Spontaneous formation of liquid crystalline phases and phase transition in highly concentrated plasmid DNA

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Abstract

We have studied liquid crystalline (LC) properties of supercoiled plasmid DNA samples: pBSK (2958 bp) and pGEM (3000 bp), using Polarized Light Microscopy (PLM), Circular Dichroism (CD) and UV-Vis spectroscopy techniques. We report the influence of isolation methods on plasmid LC behaviour. Using PLM, we show the spontaneous formation of cholesteric fingerprint like textures. Preliminary studies on liquid crystalline phase transitions in pGEM showed the irreversibility of LC phase formation as an effect of changes in the tertiary structure of supercoiled plasmids. In UV-Vis spectroscopy measurements a hyperchromic effect was observed as a function of increasing temperature. CD spectra clearly show structural changes and probably mismatching of DNA bases during the cooling process. Finally we observe an irreversibile phase transition in plasmid DNA which is very different from that previously reported for linear DNA.

Introduction

Reports concerning liquid crystalline phases in DNA solutions started to appear in 1950 (1). Investigations were subsequently extended to the formation of lyotropic liquid crystalline

phases of linear DNA (2), other helical biological polymers (3), and synthetic polymers which also possess helical structures (4). The properties of observed anisotropic phases were found to strongly depend on temperature and polymer concentration and to a smaller extent on the ionic strength and other characteristics of the polymer chain (e.g. persistence length) (2), (5). However, interest in liquid crystalline phases in plasmid DNA waned until 1989 when Jim Torbet and Elisabeth DiCapua described the liquid crystalline behaviour of supercoiled DNA (6). The essential results of this seminal study were that negatively supercoiled DNA could form interwound configurations in liquid crystals at concentrations between 30 to 90 mg/ml with little evidence for the presence of toroidal configurations.

The molecular organization of DNA *in vivo* bears some resemblance to liquid crystal DNA phases observed *in vitro* (2). However, few reports have been published concerning LC phase formation in supercoiled DNA (7), (8), (9). At the primal level of DNA structure in relaxed linear DNA the two DNA strands twist around the helical axis on average every 10.4 - 10.5 base pairs, if the DNA is constrained into a closed circular form then the molecule will form a supercoil characterised by a specific writhe. The number of helical turns around the helical axis is the twist (Tw). A useful parameter is the linking number (Lk) which is the number of base pairs divided by the helical pitch (10.4 in canonical B DNA) such that Lk_0 = bp/10.4. The number of times that the DNA molecule crosses over itself (the interwindings) defines the supercoil and is referred to as the writhe (Wr). Writhe and twist are equivalent and Lk = Tw + Wr (10) .A consequence of this is that $\Delta Lk = Lk - Lk_0 = \Delta Tw + Wr$ and thus any change in the linking number may be accomodated by changing the helical twist or by forming interwound structures (Wr). Supercoiling therefore depends upon the addition or subtraction of twist, extra twist results in positive supercoiling and the removal of twist produces negative supercoiling. Supercoiled DNA exists in two helical forms either as a toroid configuration or a plectonemic configuration. A negative supercoil for example can form either a left handed toroid or a right handed two start plectoneme. Closed circular DNA usually exists *in vivo* in a plectonemic negative supercoiled configuration and right-handed interwinding is the most common conformation. Supercoiling in bacterial chromatin is involved in many cellular mechanisms such as replication, transcription and DNA repair and is associated with the formation of nucleoprotein complexes (11). Although supercoiling is also involved in the packaging of plasmid DNA in the bacterial cytoplasm, until now there have been reports showing spontaneous formation of cholesteric fingerprint like textures only at concentrations up to 25 mg/ml (9)

