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Anthracycline Gels. II. Spectroscopic study on doxorubicin–lecithin association products

Marcello Giomini [a], Anna Maria Giuliani [b], Mauro Giustini [c] and Edoardo Trotta [d]

[a] *Dipartimento di Chimica, Università "La Sapienza", Rome (Italy)*
[b] *Dipartimento di Chimica Inorganica dell'Università, Palermo (Italy)*
[c] *ETH, Institut für Polymere, Zürich (Switzerland)*
[d] *CNR, Istituto di Medicina Sperimentale, Rome (Italy)*

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Abstract

Thixotropic and thermally reversible gels have been prepared from doxorubicin–lecithin association products (DL12) by addition of salts to their aqueous solutions. The gel formation and the melting profiles have been followed by several spectroscopic techniques (^{1}H NMR, UV-Vis, Circular Dichroism). The transition temperatures increase as the concentration of both the salt and the DL12 is increased, suggesting a progressive closer approach of the gel forming species. The process of the gel formation is cooperative and causes immobilization of the doxorubicin molecules of DL12.

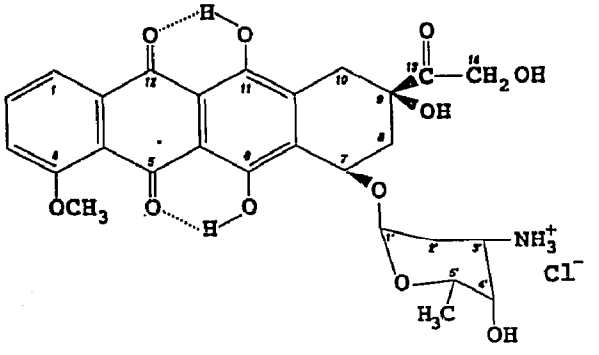
Keywords: Thermally reversible gel; Doxorubicin–lecithin association; Gel formation spectroscopy

1. Introduction

A growing body of evidence is available implicating membranes, and specifically membrane lipids [1–3], as a target, for the anthracycline cytotoxic action, alternative to DNA. These drug-phospholipid interactions appear to be involved both in the antiblastic activity and in the cardiotoxic side effects of the anthracyclines. A knowledge, as comprehensive as possible, of such interactions appears therefore of primary interest.

We have already reported [4,5] on a doxorubicin–egg lecithin 1:2 associate with unusual physico-chemical characteristics, which has been shown to retain the same antitumor potency of the drug, both *in vitro* and *in vivo*, and to produce less severe and delayed acute and long-term cardiotoxic effects [6]. Recently, the preparation and some physico-chemical properties of thermally reversible gels of anthracyclines obtained from aqueous electrolytic solutions of the drug have been described [7,8]. We report here on the preparation and characterization of gels from

Correspondence to: M. Giomini, Dipartimento di Chimica, Università "La Sapienza", P.le Aldo Moro 5, I-00185 Rome (Italy).



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aqueous solutions of the associate, obtained by addition of simple electrolytes.

2. Materials and methods

2.1 Reagents

Doxorubicin (1) was a kind gift of Farmitalia Carlo Erba; egg yolk L-α-phosphatidylcholine (EPC) from Sigma Chemicals Co. (type VII-E, chloroform solution) was used after the evaporation of the solvent under vacuum. The average molecular weight of the product, as determined by ^{1}H and ^{13}C NMR data, was found to be 819 dalton (this includes between three to four water molecules per polar head, which were not removed). Absence of impurities or degradation products was checked by TLC.

The salts (Merck or Carlo Erba analytical reagent grade), necessary for the preparation of the gels, were oven (dried at 378 K and used without further purification. When necessary (Ca^{2+} and Mg^{2+}), the amount of cations in the commercial hydrated salts was checked by EDTA titration [9].

Twice distilled water was always used in the preparations; Merck UVASOL^{TM} $^{2}H_2O$ (> 99.5% deuteriation) was employed for NMR measurements.

The 1 : 2 doxorubicin–lecithin association product was prepared by adding to a methanolic solution of the drug the stoichiometric amount of the phospholipid dissolved in methanol and taking the solution to dryness under vacuum [4].

All other chemicals were analytical reagent grade Carlo Erba products.

2.2 Preparation of the gels

Having in mind the procedure used for doxorubicin alone [7,8], we tried to prepare gels from the doxorubicin–lecithin 1 : 2 association product (hereinafter called DL12) by dissolving it in water and then adding, under stirring and gently heating, small amounts of concentrated saline solutions to reach the established final concentration of both species. Upon cooling, the desired gels were obtained as in the case of the pure drug.

Gels were prepared at constant DL12 concentration (15 m*M* in doxorubicin) and 0.9% (w/v) concentration of different electrolytes, or constant DL12 concentration (10 m*M* in doxorubicin) and variable concentration of the same electrolyte (namely NaCl from 0.15 to 0.80 *M*).

