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## Liquid Crystals

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

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

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The liquid crystalline (LC) properties of two supercoiled plasmid DNA samples, pBSK (2958 bp) and pGEM (3000 bp), have been studied using polarised light microscopy (PLM), circular dichroism (CD) and UV–Vis spectroscopy. The influence of methods of isolation on plasmid LC behaviour is described, and using PLM we have demonstrated the spontaneous formation of cholesteric fingerprint-like textures. Preliminary studies of LC phase transitions in pGEM show the irreversibility of LC phase formation, as a consequence of changes in the tertiary structure of supercoiled plasmids. Using UV–Vis spectroscopy a hyperchromic effect was observed with increasing temperature. The CD spectra clearly showed structural changes, and probably mismatching of DNA bases, during cooling. Finally, we have observed an irreversible phase transition in plasmid DNA which is very different from that previously reported in linear DNA.

**Keywords:** plasmid DNA; liquid crystal; cholesteric phase; phase transition; fingerprint-like texture; supercoiling; hyperchromic effect; DNA isolation techniques; circular dichroism

### 1. Introduction

Reports concerning the presence of liquid crystalline (LC) phases in DNA solutions began to appear as early as 1950 [1]. Investigations were subsequently extended to include the formation of lyotropic LC phases in linear DNA [2], in other helical biological polymers [3], and in synthetic polymers containing helical structures [4]. The properties of the anisotropic phases observed were found to depend strongly on temperature and polymer concentration, and to a lesser extent on the ionic strength and other characteristics (e.g. persistence length) of the polymer chain [2, 5]. However, interest in liquid crystalline phases in plasmid DNA waned, until Torbet and DiCapua in 1989 described the LC behaviour of supercoiled DNA [6]. The results of this seminal study were essentially that negatively supercoiled DNA could form interwound configurations in LCs at concentrations between 30 and 90 mg ml<sup>-1</sup>, with little evidence of the presence of toroidal configurations.

The molecular organisation of DNA *in vivo* bears some resemblance to the LC DNA phases observed *in vitro* [2]. However, few reports have been published concerning LC phase formation in supercoiled

DNA [7–9]. At the primal level of structure in relaxed linear DNA the two DNA strands twist around the helical axis on average every 10.4–10.5 base pairs, and if the DNA is constrained into a closed circular form the molecule will form a supercoil, characterised by a specific writhe. The number of helical turns around the helical axis is described as 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  $Lk_0 = \text{base pairs (bp)}/10.4$ . The number of times the DNA molecule crosses over itself (known as 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 any change in Lk may therefore be accommodated by changing the helical twist or by forming interwound structures. 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 a toroid or a plectonemic configuration. A negative supercoil, for example, can form either a left-handed toroid

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or a right-handed two-start plectoneme. Closed circular DNA usually exists *in vivo* in a plectonemic negative supercoiled configuration, and the most common conformation is right-handed interwinding. Supercoiling in bacterial chromatin is involved in many cellular mechanisms, including 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, at the present time the reports of spontaneous formation of cholesteric fingerprint-like textures have only referred to concentrations below  $25 \text{ mg ml}^{-1}$  [9].

In the case of plasmid DNA, supercoiling plays an important role in the formation of LC phases. Supercoiling responds to changes in environmentally induced factors such as counterions [12] and temperature [13], and it therefore has an influence on the critical boundaries of the isotropic to LC 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 a fixed level at temperatures as high as  $50^\circ\text{C}$  [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 as twist and writhe are affected the Lk increases [15]. On cooling, negative supercoiling is increased in DNA and Lk decreases, as explained by Mizushima *et al.* [16]. On the other hand, if the temperature increases beyond a critical value, bacterial DNA is unable to return to the same degree of negative supercoiling. The effect of temperature on the organisation of supercoiled DNA is therefore an important factor. Investigating LC phase transitions *in vitro* may provide an insight into what happens to condensed genetic material *in vivo* during exposure to extreme conditions, such as high temperature. Since supercoiled plasmids are involved in the regulation of two critical processes in living organisms, replication and transcription [17], a better appreciation of supercoiling would have an important impact on our understanding of these essential processes, in addition to providing information concerning their control using, 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 the present study we have applied the techniques of polarised light microscopy (PLM), circular dichroism (CD) and UV-Vis spectroscopy to investigate the formation of LC phases in highly concentrated samples. As far as we are aware this is the first report of cholesteric fingerprint-like textures

occurring spontaneously in concentrations as high as  $60 \text{ mg ml}^{-1}$ . Finally, we describe some preliminary studies of phase transitions in plasmid DNA.