In the case of plasmid DNA, supercoiling plays an important role in LC phase formation. Supercoiling responds to changes in environmental induced factors such as counterions (12) and temperature (13) and thus influences the critical boundaries of isotropic to liquid crystal phase transition. Changes in plasmid DNA structure induced by these factors can affect

essential functions that are necessary for cell viability. Bacterial cells can maintain superhelical tension at fixed levels up to temperatures as high as 50^{o}C (14) using specific enzymes - topoisomerases and gyrases that are able to alter the local DNA superhelical density. With increasing temperature DNA undergoes a relaxation process and the linking number (Lk) increases as twist and writhe are affected (15). Upon cooling negative supercoiling is increased in DNA and the Lk decreases as explained by Mizushima and colleagues (16). However, if the temperature increases beyond a critical value, bacterial DNA is unable to return to the same degree of negative supercoiling. The influence of thermal changes on the organization of supercoiled DNA is therefore an important issue. Investigating liquid crystalline phase transitions *in vitro* may provide insights into what happens with condensed genetic material *in vivo* during exposure to extreme conditions such as high temperature. Since supercoiled plasmids are involved in the regulation of replication and transcription (17), two critical processes for living organisms, a better understanding of supercoiling would have an important impact on our understanding of these essential processes as well as providing information concerning their control by, for example, gene therapy. Superhelical DNA topology can also act as a biosensor to subtle changes in external conditions or as a signal transducer influencing gene expression.

In this work we applied the techniques of polarized light microscopy, circular dichroism and UV-Vis spectroscopy to investigate the formation of liquid crystalline phases in highly concentrated samples. As far as we are aware, this is the first report where cholesteric finger print like textures occur spontaneously in concentrations as high as 60 mg/ml. Finally, we present some preliminary studies on phase transitions in plasmid DNA.

Materials and Methods

Isolation of pBluescript plasmid DNA

Colonies of plasmid containing *E. coli* were bred on Petri plates containing LB agar with antibiotic. A single colony was removed to cone flasks containing Luria Bertani broth (LB) (10 g Tryptone, 5 g yeast extract, 5 g NaCl, glycerol, pH 7.5). The volume of the culture was restricted to 1/5 of the flask volume due to the shaking process during incubation under optimized conditions for bacterial growth (24 h, 185 turns per minute, 37^{o}C). Ampicillin was added to a final concentration of 100 µg/ml.

18 litres of LB medium with ampicillin were inoculated with 200 ml of *E. coli* culture and then incubated at 37^{o}C overnight under continuous shaking. Cells were then harvested by centrifugation and stored at -20^{o}C.

Pellets of *E. coli* cells were suspended in 180 ml ice-cold solution of buffer P1 (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM Glucose, 100 µg/ml RNase A) and left for 5 min. at room

temperature. The suspension was then equally divided into two flasks of the same volume and 180 ml of buffer P2 (200 mM NaOH, 1% SDS) were added to each flask. The flasks were left on ice until the solutions became transparent indicating that cell lysis had occurred. After 10 min. of incubation on ice, 135 ml of buffer P3 (3M CH_3COOK pH 5.5) were added to each flask in order to precipitate genomic DNA, cellular debris and proteins. The solutions were gently agitated for another 5 min. on ice and then centrifuged at 10000 rpm for 15 min. The supernatant was transferred to six new flasks and plasmid DNA precipitated by the addition of two volumes of ethanol. The DNA pellets were dried and dissolved in 18 ml of TE buffer (1 mM EDTA pH 8.0, 10 mM Tris pH 7.4). 100 µl of 10 mg/ml RNase were added and left for 1 h at 37^{o}C to remove RNA. Subsequently, 20 µl of 20 mg/ml Proteinase K was added for 1.5 h at 37^{o}C.

Purified samples were precipitated with 0.7 volumes of isopropanol and centrifuged at 15000 rpm for 10 min. The pellets were washed with 70% ethanol and centrifuged as described above. The pellet of pure plasmid DNA was dried under vacuum. Finally, samples were dissolved in 3 ml of water and lyophilized. In general, 30-40 mg of plasmid DNA were obtained from 3 litres of LB solution.