The preparations were carried out at a temperature just above the gel-solution transition temperature (see below), and the gels were stored at ca. 275 K for 24 hours before use, to allow equilibration. The samples were always kept in the dark and in the refrigerator. All samples were at the natural pH of doxorubicin in water (6.0 ± 0.2) and no buffer was used.

2.3 Instrumental

The NMR spectra were acquired on a Bruker AC200 instrument operating at 4.7 T and equipped with temperature control unit. ^{2}H lock on the water solvent ensured frequency stability, while a coaxial capillary, containing 20 m*M* aqueous sodium hexadeuterotrimethylsilylpropane sulphonate (TSP-d_6) was used as reference (δ = 0.00 ppm) and for relative quantitation of the resonances, i.e. its integrated intensity was set equal to 100 at each temperature.

The water resonance peak was always suppressed by presaturation, except when otherwise specified.

The series of experiments at variable temperatures were always run starting from the lowest

temperature and allowing time for the thermal equilibration of the gel after each increase of temperature; the explored range was between 280 K and 333 K.

A Perkin Elmer 555 spectrophotometer was used for the UV–Vis spectra. Temperature control (accuracy ±0.1 K) was achieved by circulating water from a Haake F3-K cryothermostat in the jacket of the cell holder. Quartz cells (0.1 mm path length) were used.

CD spectra were recorded with a Jasco J600 spectropolarimeter, connected to an IBM AT/80286 computer for the acquisition and the processing of the spectral data. A Hewlett–Packard HP 7475A plotter was used to produce hard-copies of the spectra. The temperature of the measuring cell (0.1 mm path length) was maintained stable (within 0.1 K) by circulation of thermostatic fluid in the jacket of the cell holder by means of a thermocryostat Lauda MGW-RK-20. A thermocouple with digital readout was inserted in the cell holder to control the sample temperature.

3. Experimental results

3.1 Properties of the gels

Thermally reversible aqueous gels were obtained from DL12 following a previously described procedure in detail [7,8].

The gelation process is reversible but, while melting is fast, equilibration of the system when the temperature is lowered requires a considerable time. The kinetics of the gelation process appears to be slow, although it has not been investigated in any detail.

The gel–solution transition temperature depends on the concentration, and to a lesser extent, on the nature of the salt. The influence of the nature of the salts has not yet been rational-

