High-Yield Preparation of Oligomeric C-Type DNA Toroids and Their Characterization by Cryoelectron Microscopy

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Abstract: The quantitative and reversible compaction of open circular plasmid DNA (7676 bp) into toroids containing one to 19 molecules by sequential treatment with spermine and an excess of uranyl acetate is reported. The toroidal DNA structure was proven by cryoelectron microscopy. Linearized and supercoiled variants of the DNA also gave toroids under these conditions, but yields were significantly lower. In the presence of spermine alone *no* toroids were found. Open circular plasmid B-DNA helix was converted into the C-type helical form upon compaction as was shown by CD spectroscopy (negative peak at 255 nm) and electron microscopy (1.8-nm interduplex distance instead of 2.9 nm). Addition of uranyl salt to the DNA−spermine complexes resulted in the formation of netlike assemblies which further compacted to give toroids. A model containing a hexagonal arrangement of DNA strands with extensive strand crossings is proposed. Curvature and thus toroid formation is thought to be induced by the hydrophobic DNA coating of spermine methylene groups.

Introduction

The compaction of DNA is currently an area of active research because it probably provides the most dense packing of nucleic acids for the transport of DNA in viral vectors. Linear DNA strands and their noncovalent assemblies can be compacted in bulk aqueous solution by the formation of interwound supercoils[1] or by rolling-up into toroids.[2−7] Toroidal compaction of DNA strands has repeatedly been achieved by either high electrolyte concentrations, addition of heavy metal salts, or oligoamines. Yields were however low, and electron micrographs showed only small numbers of toroids, together with large amounts of amorphous precipitates, rodlike assemblies, globules, and/or noncompacted DNA strands. We report here for the first time (i) the conditions for a quantitative and reversible conversion of open circular plasmid DNA (7676 bp) into toroids by spermine and uranyl acetate, (ii) a conversion from B- to C-DNA indicated by high resolved electron micrographs and CD spectra of the toroidal DNA, and (iii) the appearance of a toroid precursor consisting of parallel staggered bundles of DNA strands. The original supercoiled as well as the linearized DNA variants were also investigated for their ability to form toroids under these conditions but were less satisfactory.

Experimental Section

Materials. Spermine [*N*,*N*′-bis(3-aminopropyl)-1,4-butanediamine tetrahydrochloride] from Sigma (Deisenhofen, Germany) was dissolved in 1 mM cacodylate buffer to give a 30 μM solution (pH 6.5). A 1% (w/v) aqueous solution of uranyl acetate was always freshly prepared and filtered (0.22 μm Schleicher and Schuell membrane filters) before use.

Plasmid DNA Preparation. Plasmid pEGlacZ containing the bacterial β-galactosidase gene under the control of the human cytomegalovirus immediate early promoter was constructed using standard molecular cloning techniques.[8] The 7676 bp plasmid was derived by cleavage of pGFP-N1 (Clontech) with Hind III and Bcl I to remove the reporter gene fragment. The β-galactosidase coding region from pSV-β-galactosidase (Promega) was then inserted via a Hind III to Bam HI fragment. Supercoiled (>95%, estimated from gel electrophoresis) plasmid DNA was isolated using a Qiagen kit according to the manufacturer's protocol. The open circle form of plasmid DNA was obtained by limited digestion of supercoiled plasmid with DNase I, resulting in >90% of the desired form. Supercoiled plasmid DNA was digested to completion with Eag I to derive the linear form. In all preparations, a very low level (<5%) of *Escherichia coli* genomic DNA was present. The plasmid DNA used throughout has a (G + C) content of 52.7%. The base distribution is fairly uniform with a few short regions of lower (G + C) content (25%, scanned with a 50 bp window). The overall (G + C) content is close to that found in *E. coli* 51%. DNA was dissolved in 1 mM cacodylate buffer at pH 6.5 to yield a 30 μM solution.

Sample Preparation. One milliliter of the 30 μM spermine solution was added dropwise to an equal volume of the buffered solution of

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DNA within 30 min to yield a final concentration of 15 μM DNA (based on base pair molarity) and spermine (pH = 6.5). The solution can be stored at 4 °C for as long as 24 h but should be used as soon as possible. Aged solutions gave lower yields of toroids.