Purification of supercoiled pBluescript DNA by centrifugation in CsCl gradients

After standard isolation as described above, caesium chloride (CsCl) density gradient centrifugation was performed on the plasmid DNA. Freeze dried DNA was dissolved in 20 ml of milliQ water and mixed with 20 ml of 2 g/ml CsCl (final concentration CsCl = 1 g/ml). This was equally divided into two ultracentrifuge polyallomer tubes and 0.5 ml of 10 mg/ml ethidium bromide (EtBr) was added to each. The tubes were centrifuged in a Beckman rotor 70.1 Ti at 48000 rpm for 21 hours at 20^{o}C in a Beckman J5 centrifuge. 30 µl of 10 mg/ml RNase was then added and the samples were once again centrifuged under the same conditions. In order to remove ethidium bromide from the DNA, extraction with water saturated n-butanol (50 ml 0.2 M NaCl, 50 ml n-butanol) was repeated seven times. 15 ml of solution was then immersed in 3 litre TE solution. CsCl was removed by dialysis.

Purification of pGEM®-T vector on a column using CompactPrep Plasmid Giga Kit

The isolation of pGEM ®-T plasmid was carried out using standard procedures. Buffers P1, P2 and P3 were used for cell lysis as described for the pBSK plasmid. A slight difference was in the amount of prepared culture volume for the experiment. Instead of 3 litres used for isolation of the pBluescript, for pGEM ®-T plasmid only 2.5 litres could be used because of limitations imposed by further purification using the Giga-prep Kit technique. The supercoiled plasmid DNA was purified according to the manufacturer's instructions.

Plasmid purity.

To check the quality and amount of isolated pBSK (2958 bp) and pGEM (3000 bp), UV-VIS spectroscopic measurements were carried out. Samples of superhelical DNA were appropriately diluted and transferred to standard 1 ml quartz cuvettes. The maximum of absorption of nucleic acids is λ_{max} =260 nm. However, samples may be contaminated with proteins or sugars, which absorb respectively at λ=280 nm and λ=230 nm. The ratio of absorption (A_{260}/A_{280}) in the range from 1.80 to 2.00 indicates relatively pure DNA.

The measured ratios of absorbencies A_{260}/A_{280} were 1,82 and 1,65 for supercoiled pBSK and supercoiled pGEM, respectively. A small amount of protein was therefore present in the supercoiled pGEM solution. 3 mg of supercoiled pBSK and 4 mg of pGEM were obtained. Both plasmid samples were stored at 4^oC.

Polarized light microscopy

PLM is a very useful tool for the recognition of liquid crystalline phases. For PLM a 15 µl droplet of 60 mg/ml was deposited between glass slides. Photos were taken with an Olympus 60BX microscope, under crossed polarizer conditions. During observation 10x and 20x magnification objectives were used. The microscope was equipped with a hot stage and CCD camera. The observation of phases as well as phase transitions as a function of temperature was performed in the temperature range 20^oC - 80^oC.

Circular dichroism

CD is a powerful and sensitive spectroscopic technique probing secondary structure or conformation of chiral molecules such as DNA. A Circular Dichroism Spectrometer Chirascan was used in our experiments, equipped with a temperature controller which allowed

analysis of denaturation and renaturation of plasmids in the frame of LC phase transitions. Solutions were prepared by diluting the samples to a final concentration of 45 µg/ml.

Results

Plasmid characterization

pBluescript vector (DNA concentration of 0.75 µg/µl) and pGEM (DNA concentration 2.5 µg/µl) were loaded on 1.0 % agarose gel containing intercalating ethidium bromide at a concentration of 1µg/ml. Electrophoresis was carried out in 0.5x Tris-borate-EDTA (TBE) buffer at 70V and 0.5 A. The homogeneity of supercoiled DNA was confirmed and neither nicked circular nor linear DNA was observed. However, there was a distribution of topological isomers of supercoiled DNA (Figure 1A).

Fig. 1.

Quantification of the intensity of bands in a gel using SigmaGel software (provided by Jandal Scientific) allowed the estimation of the relative distribution of topoisomers present. In pBluescript plasmid DNA the predominant isoform corresponded to 96% whereas in pGEM the predominant isoform constituted 90.5%.