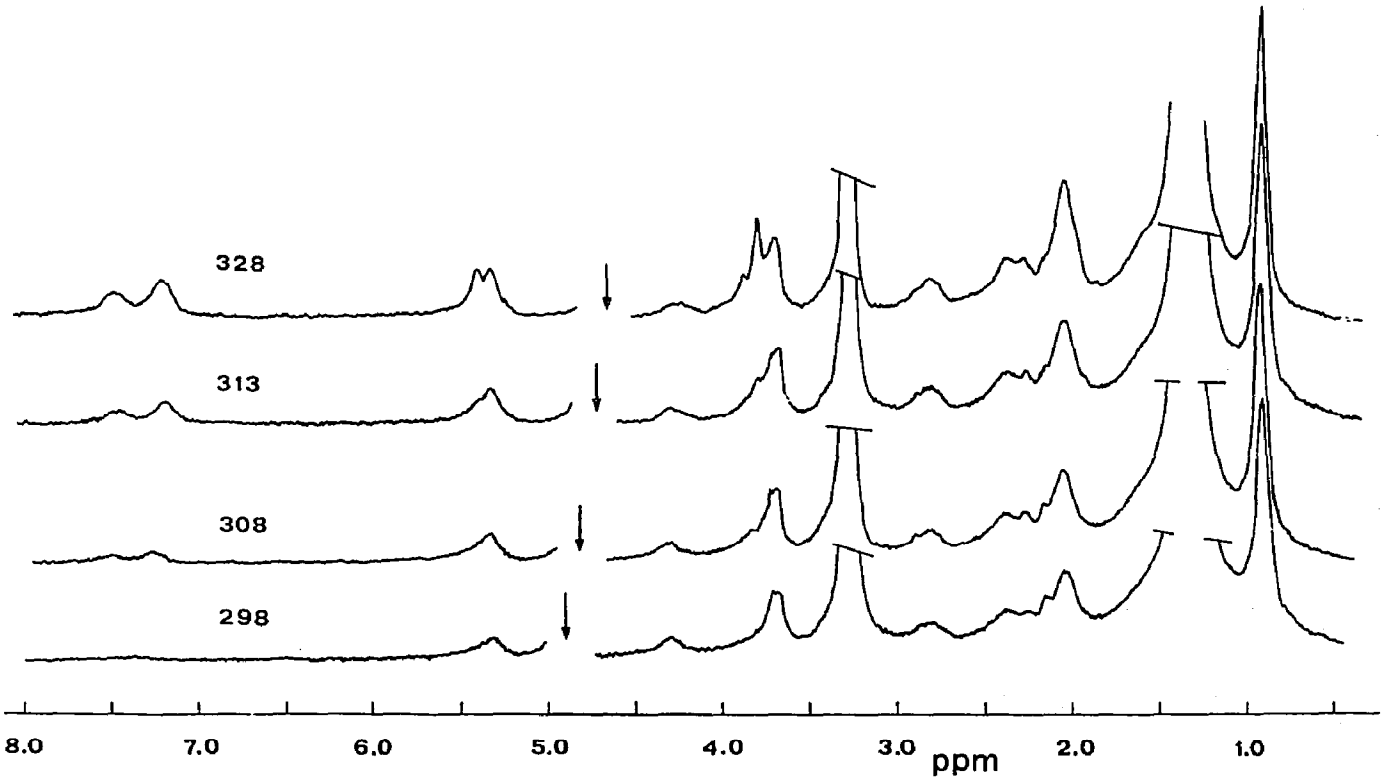

Fig. 1. Variable temperature ^{1}H NMR spectra ($B_0 = 4.7$ T) of DL12 (10 mM in doxorubicin) in 0.35 M NaCl solution. Arrows indicate the suppressed water peak; numbers on the spectra are the temperatures in K.

ized and the question is presently under study. The influence of the electrolyte concentration on the properties of the gels is connected with the hydration of the ions, which leaves less "free" solvent available for DL12. The effect is equivalent to an increase of the drug concentration, which favours intermolecular interactions.

The melting of several DL12 gels was visually followed by gradually heating the gels in a thermostatic bath (Haake F3-K thermocryostat), allowing time for thermal equilibration and observing the changes of fluidity. The observed transition temperatures are collected in Table 1: the change from a mass not moving in the test tube, to a viscous fluid, to a solution occurs within a small temperature interval of a few degrees (±5 K), as observed also for pure doxorubicin gels [7].

The gels, if kept in the dark, can be maintained for several weeks in the refrigerator (ca. 278 K), but a room temperature the phospholipid undergoes progressive degradation. A mild shaking of the gels with distilled water, converts them into fluid solutions (probably micellar solutions), their final apparent viscosity depending on the dilution ratio.

3.2 NMR measurements

The gelation process can be easily followed by NMR. The temperature effect on the proton spectra of elecrolytic solution of DL12 is twofold: a generalized broadening of the resonances and a gradual decrease of intensity of the anthracycline signals to almost complete disappearance are observed upon lowering the temperature. Typical ^{1}H NMR spectra below and above the gel–solution transition are shown in Fig. 1.

The area of the aromatic proton resonances, which are the only ones neatly separated from the rest of the spectrum and thus measurable with sufficient accuracy, has been measured relative to the area of TSP-d_6 in the concentric