Electron Microscopical Preparations. (a) Negative staining: To obtain the precursor structures in the toroid formation process (Figure 4) we proceeded as follows: A droplet of the DNA−spermine solution (5 μL) was placed on hydrophilized glow-discharged carbon grids (treated for 60 s at 10 mA (8 W) and 5×10^{-1} bar in argon plasma using a BALTEC MED 020 (BALTEC, Liechtenstein)) and blotted to generate a thin layer of the solution on the grid surface. A drop of 1% uranyl acetate was immediately added and left for 15−30 s before blotting. The grid was then dried in air. Depending on the uranyl incubation time, different stages in the toroid formation process were observed.

Almost quantitative yields of toroids were obtained on carbon grids (Figure 1a), provided that the uranyl acetate contact time was longer than 30 s, typically 45−60 s. If the samples had been already dried on the grid surface before uranyl staining solution was added, *no* toroid formation occurred; only random DNA fibers were observed, which can also be found in the absence of uranyl acetate. Ill-defined precipitates were obtained if spermine was omitted before treatment with uranyl acetate.

The above procedure was applied in the same manner to samples of the supercoiled and linearized DNA variants. The original supercoiled DNA in comparison gave a reasonable but less quantity of toroids and also showed noncompacted linear assemblies (Figure 1b). In the case of linearized DNA, the yield of toroids was almost comparable to that of the open circular variant, but the toroids showed a high tendency to cluster (Figure 1c). Nontoroidally compacted strands and rods were also found. We have therefore limited our experiments to the open circular DNA variant.

(b) Cryomicroscopical preparation: 10 μL of the described DNA−spermine solution was mixed with 20 μL of the freshly prepared and filtered 1% uranyl acetate solution corresponding to a 300-fold excess of uranyl cations with respect to DNA phosphodiester groups (final pH 4.5). The solution was allowed to equilibrate for 120 s to achieve a quantitative formation of toroids. The solution was then placed on holey-foil grids and blotted with filter paper to create ultrathin layers (100−200 nm) of the suspension spanning the holes of the carbon foil. All preparations were performed in a temperature and humidity controlled chamber (CEVS),[9] routinely operated in our laboratory to avoid unwanted effects upon evaporation of the solvent. The layers were vitrified (amorphous solidification) by propelling the grids into liquid ethane using a spring controlled shutter. The procedure takes around 3 min from the moment of uranyl salt addition, but toroids can still be found in the final solution for at least 2 h, before ill-defined precipitates start to appear.

Electron Microscopy. The vitrified samples were transferred under liquid nitrogen into a Philips CM12 transmission electron microscope (TEM) using a GATAN cryoholder (Model 623) and were directly imaged at a sample temperature of −173 °C and a primary magnification of 60000× following the microscopes' standard "low-dose" protocol. The total electron dose applied to the imaged area was 100 e/nm^2, which causes the evaporation of a water layer on the order of 0.015 nm from one surface.[10] This effect can be neglected considering the overall thickness of the specimen layer of about 150 nm. The defocus was chosen to be 0.9 μm corresponding to a first zero of the transfer function at 1.8 nm (C_s = 2 mm).

Fourier Analysis. Electron micrographs were digitized using an EMIL digitizer (Image Science GmbH, Mecklenburgische Str. 27, D-14197 Berlin, Germany) and stored on a DEC alpha workstation. Fourier transformations were calculated using IMAGIC 5 software (Image Science GmbH, Mecklenburgische Str. 27, D-14197 Berlin, Germany).

CD Spectroscopy. CD spectra were recorded on a Jasco J600 at room temperature. Open circular DNA solution (3 mL, 15 μM) was measured in and without the presence of an equimolar ratio of spermine (Figure 3a,b). After again addition of 10 μL of 1% uranyl acetate to the DNA−spermine mixture, corresponding to an equimolar ratio of uranyl cations with respect to the DNA phosphodiester groups, the measurement was repeated (Figure 3c).