## 2. Materials and methods

### 2.1 Isolation of *pBluescript plasmid DNA*

Colonies of plasmid containing *Escherichia coli* were bred on Petri plates containing Luria Bertani broth (LB) agar with antibiotic. A single colony was transferred to conical flasks containing LB (10 g Tryptone, 5 g yeast extract, 5 g NaCl, glycerol, pH 7.5). The volume of the culture was restricted to one-fifth of the volume of the flask to accommodate the shaking process during incubation under optimal conditions for bacterial growth (24 h, 185 turns per min,  $37^\circ\text{C}$ ). Ampicillin was added to give a final concentration of  $100 \mu\text{g ml}^{-1}$ . 18 l of LB medium containing ampicillin were inoculated with 200 ml of *E. coli* culture and incubated at  $37^\circ\text{C}$  overnight with continuous shaking. Cells were then harvested by centrifugation and stored at  $-20^\circ\text{C}$ .

Pellets of *E. coli* cells were suspended in 180 ml of an ice-cold solution of buffer P1 (25 mM Tris-HCl, pH 8.0; 10 mM EDTA; 50 mM glucose;  $100 \mu\text{g ml}^{-1}$  RNase A) and allowed to stand for 5 min at room temperature. The suspension was then divided equally between two flasks of similar volume and 180 ml of buffer P2 (200 mM NaOH, 1% sodium dodecyl sulphate) added to each flask. The flasks were left for 10 min in ice until the solutions became transparent, indicating that cell lysis had occurred. 135 ml of buffer P3 (3M  $\text{CH}_3\text{COOK}$ , pH 5.5) were then added to each flask to precipitate genomic DNA, cellular debris and proteins. The solutions were gently agitated for a further 5 min on ice and then centrifuged at 10,000 rpm for 15 min. The supernatant layer was transferred to six new flasks and plasmid DNA precipitated by 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).  $100 \mu\text{l}$  of  $10 \text{ mg ml}^{-1}$  RNase were added and left for 1 h at  $37^\circ\text{C}$  to remove RNA. Subsequently,  $20 \mu\text{l}$  of  $20 \text{ mg ml}^{-1}$  Proteinase K was left to stand for 1.5 h at  $37^\circ\text{C}$ .

Purified samples were precipitated using 0.7 volumes of isopropanol and centrifuged at 15,000 rpm for 10 min. The pellets were washed with 70% ethanol and centrifuged as described above. The pellet of pure plasmid DNA was dried *in vacuo*. Finally, the samples were dissolved in 3 ml water and lyophilised. In general, 3 l of LB solution yielded 30–40 mg of plasmid DNA.

## 2.2 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 Milli-Q water and mixed with 20 ml of 2 g ml<sup>-1</sup> CsCl (final concentration CsCl = 1 g ml<sup>-1</sup>). This was equally divided between two ultracentrifuge polyallomer tubes and 0.5 ml of 10 g ml<sup>-1</sup> ethidium bromide added to each. The tubes were centrifuged in a Beckman rotor 70.1 Ti at 48,000 rpm for 21 h at 20°C in a Beckman J5 centrifuge. After centrifugation, RNase enzyme was added and the samples once more centrifuged in similar 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 l TE solution, and the CsCl removed by dialysis.

## 2.3 Purification of pGEM<sup>®</sup>-T vector on a column using CompactPrep Plasmid Giga-prep kit

The isolation of pGEM<sup>®</sup>-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 made in the quantity of culture volume prepared for the experiment. Instead of 3 l used for isolation of the pBluescript, only 2.5 l could be accommodated for pGEM-T plasmid due to the limitations imposed by further purification using the Giga-prep kit technique. The supercoiled plasmid DNA was purified according to the manufacturer's instructions.