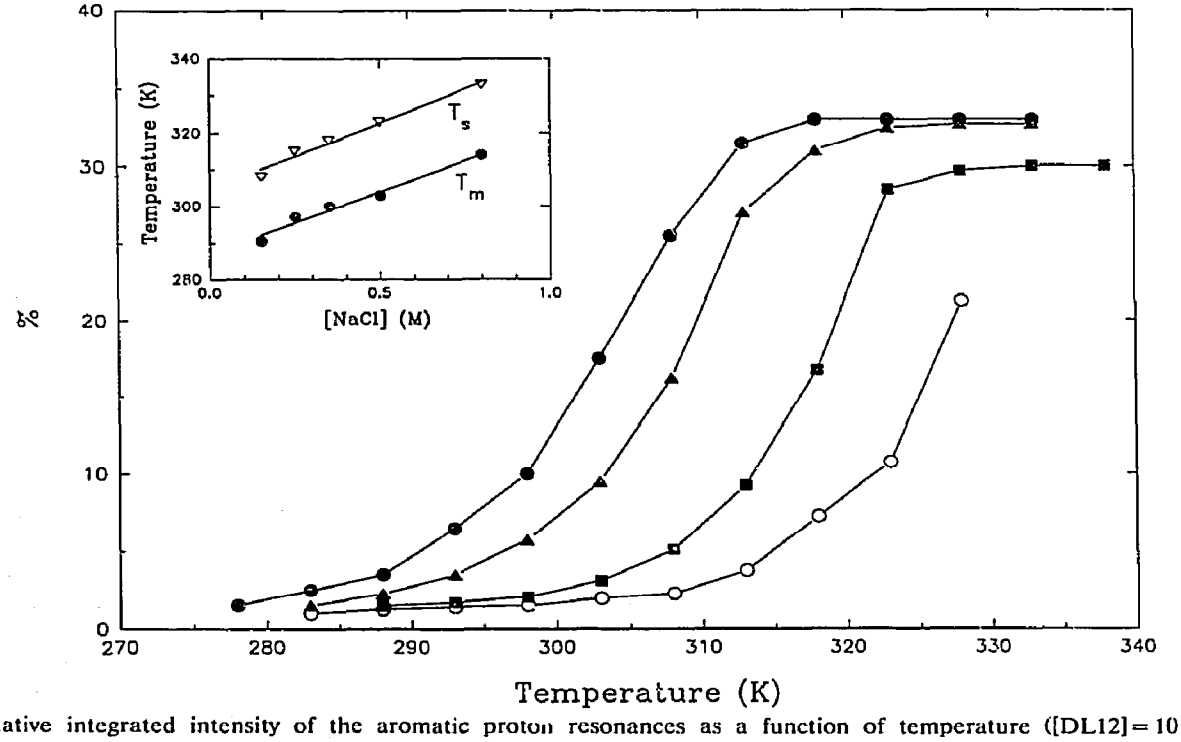

Fig. 2. Relative integrated intensity of the aromatic proton resonances as a function of temperature ([DL12] = 10 mM in doxorubicin; (●) [NaCl] = 0.15 M; (▲) [NaCl] = 0.25 M; (■) [NaCl] = 0.50 M; (○) [NaCl] = 0.80 M). The area of the external TSP-d_6 reference is taken as 100. The inset shows the salt concentration dependence of the melting temperature of the DL12 gels as determined visually (T_m) and from ^{1}H NMR data (T_s).

capillary, set equal to 100. The area changes with temperature following an "S-shaped" curve (Fig. 2), indicating cooperativity of the aggregation process responsible for gelation.

It is possible, from these NMR data, to identify a melting interval, with a temperature T_i at the foot of the rising portion of the curve, which corresponds to the beginning (inception) of the "melting" process, and a saturation temperature T_s, at which the curve begins to level off, corresponding to completion of the melting. The values of T_i and T_s for systems at constant DL12 concentration (10 mM in doxorubicin) and variable NaCl concentration are shown in Table 1.

Also the chemical shift values of the doxorubicin resonances, and in particular those of the aromatic protons, are affected by the gelation process. As already observed in the case of pure doxorubicin gels [7], when the temperature is increased, the resonances move upfield, if the sample is below the transition temperature (Fig. 3), and downfield if above; the melting temperature of the gels can therefore be evaluated with some accuracy.

It appears that the ring system is involved in the gel formation process to a greater extent than the rest of the doxorubicin molecule, since the aromatic resonances chemical shifts are the most affected ones by any temperature variation.

On the other hand, the proton resonances of lecithin are not shifted by varying the temperature, and their relative integrated intensities show only a minor decrease upon gelation of the system. These findings suggest that the phospholipid molecules are involved in the formation of the gel structure only marginally. However, the ^{31}P NMR data indicate that the structure and mobility of the lecithin polar head are somewhat modified upon gel formation. Indeed, an upfield shift of the phospholipid ^{31}P NMR resonance is observed as the temperature increases, while its linewidth decreases monotonically to reach a constant value when the gel melts (data not shown).

Experiments run without presaturation of the water signal show that, while the signal moves regularly downfield as the temperature is lowered, its full width at half height remains constant

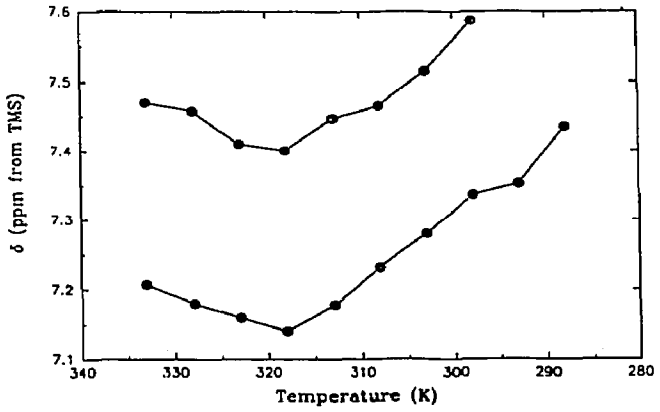

Fig. 3. Temperature dependence of the aromatic proton chemical shifts for a 10 mM in doxorubicin DL12–0.35 M NaCl system (B_0 = 4.7 T, TSP-d_6 reference δ = 0.00 ppm).