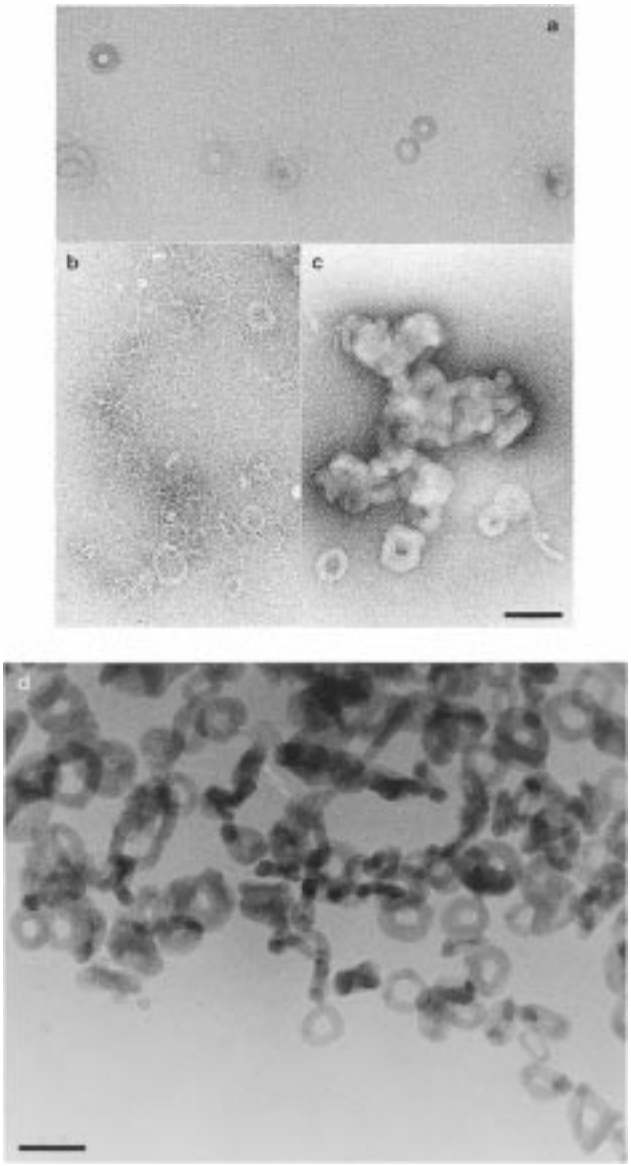

Figure 1. (a−c) Air-dried preparations of open circular, super coiled, and linearized DNA (pEGlacZ) in the presence of an equimolar ratio of spermine and an excess of uranyl cations examined r to determine differences in terms of yield of compacted toroids: (a) a uniform distribution of toroids from open circular DNA; (b) a lower population of toroids occurring together with a variety of linear assemblies from the supercoiled DNA variant; (c) clustering of toroids and rodlike assemblies frequently observed from linearized DNA preparations; **(**d**)** representative cryoelectron micrograph showing toroidally compacted open circular plasmid DNA (15 μM) formed in the presence of an equimolar ratio of spermine and an 300-fold excess of uranyl cations. No unassembled DNA strands can be detected (bars are 100 nm).

Results

A buffered 30 μM solution of spermine was added over 30 min to a buffered solution (1 mM sodium cacodylate at pH 6.5) of 30 μM (based on base pair molarity) 7.7 kbp (i) open circular plasmid DNA, (ii) linearized DNA, and (iii) supercoiled DNA (pEGlacZ). The 300-fold excess of uranyl acetate with respect to DNA phosphate groups was then added. The final pH was 4.5. Air-dried samples of all three preparations were then

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analyzed by electron microscopy. Open circular DNA gave an essentially quantitative yield of toroids, individual toroids being nicely separated from each other (Figure 1a). No linear strands or rodlike structures could be detected over the whole area of the grids, although they were seen in all probes, which were not pretreated with spermine. Supercoiled DNA preparations also gave fully compacted toroids. Linear strands and thin looplike structures were also frequently detected and point to incomplete compaction under the conditions reported (Figure 1b). Preparations of linearized DNA again showed high yields of toroids, which rather tend to cluster (Figure 1c). Rods were also observed occasionally. Circular plasmid DNA was obviously most easy to handle, and we therefore selected this type for further characterization of the toroids. Optimization of the toroid preparation for the two other DNA types has not been attempted.