## 2.4 Plasmid purity

To check the quality and amount of isolated pBSK (2958 bp) and pGEM (3000 bp), UV-Vis spectroscopic measurements were made. Samples of superhelical DNA were appropriately diluted and transferred to standard 1 ml quartz cuvettes. The absorption maximum ( $\lambda_{\max}$ ) of nucleic acids occurred at 260 nm. However, samples might be contaminated with proteins or sugars, which absorb respectively at 280 nm and 230 nm. If the ratio of the absorption 260:280 and 260:230 was within the range 1.80 to 2.00 this would indicate relatively pure DNA.

The ratios of the absorbency 260:280 nm for supercoiled pBSK and pGEM were 1.82 and 1.65, respectively. A small amount of protein was evidently

present in the supercoiled pGEM solution. 3 mg of supercoiled pBSK and 4 mg of pGEM were obtained, and the two plasmid samples were stored at 4°C.

## 2.5 Polarised light microscopy

PLM is a very useful tool for the recognition of liquid crystalline phases. A 15  $\mu$ l droplet of 60 mg ml<sup>-1</sup> concentration was deposited between glass slides. The microscope was equipped with a hot-stage and CCD camera. Photographs were recorded under crossed polarisers under an Olympus 60BX microscope, at 10 $\times$  and 20 $\times$  magnification. The observation of phases, and phase transitions as a function of temperature, was performed within the temperature range 20–80°C.

## 2.6 Circular dichroism

CD is an additional powerful and sensitive spectroscopic technique for probing secondary structures or the conformation of chiral molecules such as DNA. A CD Chirascan spectrometer (Applied Photophysics Ltd, Leatherhead, UK) was used in our experiments, equipped with a temperature controller which allowed analysis of denaturation and renaturation of plasmids within the frame of LC phase transitions. Solutions for examination were prepared by diluting samples to a final concentration of 45  $\mu$ g ml<sup>-1</sup>.

## 3. Results

### 3.1 Plasmid characterisation

pBluescript vector (DNA concentration 0.75  $\mu$ g  $\mu$ l<sup>-1</sup>) and pGEM (DNA concentration 2.5  $\mu$ g  $\mu$ l<sup>-1</sup>) were loaded on 1.0% agarose gel containing intercalating ethidium bromide at a concentration of 1  $\mu$ g ml<sup>-1</sup>. Electrophoresis was carried out in 0.5  $\times$  Tris-borate/EDTA buffer at 70 V and 0.5 A. The homogeneity of the supercoiled DNA was confirmed, and neither nicked circular nor linear DNA could be observed. On the other hand, a distribution of topological isomers of supercoiled DNA was detected (Figure 1(A)).

Quantification of the intensity of bands in a gel using SigmaGel software (SPSS Ltd, Winchester, UK) allowed the estimation of the relative distribution of topoisomers present. In pBluescript plasmid DNA the predominant isoform corresponded to 96%, whereas in pGEM it constituted 90.5% of the total.