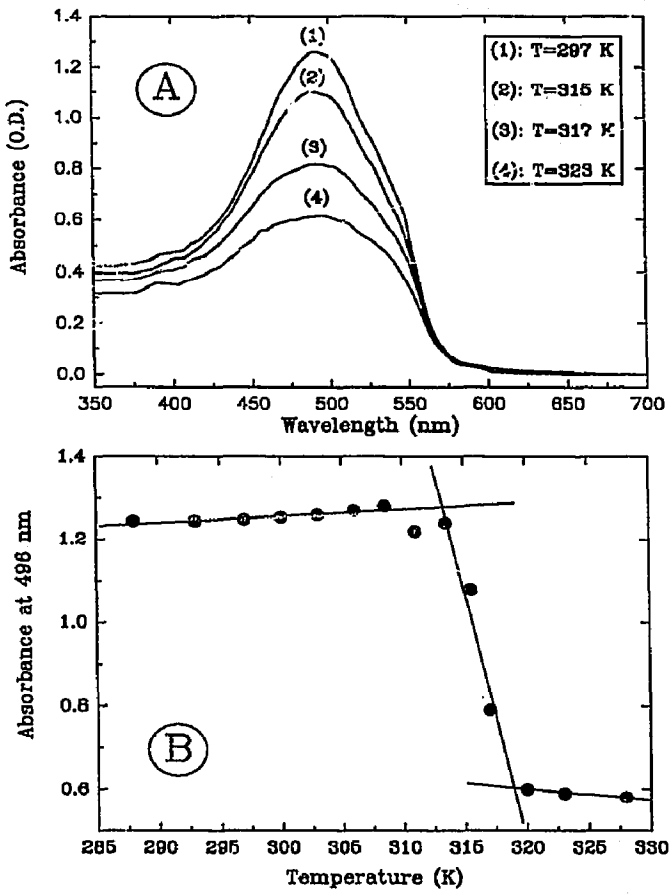

Fig. 4. Variable temperature electronic spectra (A) and temperature dependence of the absorbance at 496 nm (B) for a 10 mM in doxorubicin DL12–0.80 M NaCl system.

through the gelation temperature. A slight broadening occurs only at the temperatures near the freezing point ($T \lesssim 283$ K) due to changes of viscosity and hydrogen bonding, but it is independent of the melting temperature of the gels. The micromobility of the solvent is not affected by the gelation process.

3.3 Electronic spectra

A characteristic temperature dependence is exhibited by the UV–Vis spectra of the DL12 gels. The shape of the spectrum remains essentially unchanged at all temperatures, with only minor shifts of the absorption maxima. However, the intensity of the main absorption at about 496 nm drops markedly upon melting of the gel (Fig. 4).

The marked and rather abrupt changes observed in the visible spectra when the gels melt, indicate that the chromophore of the anthracycline is strongly involved in the gelation process. Such evidence is in agreement with that obtained by NMR spectroscopy.

3.4 Circular dichroic spectra

The CD spectrum of doxorubicin markedly depends on the association state of drug in solution [10,11]: at the concentrations used in the present work doxorubicin is associated and the CD spectrum in water exhibits the characteristic two negative bands at 545 nm and 515 nm, two positive bands at 450 nm and 350 nm, and a negative band at 298 nm [12].

The CD spectra of doxorubicin alone and in association with lecithin in electrolytic solution are clearly different from its spectrum in distilled water (Fig. 5a), but more conspicuous differences are observed between the CD spectra of the gels, of either doxorubicin or DL12, and of the anthracycline solution (Fig. 5b). The CD spectra of DL12 in electrolytic solutions are indeed very sensitive to the gelation process, *i.e.* very strongly temperature dependent (in the visible as well as in the UV spectral region): typical CD spectra of DL12 at various temperatures above and below the gelation temperature are reported in Fig. 6a, while in Fig. 6b representative CD spectra of DL12 and of doxorubicin alone, above and below the transition temperature, are shown in comparison.

Above the melting point of the DL12 gel, the spectrum is characterized by a broad negative band at 515 nm with a ill defined shoulder at around 550 nm, a positive band at 450 nm and another negative band at 295 nm. As the temperature is decreased trough the gelling point, the

Table 1
Melting temperatures of DL12 gels

Composition of the gel	T_m (K) [a]	T_i(K) [b]	T_s(K) [b]	t_s(K) [c]
15 m*M* DL12+0.9% LiCl (0.212 *M*)	302–304			
15 m*M* DL12+0.9% NaCl (0.154 *M*)	302–304			
15 m*M* DL12+0.9% KCl (0.121 *M*)	298–300			
15 m*M* DL12+0.9% $MgCl_2$ (0.095 *M*)	291–293			
15 m*M* DL12+0.9% $CaCl_2$ (0.081 *M*)	298–300			
10 m*M* DL12+0.15 *M* NaCl	288–290	295	311	308
10 m*M* DL12+0.25 *M* NaCl	296–298	301	315	316
10 m*M* DL12+0.35 *M* NaCl	299–301	309	319	318
10 m*M* DL12+0.50 *M* NaCl	302–304	312	323	323
10 m*M* DL12+0.80 *M* NaCl	314–313	322	≥ 333	≥ 333

[a] Visually determined.
[b] From ^{1}H NMR, aromatic proton area.
[c] From ^{1}H NMR, δ of aromatic protons.

following changes are observed:

(i) the 450 nm band increases and slightly shifts to higher wavelength;
(ii) the negative bands between 550 nm and 500 nm become positive;
(iii) two negative bands appear at *ca.* 350 nm and at 323 nm.