For the cryomicroscopical examination, the freshly mixed solution of open circular DNA, spermine, and uranyl acetate was left to equilibrate for about 120 s, allowing the complete formation of toroids before freezing in liquid ethane was performed. Cryoelectron micrographs of vitrified samples showed exclusively toroidally compacted DNA (Figure 1d). No unassembled or nontoroidal DNA strands were detectable. Upon addition of excess EDTA to this solution (final concentration 2 mM), the toroids disappeared and similar disorganized DNA strands were found under the electron microscope as before the addition of uranyl salt. *Without uranyl acetate no toroids were found*. The spermine−uranyl−DNA toroids were stable for about 2 h and then irreversibly rearranged to form ill-defined aggregates. When uranyl acetate was added before the spermine, such precipitates were formed immediately.

Due to rotation in solution, different projections of the toroids were detectable on cryoelectron micrographs. Tilting of the specimen allowed the determination of the diameter of *individual* torus cylinders, which was found to be constant from several view angles (Figure 2d). This proves that DNA toroids have a circular cross section in aqueous solution. Earlier freeze-etching experiments by Marx[11] suggested the same shape. Furthermore, high-resolution analysis of the images revealed large numbers of strands running concentrically (Figure 2a). The Fourier transformation of the micrograph clearly indicates 1.8 ± 0.1 nm interstrand spacings. This corresponds to the center-to-center interhelix distance found in Z-[12] and C-DNA[13] in crystals, whereas the spermine condensed B-DNA strand distance is known to be on the order of 2.9 nm.[14]

To differentiate between Z- and C-DNA we obtained CD spectra. Upon addition of spermine, the B-type spectrum of DNA (Figure 3a) only shifted slightly to longer wavelengths (Figure 3b). However, upon again addition of an equimolar amount of uranyl acetate, the positive 273-nm band disappeared and was replaced by a negative band at 255 nm (Figure 3c). This CD-band spectrum is identical with the spectrum reported for C-DNA spectra[15,16] and is different from Z-DNA spectra.

The central hole diameter of toroids on cryomicrographs varied between 15 and 40 nm, the external diameter from 50 to 110 nm, and the thickness of the toroidal cylinder from 11 to

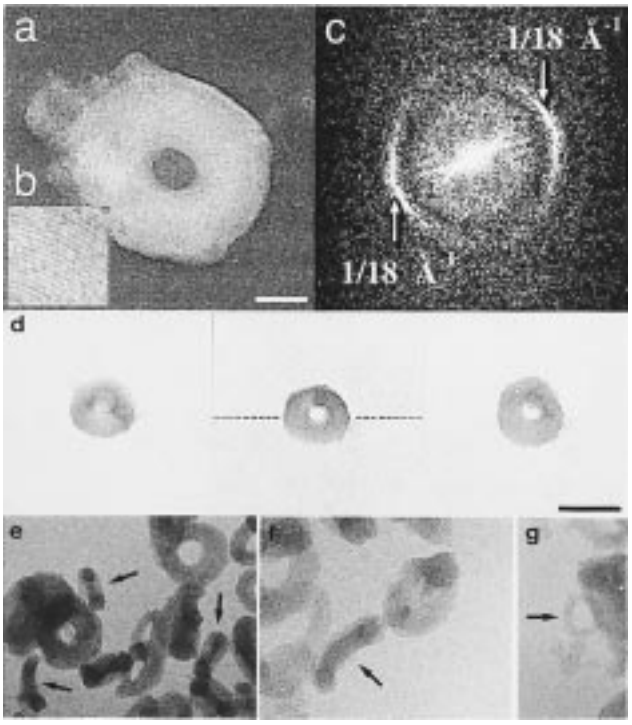

Figure 2. (a) Well-preserved torus of an air-dried sample clearly indicating almost circumferential striations. Volume calculations suggest a content of 19 DNA molecules. (b) 2× enlarged area taken from part a. (c) Fourier transform of part a indicating spots at 1.8 ± 0.1 nm spacings. (d) Tilt series of an individual torus at tilt angles of 45°, 0°, and −45°. The tilt axis is indicated. (e) Cryoelectron micrograph showing side-view projections of several tori (arrows). (f) Cryoelectron micrograph showing the smallest toroid found, whose dimensions correspond to one plasmid DNA molecule (bar is (a) 30 nm and (d−f) 50 nm).