LC cells of supercoiled pGEM and pBSK

Plasmids: pGEM and pBSK isolated using the column technique and CsCl centrifugation respectively were further used for LC cells preparation. The same concentrations of up to 60 mg/ml of purified materials were placed between two glass plates and closed without a spacer for microscopic analysis. Both samples were kept at room temperature for the same time period. No phases were formed in pBSK samples (results not shown) though birefringent domains were clearly observed in the 60 mg/ml pGEM plasmid DNA LC between crossed polarizers of the microscope [Fig. 2].

Fig. 2.

LC phases in samples of highly concentrated supercoiled DNA have been reported in (6), where it was postulated that superhelical plasmids form liquid crystals at concentrations between 30 mg/ml up to the highest concentration studied, 80 mg/ml. The microscopy photographs presented here confirm related experiments with Small Angle Neutron Scattering and X-Ray diffraction (6). The observation of translucent birefringent domains under PLM was continued over a period of several weeks. Three months after the preparation of the liquid crystal cell a fingerprint-like texture appeared [Fig. 2]. This type of texture is characteristic of

a cholesteric phase with a homeotropic alignment of molecules. The cholesteric pitch, measured for twice the distance between the fringes was found to be 4 μm, in agreement with scientific reports concerning cholesteric phases in supercoiled DNA (12). This observation indicates that in addition to a macroscopic anisotropic organization observed directly after the preparation of LC cells, closed-circular supercoiled DNA molecules spontaneously form cholesteric microdomains.
These experimental data concur with the prediction from theory that the cholesteric phase of fingerprint texture results from the chiral nature of the supercoiled plasmids.

Phase transition in pGEM

A hot stage with temperature controller was used to investigate phase transitions in plasmid DNA. Heating the sample of pGEM (c_{DNA} = 60 mg/ml) up to 70^{o}C did not affect the sample, but higher temperatures very rapidly changed the phase from anisotropic to isotropic, in agreement with published data reporting the high sensitivity of supercoiled DNA boundaries to temperature (12). However, the process was not reversible, since upon cooling the cholesteric fingerprint-like texture did not reappear in microdomains. Although the birefringence, at around 30^{o}C was observed over the entire cell area, there was no specific LC texture [Fig. 3].

Fig. 3.

Circular dichroism measurements as a function of temperature were carried out to investigate the behaviour of DNA structure. The isotropic phase appeared when supercoiled plasmid was slowly heated to 75^{o}C (Δ1^{o}C per minute) and anisotropy was restored when the sample was cooled to 25^{o}C (Δ1^{o}C per min.). Measurements were carried out stepwise every 5^{o}C. Due to thermal variations structural changes were observed [Fig. 4A]. High temperature induces untwisting of the plectonemic structure and denatures DNA. For highly concentrated samples of supercoiled plasmid there is a high probability that, after slow cooling, the DNA may not return to the same liquid crystalline organization as before heating.
In parallel with dichroic spectra measurements, changes in absorbance were also followed as a function of temperature. Supercoiled DNA was quite stable up to 50^{o}C and from 55^{o}C rapid denaturation began as shown in the absorption spectra [Fig. 4B]. At 75^{o}C DNA strands were fully separated as confirmed by the observation of the hyperchromic effect already well described in (18) and (19). Upon slow cooling to 25^{o}C the absorbance decreased but was still much higher than before sample heating. This may be due to mismatching of DNA bases upon renaturation in very highly concentrated samples.

These observations of a hysteresis type behaviour show that the tertiary structure of a supercoiled plasmid is difficult to restore after heating of samples since the changes are partially irreversible.

Fig. 4.

Discussion and conclusions

We observe that supercoiled DNA spontaneously forms liquid crystalline phases at high concentrations up to 60 mg/ml. Spontaneous self-ordering permits direct observation of the cholesteric fingerprint-like textures in microdomains of supercoiled DNA without the need for additional manipulation (orientation in high magnetic fields, pretreatment of glass plates of LC cell etc.). Our data presented here support the idea that helical structure can promote the formation of this type of LC phase. Reports in the literature about the formation of cholesteric fingerprint-like textures in synthetic helical polymers such as poly(4-(N,N-diisopropylaminomethyl)phenylacetylene) (4) and biopolymers like linear DNA (2) also suggest that in supercoiled DNA this type of phase should appear spontaneously.