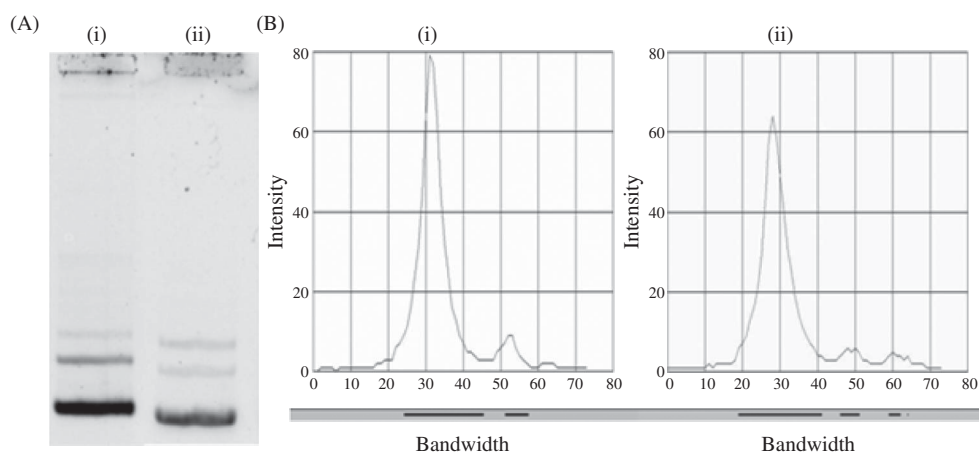


Figure 1. (A) Electrophoretic gels of (i)  $0.75 \mu\text{g } \mu\text{l}^{-1}$  supercoiled pBSK and (ii)  $5.0 \mu\text{g } \mu\text{l}^{-1}$  supercoiled pGEM. (B) Band intensities analysed by SigmaGel software: (i)  $0.75 \mu\text{g } \mu\text{l}^{-1}$  supercoiled pBSK, and (ii)  $5.0 \mu\text{g } \mu\text{l}^{-1}$  supercoiled pGEM.

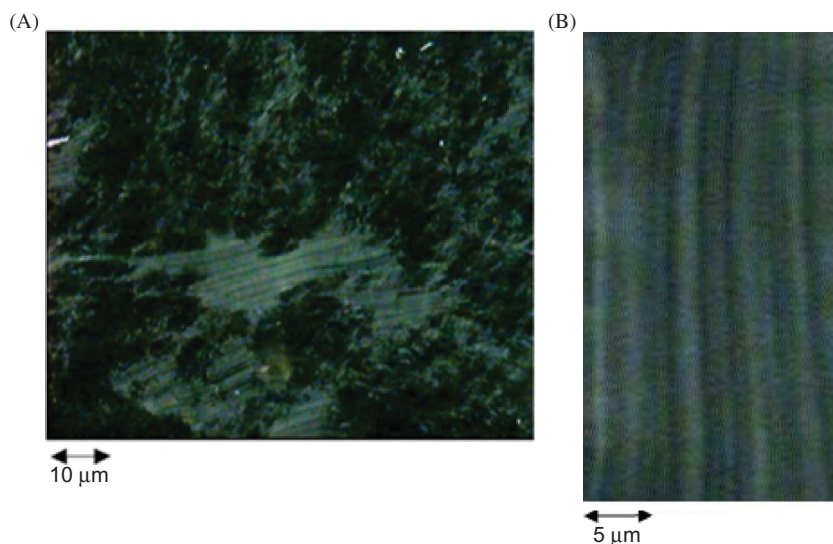


Figure 2. PLM photographs of (A) birefringent domains in  $60 \text{ mg ml}^{-1}$  supercoiled pGEM plasmid DNA,  $10\times$  magnification, (B) magnified domain with characteristic cholesteric fingerprint-like texture,  $20\times$  magnification (colour version online).

### 3.2 LC cells of supercoiled pGEM and pBSK

Plasmids pGEM and pBSK were isolated using the column technique and CsCl centrifugation, respectively, and were used in the preparation of LC cells. Similar concentrations, up to  $60 \text{ mg ml}^{-1}$ , of purified materials were placed between two glass plates and closed without spacer for microscopic analysis. Each sample was held at room temperature over a similar time period. No phases were formed in pBSK samples (results not shown), but birefringent domains were clearly observed under the microscope in the  $60 \text{ mg ml}^{-1}$  pGEM plasmid DNA LC cell between crossed polarisers (Figure 2).

LC phases in samples of highly concentrated supercoiled DNA have previously been reported by Torbet and DiCapua [6], who postulated that

superhelical plasmids formed LCs at concentrations between  $30 \text{ mg ml}^{-1}$  and the highest concentration studied,  $80 \text{ mg ml}^{-1}$ . Our photomicrographs confirmed the results reported by these authors using small-angle neutron scattering and X-ray diffraction techniques [6]. Observation of translucent birefringent domains under PLM was extended over several weeks. Three months after the preparation of the LC cell a fingerprint-like texture appeared (Figure 2). This type of texture is characteristic of a cholesteric phase with homeotropic alignment of the molecules. The cholesteric pitch, measured over twice the distance between the fringes, was found to be  $4 \mu\text{m}$ , in agreement with earlier reports of cholesteric phases in supercoiled DNA [12]. This observation indicates that, in addition to

the macroscopic anisotropic organisation 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 supercoiled plasmids.