The high temperature spectra resemble, to a certain extent, those of pure doxorubicin (Fig. 5a) though the 350 nm band has practically disappeared and lesser modifications are observed in the position and intensity of the other bands.

On the other hand, the gel spectra, which are non-conservative [13], show major changes.

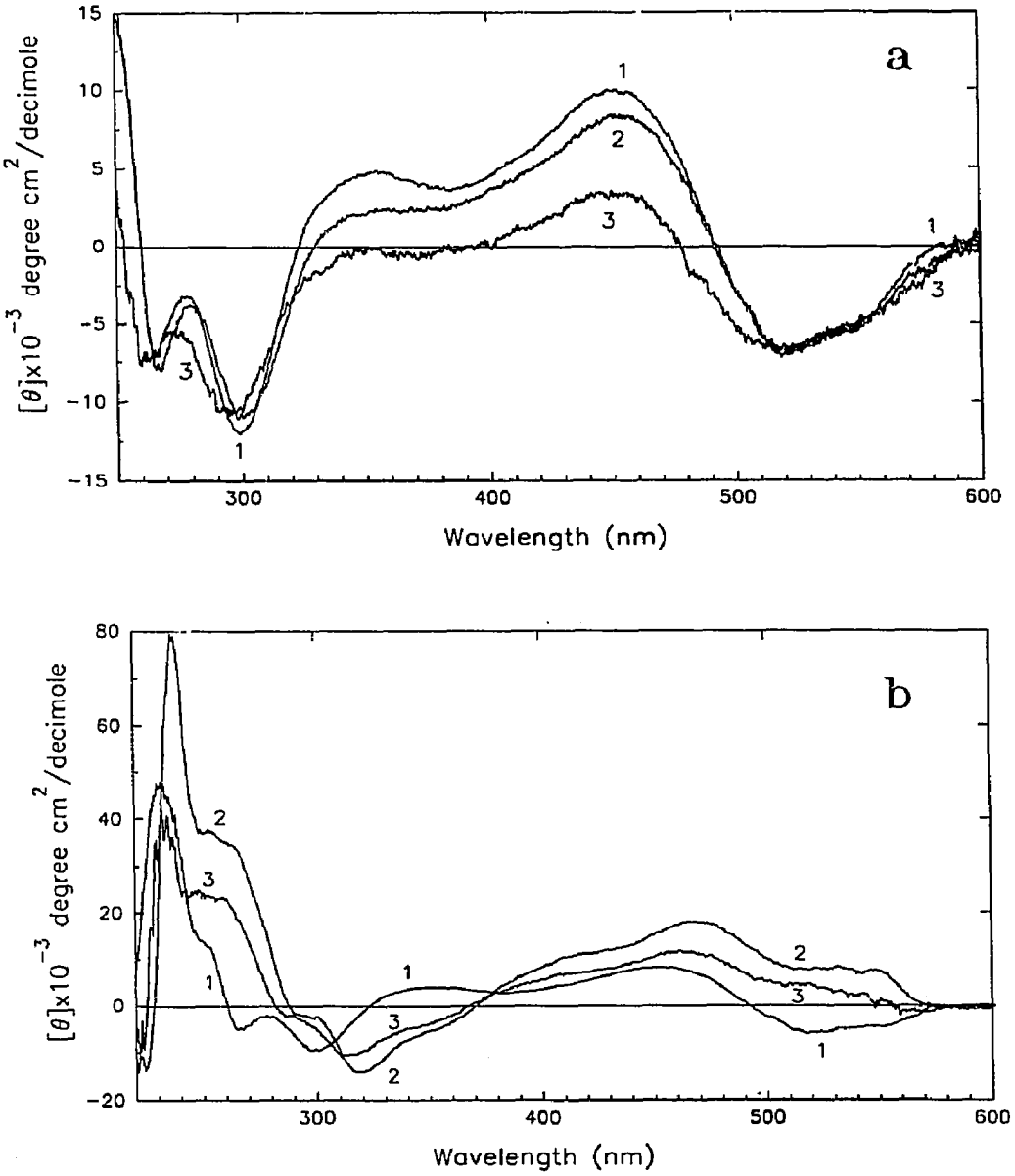

Fig. 5. (a) CD spectra of doxorubicin containing solutions, $T = 318$ K: 1 – doxorubicin 10 mM in water; 2–doxorubicin 10 mM in 0.25 M NaCl solution; 3–DL12 (10 mM in doxorubicin) in 0.25 M NaCl solution. (b) CD spectra of doxorubicin containing gels, $T = 298$ K: 1–doxorubicin 10 mM solution in water; 2–doxorubicin 10 mM in 0.25 M NaCl; 3–DL12 (10 mM in doxorubicin) in 0.25 M NaCl.