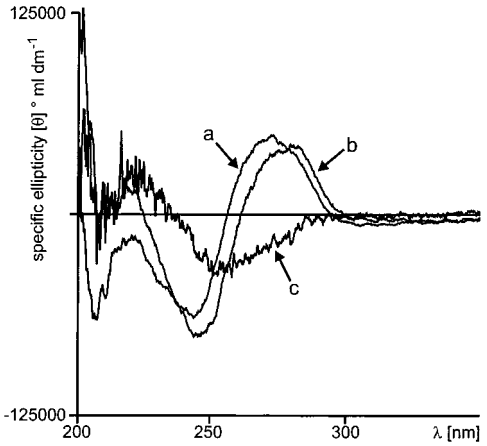

Figure 3. CD spectra of (a) 15 μM buffered open circular plasmid DNA; (b) 15 μM plasmid DNA in the presence of an equimolar ratio of spermine; (c) 15 μM plasmid DNA in the presence of equimolar ratios of spermine and uranyl acetate.

28 nm. The calculated hollow volume V of the toroids ($V = 2\pi^2 r^2 R$ (cf. Figure 6a)) ranged from 0.8×10^4 to 1.7×10^5 nm^3. These dimensions are similar to earlier reports,[17] although the numbers of DNA base pairs per plasmid (1350 and 2700 bp) in these cases were lower.

With a 1.8-nm center-to-center distance of the C-DNA helices[13] and a distance between base pairs of 0.33 nm,[15] a 7676 bp cylinder would have a length of about 2.5 μm and a volume

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of 6300 nm^3. If we assume a hexagonal packing of the DNA and a packing fraction for parallel cylinders of 0.74 (ratio of the strands cylinder volume and the total toroid volume), the calculated toroid volume for a single DNA molecule would then be 8500 nm^3. This is in agreement with the measured volume of the smallest toroid we have detected (Figure 2f). A single plasmid DNA molecule winding its duplex pairwise for 10 times around a central hole, with an average diameter of 15 nm, would agree with the model proposed in Figure 7. Most toroids contained between three and 19 plasmids, four to five plasmids being found most frequently.

We tried to detect intermediates between linear and toroidal DNA assemblies by reducing the incubation time of uranyl acetate to less than 30 s. Extended nets of parallel DNA fibers were observed, which further annealed to form cylindrical bundles which then coiled up to yield toroids of different diameters (Figure 4a). Smaller assemblies of DNA strands were also found. Figure 4b−e shows examples of such early stages of compaction. "Supercoiling" of the oligomeric circular plasmids leads to the formation of terminal loops (Figure 4c,d), and contractions of the assemblies initiate a stacking of the terminal loops as is indicated in Figure 4f. An alternative pathway is the successive winding around a preformed loop at one end of the circular DNA (Figure 4e).

The spermine−uranyl−DNA toroids were tested for their ability to transfect COS-1 cells (SV40 transformed African Green Monkey kidney cells). Strongly reduced cell viability was observed in all samples due to the toxicity of the uranyl salts.[18] All samples containing uranyl salts showed significantly reduced protein levels, less than one-third of that seen in control samples (data not shown). Various other transition metal salts have been used to compact DNA in the literature, but these are also toxic *in vitro*[19] and *in vivo*.[20]

Discussion

Cryoelectron microscopy has proven for the first time the three-dimensional torus structure of the multistranded DNA in the vitrified state. The formation of the toroids only occurs, if spermine is added first to form linear assemblies followed by uranyl salt. The latter induces curvature within minutes. Many earlier preparations involved first treatment with oligoamines or salts in solution, followed by contrast staining with uranyl acetate on the electron microscope grid. Since the DNA−spermine sample was probably partially dried before addition of the staining solution, a large variety of assemblies were obtained in addition to a low number of toroids. Other metal ions, e.g. cobalt, in the presence of ammonia or oligoamines have produced similar effects,[7] but the yields of toroids were invariably low. In one case manganese was applied without oligoamines.[21] Compaction occurred slowly and some clustered toroids were found.