### 3.3 Phase transition in pGEM

A hot-stage with temperature controller was used to investigate the phase transitions present in plasmid DNA. Heating a sample of pGEM ( $c_{\text{DNA}} = 60 \text{ mg ml}^{-1}$ ) to  $70^\circ\text{C}$  did not affect the sample, but higher temperatures rapidly caused the phase to change from anisotropic to isotropic, in agreement with published data reporting the high sensitivity of supercoiled DNA boundaries to temperature [12]. The process was not reversible, however, and on cooling the cholesteric fingerprint-like texture did not reappear in the microdomains. Although birefringence was observed at around  $30^\circ\text{C}$  over the entire cell area, no specific LC texture was seen (Figure 3).

Circular dichroism measurements were carried out to investigate the behaviour of DNA structure with change in temperature. The isotropic phase appeared when a supercoiled plasmid was slowly heated to  $75^\circ\text{C}$  (at  $1^\circ\text{C min}^{-1}$ ) and anisotropy was restored when the sample was cooled at a similar rate to  $25^\circ\text{C}$ . Measurements were carried out stepwise every  $5^\circ\text{C}$ . Change in thermal conditions led to structural changes (Figure 4(A)). Higher temperature was seen to induce untwisting of the plectonemic structure and caused denaturing of the DNA. In highly concentrated samples of supercoiled plasmid there is an increased probability that on slow cooling the DNA may not return to the same LC organisation as beforehand.

In parallel with measurement of dichroic spectra, changes in absorbance were also determined as

a function of temperature. Supercoiled DNA was shown to be quite stable up to  $50^\circ\text{C}$ , but above  $55^\circ\text{C}$  rapid denaturation began, as shown by the absorption spectra (Figure 4(B)). At  $75^\circ\text{C}$  the strands of DNA had become fully separated, confirming observations of the hyperchromic effect described by Felsenfeld and Sandeen [18] and by Cox [19]. On slowly cooling to  $25^\circ\text{C}$  the absorbance decreased but was still much higher than before heating. This may be due to mismatching of DNA bases on renaturation in highly concentrated samples.

These observations of hysteresis-type behaviour show that the tertiary structure of a supercoiled plasmid is difficult to restore following the heating of a sample and that the changes are partially irreversible.

## 4. Discussion and conclusions

We have observed that supercoiled DNA spontaneously forms LC phases at high concentrations, up to  $60 \text{ mg ml}^{-1}$ . Spontaneous self-ordering allows the cholesteric fingerprint-like textures in microdomains of supercoiled DNA to be observed without the need for additional manipulation, such as orientation in high magnetic fields or pretreatment of the glass plates of the LC cell. The data presented here support the view that helical structures are able to promote the formation of LC phases. Reports in the literature of the formation of cholesteric fingerprint-like textures in synthetic helical polymers such as poly(4-(*N,N*-diisopropylaminomethyl)phenylacetylene) [4] and in biopolymers, including linear DNA [2], also suggest that in supercoiled DNA this type of phase may appear spontaneously.

Phase formation in supercoiled DNA is a relatively slow process in comparison to either of the above examples. LC phase formation in plasmids can extend over at least three months, presumably due to the weak ordering properties of the plectonemic structure of DNA, or perhaps to the dynamic