Phase formation in supercoiled DNA is a relatively slow process compared to both poly(4-(N,N-diisopropylaminomethyl)phenylacetylene) and to linear DNA. Liquid crystalline phase formation in plasmids can last at least for three months, presumably due to the weak ordering properties of the plectonemic structure of DNA, or to the dynamic nature of the plectoneme itself. Although the samples were purified and contained neither linear nor nicked DNA they were not uniform as they contained a number of supercoiled topoisomers. It seems that better defined phases might be obtained by preparing samples with a smaller distribution in the number of superhelical turns. CsCl gradient purification provides a better quality and higher purity of material (260/280 nm =1.82) than samples isolated by CompactPrep plasmid Giga Kits (260/280 nm = 1.65). However, as shown, purity is not the essential condition to obtain liquid crystalline phases since no phases were observed in samples with pBSK plasmids. Indeed, according to studies on a number of liquid crystal systems, a degree of impurity could increase the rate of LC phase formation (20).

Liquid crystalline phase formation could also be influenced by a number of factors including cell growth conditions (temperature, pH) or isolation techniques. The conformation or superhelicity of supercoiled DNA of *E. Coli* grown at 37°C may differ from DNA isolated from cells grown at room temperature (21). Also CsCl gradient purification may be more detrimental to maintaining plasmid tertiary structure due to the intercalation with ethidium bromide during the process. The choice of purification strategy is thus crucial for investigating highly condensed plasmid DNA. Our studies of pBSK *in vitro* reveals that even a slight change in external conditions may have a large impact on cell DNA structure *in vivo*.

Nevertheless the reproducibility of the formation of LC domains in different polymers and DNA indicates that cholesteric fingerprint-like textures should be a feature of molecules with helical structures. The theoretical background supporting the model of chiral phase formation by helical macromolecules is described in (22).

Experiments with phase transitions showed that liquid crystalline phases are irreversible since no cholesteric fingerprint textures were observed after heating and then cooling of the sample. The tertiary structure of supercoiled plasmids is very sensitive and does not renature in the same manner during temperature changes. Direct observations of denaturation and renaturation processes of plasmid DNA were performed using AFM (23). They revealed that fractal aggregates can be formed upon heating and then cooling (24). However, conditions in highly packed LC phases of plasmid DNA certainly impose more complicated intermolecular interactions that need to be taken into account in the determination of temperature induced changes in plasmid conformation. Circular dichroism and absorbance measurements as a function of temperature confirmed our observation from PLM. Changes in the structure of supercoiled plasmids are not reversible as the absorption of left and right circularly polarized light differs upon heating and further cooling of DNA samples. This indicates that the relaxed form does not twist in the same tertiary structure upon slow cooling, possibly due to changes in superhelicity. The phases reported before claimed the appearance of birefringence while superhelicity was in the range -0.03 to -0.05, as found in living organisms (6), (7), (9). Other values of this parameter seem to affect the ordering properties at high concentrations and no birefringent domains are visible.

Another reasonable explanation could be that there is nicking of supercoiled DNA during the renaturation process. Mismatching of DNA bases in highly packed liquid crystalline cells may promote nicking that in turn affects the tertiary structure and perhaps the LC properties of plasmid DNA. Nicked circular DNA in highly concentrated DNA samples corresponds to linear DNA and its behaviour is significantly different from supercoiled DNA (25). To our knowledge, no straightforward technique exists to investigate the conformation of DNA in LC phases. Recently, two photon microscopy with polarization analysis was successfully introduced to visualise the orientation of linear DNA chains stained with fluorescent dyes in LC phases (26). Although doping of plasmids with intercalators and groove binders changes their structural properties, this technique provides the means to investigate organized DNA systems at very good resolution in three dimensions. Such a precise study of DNA LC phases is currently in progress in our group.

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