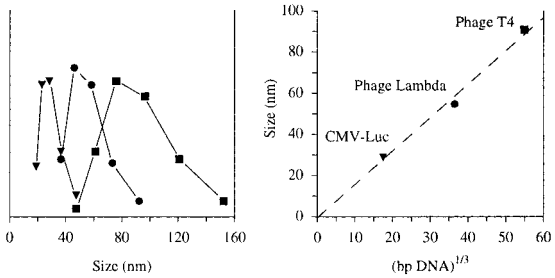

Figure 5. Particle size varies as the cubic root of the DNA size. The sample solution was prepared by adding 5 μL of detergent from a 6 mM stock solution to 10 μg of DNA contained in 1 mL of 15 mM Tris-HCl buffer, pH 8.4. After 24 h, particle size was determined by light scattering as specified in Figure 3. pCMV−Luc complexes had to be vacuum-concentrated twice to scatter enough light.

have a negative surface charge which ensures colloid stability and in vivo diffusion, yet makes them unsuitable for carrying DNA into cells.[1] To become functional as a gene-delivery system, these minimal virus-like particles will now have to be grafted with cell-surface binding residues.

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