If one arrests the toroid formation shortly after the addition of uranyl salts, intermediate states were obtained. The most prominent structures were polymeric assemblies of parallel overlapping DNA strands (Figure 4a). They might correspond to the parallel arrangement of DNA−spermidine complexes observed in liquid crystalline phases.[22] The addition of spermine to B-DNA presumably leads to partial dehydration, but the formation of C-type double helices requires uranyl salts. We speculate that the sperminium counterions to the phosphate groups are replaced by uranyl ions, the spermine remaining, however, bound by hydrogen bonds to both the phosphate and

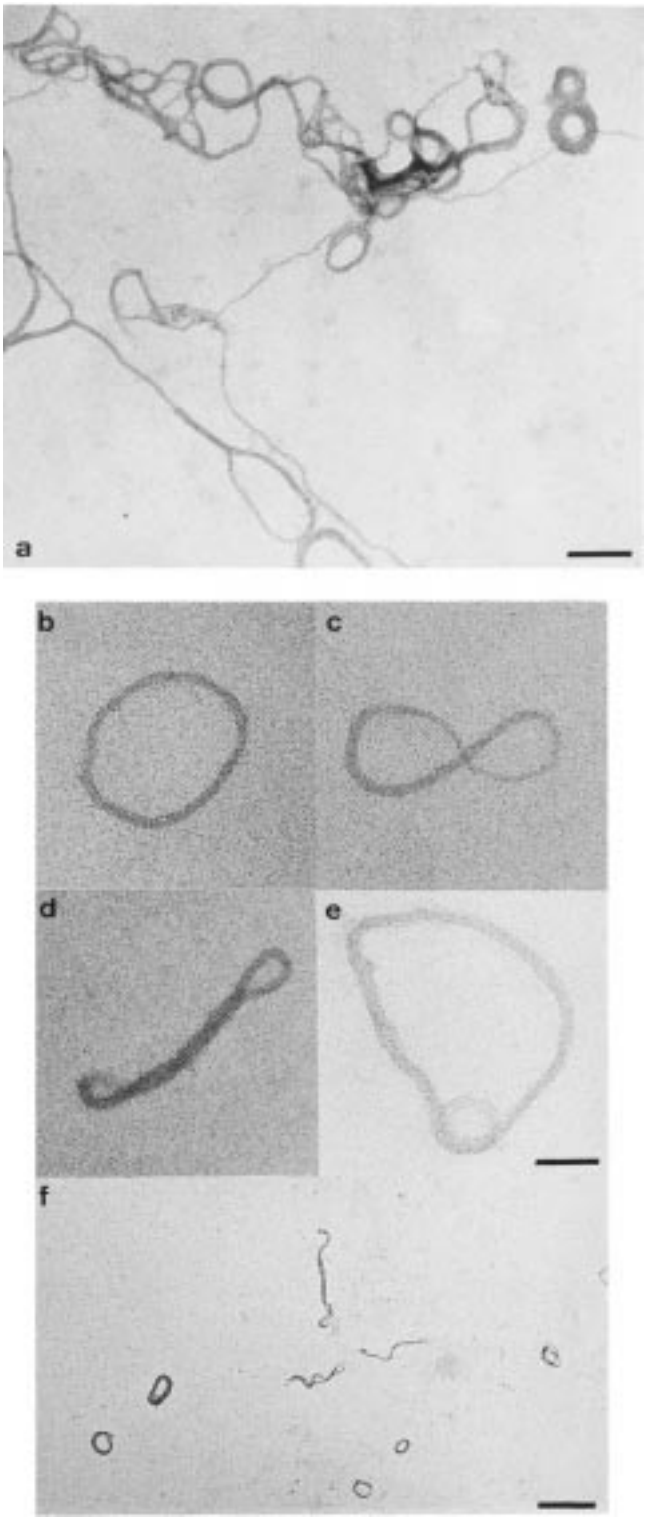

Figure 4. Electron micrographs of open circular DNA molecules at various stages in the formation process of toroidal compaction: (a) network of end-on and side-on oligomerized DNA strands and isolated toroids; (b) circular oligomer of open circular plasmid DNA; (c) circular assembly containing several DNA molecules undertaking a first crossover; (d) supercoiled oligomeric assembly of DNA showing several crossovers; (e) initial terminal spooling of a circular DNA assembly; (f) different stages of torus formation in a single preparation (bar is (a) 100 nm, (b−d) 50 nm, and (f) 150 nm)

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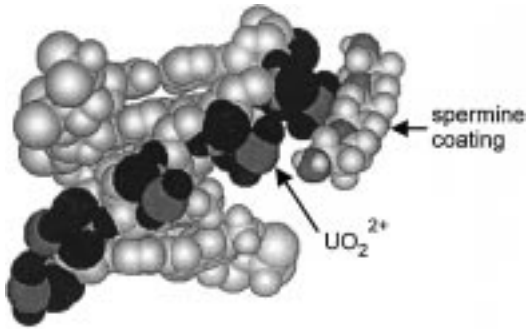