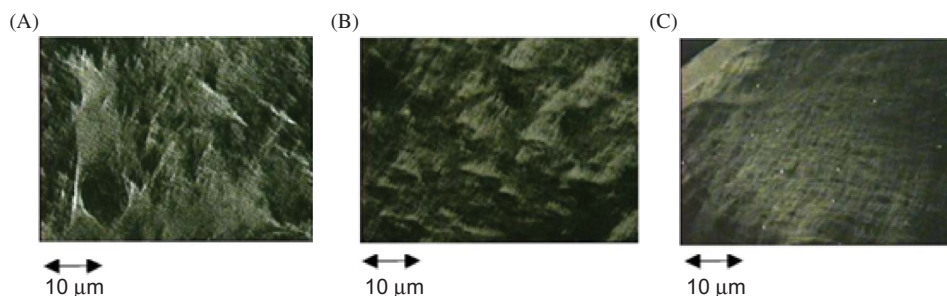


Figure 3. Birefringent domains after heating and cooling on a hot-stage under PLM: (A)  $60 \text{ mg ml}^{-1}$  after heating,  $10\times$  magnification, (B)  $60 \text{ mg ml}^{-1}$  after second heating/cooling cycle,  $10\times$  magnification, (C)  $60 \text{ mg ml}^{-1}$  two weeks later,  $10\times$  magnification (colour version online).

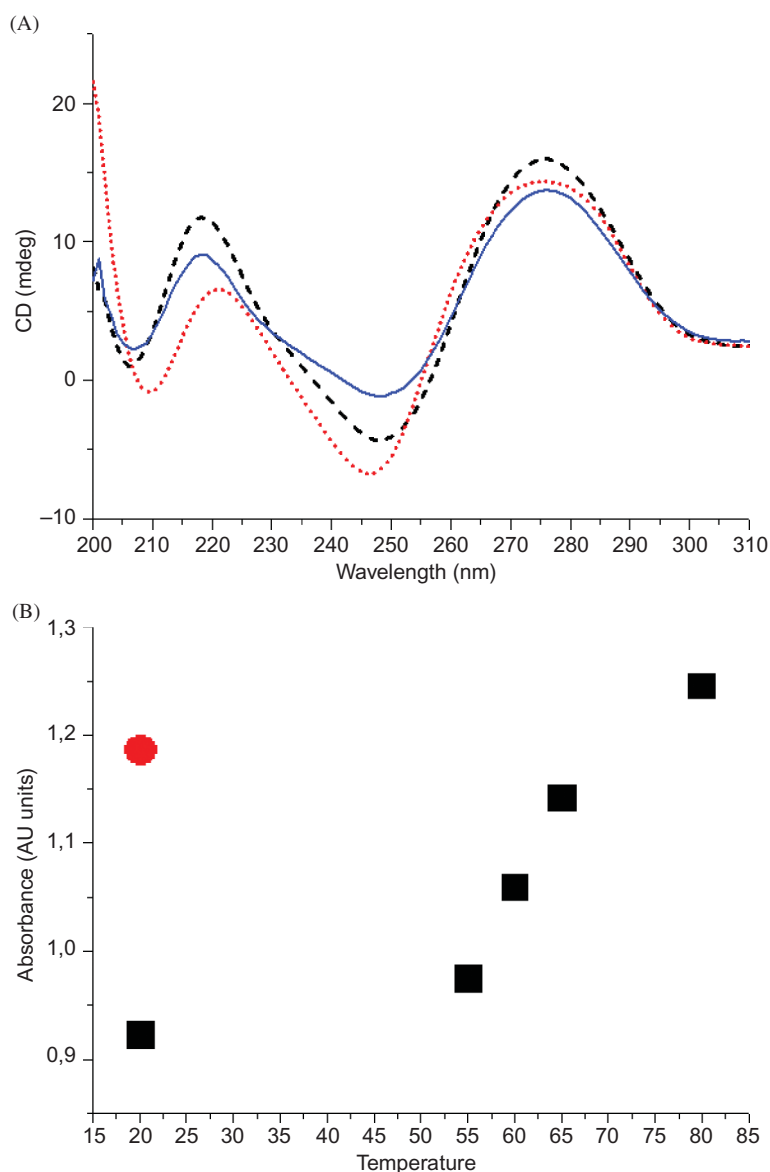


Figure 4. (A) Circular dichroism spectra of  $45 \mu\text{g ml}^{-1}$  of pGEM plasmid under changing temperature conditions (●●●)  $25^\circ\text{C}$  before heating, (—)  $75^\circ\text{C}$ , (- - -)  $25^\circ\text{C}$  after heating, (B) absorbance in temperature function of supercoiled pGEM ( $45 \mu\text{g ml}^{-1}$ ) at 260 nm measured in parallel with the CD experiment. The 40% increase in absorbance indicated a hyperchromic effect and separation of DNA strands: (■) changes in absorbance upon increase of temperature, (●) absorbance at  $20^\circ\text{C}$  after heating and further cooling (colour version online).