Among others, the Cotton effect at 350 nm and 300 nm, for which the chiral centers at C_9 and C_7 are held responsible [14], appear markedly modified: the band at 300 nm is shifted to higher wavelengths, while the 350 nm band has negative ellipticity and becomes more prominent as the temperature is lowered.

Inversion of ellipticity and other conspicuous modifications are observed also in the visible wavelength region of the CD spectrum.

All these findings lead us to the conclusion that a supramolecular chirality could exist, induced by the change of the electric properties of the solvent upon addition of electrolytes.

Such hypothesis could explain some of the experimental evidences obtained form other

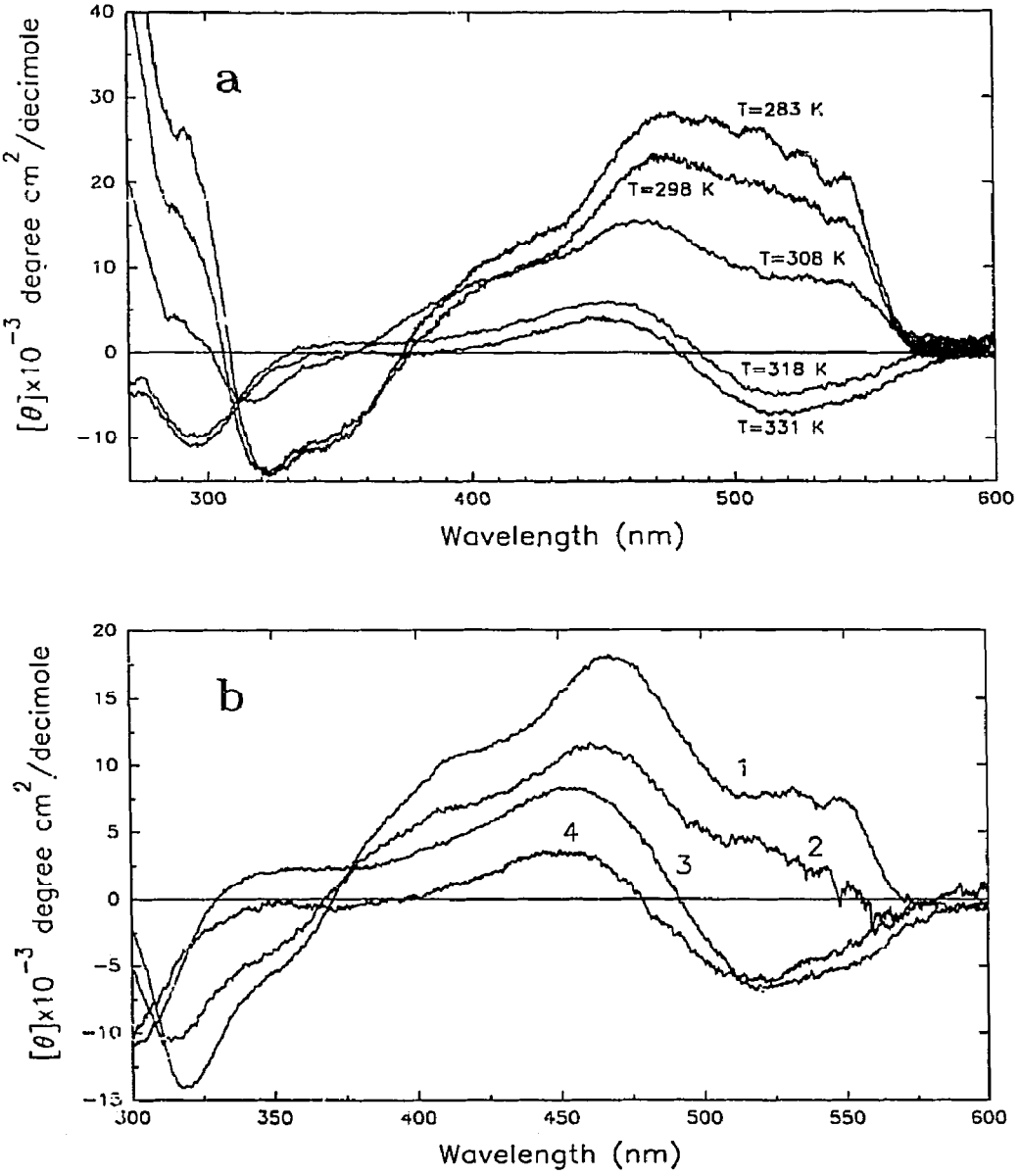

Fig. 6. (a) Variable temperature CD spectra of DL12 (10 mM in doxorubicin) in 0.5 M NaCl. (b) Matched pairs of CD spectra of doxorubicin (10 mM) and DL12 (10 mM in doxorubicin) in 0.25 M NaCl, above and below the gel transition temperature: 1–doxorubicin $T = 298$ K; 2–DL12, $T = 298$ K; 3–doxorubicin, $T = 318$ K; 4–DL12, $T = 318$ K.

spectroscopic techniques, and is now under investigation.