Figure 5. Proposal for the molecular arrangement of the uranyl−spermine coating in C-DNA helices.

the oxygens of the uranyl group (Figure 5). The phosphate/uranyl ratio within the assembly should be close to 1:1. This should lead either to electroneutral strands or even to an overall positive charge of the nucleic acid complex.[23] Cross-linking by the divalent uranyl ions to neighboring strands should be prevented by the spermine coating. The double-helix−polyamine−uranyl complex should now have a largely hydrophobic surface formed by the spermine methylene groups. Several DNA strands are already linearly connected and are now rolled-up to oligomeric toroids by the hydrophobic effect. They provide the smallest possible surface for the relatively rigid fibers. The situation is similar to double-chain lipids containing a rigid diyne unit, where similar toroids have been seen under the light microscope.[24] They are different from DNA probes with a large excess of cetyltrimethylammonium coating, which tend to appear as micrometer-sized globules[25] or with equimolar double-chain cationic lipids, which lead to micrometer-long fibers.[26] The polyelectrolyte DNA core obviously rigidifies lipid bilayers; long-chain lipid counterions on the other hand inhibit rolling-up effects of the DNA units.

With respect to the detailed structure of the DNA toroids, there exists two different models in the literature: a hexagonal arrangement of circular DNA strands with varying degrees of bending in a spool[11] and the constant DNA-radius model.[7] Side-view projections of both models are shown in Figure 6c,d. The latter model, although elegantly overcoming the logistic problem of several crossovers while packing, predicts V-shaped side views which are not found on the cryomicrographs (Figure 2e). Only the older spool model, implying unfavorable crossovers and a cylindrical side view, is consistent with the micrographs.

If the incident electron beam strikes the toroids parallel to the direction of the hexagonal layers, the 1.8-nm interhelix spacings can be clearly resolved and measured. These patterns are, however, never resolved in a complete circle and 5−10% of the torus circumference is always nonordered (Figure 2a). We interpret this region as one of multiple synchronized DNA crossovers, which are a geometrical necessity to allow the hexagonal packing of the strands for the remaining part of the torus. The model (Figure 7) shows a possible way to arrange the circular plasmid in a toroidal pattern: two double helices run parallel through the torus and finally end up in a terminal loop.

CD spectra of DNA solutions indicate that the B-form is retained if only spermine is added to the DNA solution (Figure 3b). This finding confirms earlier studies which indicated the

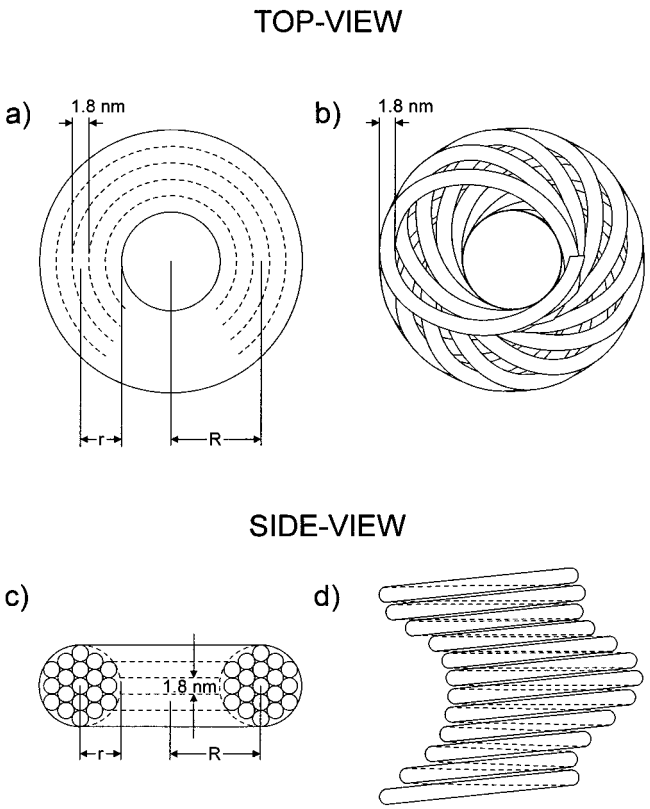

Figure 6. Comparison of "spool" [11] and "constant loop" [7] models for the compaction of DNA into toroids: (a) top view and (c) side view of the spool model; (b) top view and (d) side view of the constant loop model. The V-shaped side view (d) of the latter model is not in accordance with the cryoelectron microscopical images in Figure 2e.