nature of the plectoneme itself. Although the samples used had been purified and contained neither linear nor nicked DNA, they were not completely uniform and they contained a number of supercoiled topoisomers. It seems that better defined phases might be obtained by preparing samples containing a smaller distribution in the number of superhelical turns. CsCl gradient purification provided a better quality and higher purity of material (ratio of absorption at  $260 : 280 \text{ nm} = 1.82$ ) than samples isolated by CompactPrep plasmid Giga kits, when the corresponding ratio was 1.65. However, it is clear that

purity is not an essential prerequisite for the formation of LC phases, since no phases were observed in samples of pBSK plasmids. Indeed, according to previous studies on a number of liquid crystal systems, a degree of impurity may actually increase the rate of LC formation [20].

LC phase formation is also influenced by a number of other factors, including cell growth conditions (temperature, pH) and isolation techniques. In addition, the conformation or superhelicity of supercoiled DNA of *E. Coli* grown at  $37^\circ\text{C}$  may differ from the DNA isolated from cells grown at room

temperature [21]. Moreover, CsCl gradient purification may have been detrimental to maintaining the plasmid tertiary structure, due to intercalation with ethidium bromide during the procedure. The choice of purification strategy is thus crucial in the investigation of highly condensed plasmid DNA. Our studies of pBSK *in vitro* revealed that even a slight change in external conditions might have a large impact on cell DNA structure *in vivo*.

Nevertheless, the reproducibility of the formation of LC domains in different polymers and in DNA indicated that cholesteric fingerprint-like textures tend to be a feature of molecules with helical structures. The theoretical background supporting the model of chiral phase formation by helical macromolecules has previously been described by Cherstvy [22].

In the present study phase transitions in the LC phase were seen to be irreversible, since no cholesteric fingerprint textures were observed on heating and then cooling the sample. The tertiary structure of supercoiled plasmids is very sensitive and does not re-nature similarly during temperature changes. Direct observations of the denaturation and renaturation processes of plasmid DNA were performed using atomic force microscopy (AFM) [23]. These revealed that fractal aggregates can be formed during heating followed by cooling [24]. However, conditions in highly packed LC phases of plasmid DNA certainly impose more complicated intermolecular interactions, and these need to be taken into account in the determination of temperature-induced changes in plasmid conformation. CD and absorbance measurements as a function of temperature confirmed the observations made with PLM. Changes in the structure of supercoiled plasmids are not reversible, as the absorption of left- and right-circularly polarised light differs during the heating and subsequent cooling of DNA samples. This indicates that the relaxed form does not twist in the same tertiary structure during slow cooling, possibly due to changes in superhelicity. The phases reported previously have claimed the appearance of birefringence with superhelicity in the range  $-0.03$  to  $-0.05$ , as found in living organisms [6, 7, 9]. Other values for this parameter seem to affect the ordering properties at high concentrations, and no birefringent domains are visible.

Another reasonable explanation could be that nicking of supercoiled DNA occurs during the renaturation process. Mismatching of DNA bases in highly packed liquid crystalline cells may promote nicking, which in turn affects the tertiary structure

of plasmid DNA, and possibly also its LC properties. Nicked circular DNA in highly concentrated DNA samples corresponds to linear DNA, and its behaviour is significantly different from that of supercoiled DNA [25]. To our knowledge there is no straightforward technique that allows investigation of the conformation of DNA in LC phases. Two-photon microscopy with polarisation analysis has recently been 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 a means of investigating organised DNA systems with very good resolution in three dimensions. Such a precise study of DNA LC phases is currently in progress.

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