4. Discussion and conclusions

The ^{1}H NMR results indicate that the process of gel formation is cooperative and causes immobilization of the anthracycline molecules of the DL12 as evidenced by the disappearance of the relevant resonance peaks from the spectra upon lowering the temperature. On the other hand the solvent water and the phospholipid moiety of DL12, except the polar heads, do not show signs of reduced mobility as can be inferred from their bandwidth at half height, which remains essentially unchanged through the gel–solution transition.

The transition temperature becomes higher as the concentration of either the salt or the DL12 is increased, in agreement with the possibility of a progressively closer approach — and thus of stronger mutual interactions — of the gel forming species, as already noticed for the doxorubicin alone gels [7].

The ^{1}H chemical shifts of the aromatic protons, too, follow the temperature trend observed for pure doxorubicin gels [7], pointing to phenomena different from the regular stacking interactions as responsible for the gel structure. As mentioned for pure doxorubicin gels, such behaviour indicates that upon gelation the aromatic protons of one drug molecule find themselves in the deshielding region of the aromatic ring system of other anthracycline molecules. In the present case, formation of large molecular aggregates via weak interactions, together and in competition with stacking through π-interactions, between the aromatic ring systems of two adjacent molecules, should be envisaged as well.

On the other hand the sudden marked decrease of absorbance which accompanies the melting of the gels in the visible spectra, not only supports the view that gelation is a cooperative process, but indicates also that there is an increase in the effective chromophoric area of the ring system upon gelling of the solutions.

All the above discussed experimental evidences point to the formation, as in the case of pure doxorubicin gels [7], of a three-dimensional network where the drug molecules are helically stacked with the plane of the aromatic rings of one molecule slightly staggered with respect to the next, as in cholesteric liquid crystals, and with the solvent encapsulated in the microcompartments of the network. As already mentioned, the non-conservativity of the CD spectra suggests irregularity in the helical arrangement: short-range order should be envisaged, with regions of regular helix separated by more disordered ones.

The sugar moiety with the charged $-NH_3^+$ groups and the functional groups of doxorubicin would project out of te molecular plane. These groups are involved in specific weak intermolecular interactions (e.g. hydrogen bonding) through which the gel network is build together. Moreover, the phospholipid molecules should be bound through the polar heads to the periphery of the helix via hydrogen bonding and via electrostatic interaction of the negatively charged phosphoryl group with the protonated amino group of the drug [4].

Such an arrangement of the molecules, where twisting of the ring plane from one molecule to the next occurs, can also explain the temperature behaviour of the electronic spectra. In water solution, DL12 gives rise to micellar aggregates [4] and its aromatic moiety is certainly confined in the core of the aggregates, screened from solvent and not exposed, and with a high degree of chromophore stacking; when the gel forms the micellar structure is disrupted and the hydrophobic chromofores, now arranged in the helix, become exposed, and the absorbance increases. Similar hyperchromicity is shown by the 260 nm band of DNA when the degree of base pairing decreases and the bases become exposed to the solvent [15].

The intense effects produced by gelation in the CD spectra of DL12, support the proposed helical structure of the gels. It is indeed well known that assembly of chromophoric groups in a supramolecular, regular and asymmetric ordered array causes very large modifications in the CD spectra, which are associated with the absorption bands [16].

Thus, the CD results on one side, with the dramatic changes observed in the visible region, corroborate our proposal of the gel structure involving supramolecular chromophore organization, and on the other, with the marked modifications exhibited in the ultraviolet spectral region indicate that the chiral centers bearing hydroxyl groups are heavily involved in the process.

This is in bearing with the proposed weak interactions at the periphery of the helix involving the polar and charged groups of doxorubicin, either in building the gel structure or in binding to the lecithin molecules. It emerges, moreover, from the CD data (Figs. 5a,b and 6b) that the lecithin molecules are not simple "guests" in the gel structure basically formed by the anthracycline molecules, but instead their presence modulates the properties of the gels. These findings support previous experimental evidences of the existence of specific interactions between the phospholipid and the drug, which modify the physico-chemical [4,5] and pharmacological [6] properties of the anthracycline.

In conclusion, DL12 in water in the presence of salts can act as a "gelator", giving rise to a cholesteric liquid–crystalline structure, similar to the gels formed by the ALS molecules [17,18].

The doxorubicin molecules are immmobilized in a helically stacked structure, with exposure of the chromophore to the solvent, while the micromobility of the solvent water and of the interacting lecithin is unchanged. An increase of temperature causes a reversible melting of the gels and addition of water destroys them because the average intermolecular distances become greater.

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