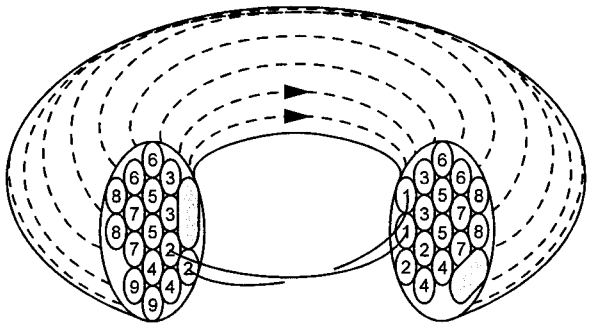

Figure 7. Spool model torus cut in the region where several crossovers of DNA strands occur to guarantee the hexagonal packing for the remaining part of the torus. The crossover pattern is intelligible by pursuing opposite strands in continuous numbers.

retention of the B-conformation upon compaction.[22,27] These preparations contained, however, large amounts of nontoroidal structures as was revealed by electron microscopy. Upon addition of uranyl acetate, we achieved for the first time a quantitative yield of toroids in cryopreparations, and a transition from the B- to C-form was suggested by a combination of Fourier analysis of electron micrographs and CD spectra. This transition, a result of DNA dehydration, was deduced earlier from the CD spectra of intact phages[28] and was also repeatedly found in dried B-DNA samples.[13] The alternative B−Z transition was not observed. It is also unlikely since it usually only occurs in longer alternating CG sequences.[29,30] This is

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not the case in our probe. Here the base distribution is fairly uniform with only few short regions of lower (G + C) content.

The quantitative differences which occur in toroid formation between the three kinds of DNA, all having essentially the same molecular mass, presumably reflect the DNA stiffness. The most rigid supercoiled DNA gives linear or looplike assemblies in addition to the toroids, the most flexible linearized DNA gives high yields of toroids which tend to cluster. Nicked circular DNA is intermediate in terms of flexibility and gave the best results, namely quantitative yields of individual toroids. The "lack of ends" in supercoiled DNA may also play a role in its resistance toward compaction.[31]

However, this study shows that the formations of all these assemblies depend strongly on the preparation conditions, namely the successive addition of spermine and the 300-fold excess of uranyl ions, the pH, and the incubation time. Different experiences of other groups, e.g. Bloomfield's[21] success with supercoiled DNA (pUC 18) and manganese salt (no spermine), are presumably related to the different time courses of compaction. Spermine and uranyl ions initiate the toroid formation within 60 s, and the manganese-induced process takes roughly 1 h at comparable concentrations. The relatively fast and drastic effect of the hydrophobic interaction is thought to be the main cause for the high yield of compaction and the B−C helix rearrangement upon spermine−uranyl treatment. Manganese-(II) ions obviously cause a relatively slow dehydration and leave the B-DNA conformation unchanged.

As a possible mechanism for the formation of C-DNA toroids from B-DNA, we propose the three consecutive steps:

(i) Spermine neutralizes the DNA surface and triggers the formation of parallel and staggered bundles of DNA strands. These assemblies tend to crystallize.[13]

(ii) If uranyl salts are added, they substitute for spermine as a counterion for the phosphodiester groups. The spermine remains, however, bound to the DNA and is attached to the uranyl and phosphate oxygens by hydrogen bonds. It thus prevents precipitation by the bivalent uranyl ions, which would otherwise bind to a second phosphate group of an adjacent DNA strand.

(iii) The outer spermine coating renders the DNA surfaces largely hydrophobic, in the core all charges are neutralized and the strands become more flexible. The hydrophobic effect now causes a rolling-up to form toroids in the same manner as has been observed for lipid bilayers.[24] A variable number of DNA strands can be involved in the early network, before the spooling begins.

Quantitative compaction into individual toroids has been achieved with open circular DNA. For other types of DNA, the procedure has to be optimized to get rid of unwanted clustering or rod formation.

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