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Anthracycline gels *

Preparation and some physico-chemical properties

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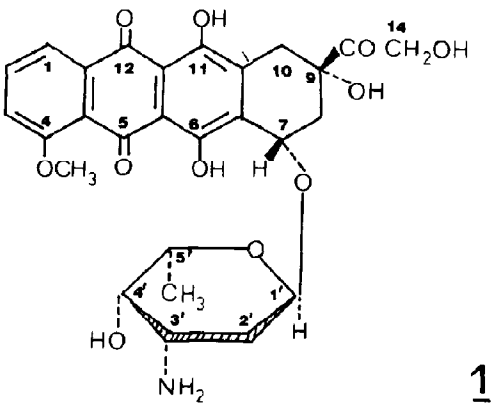
Gels have been prepared from aqueous solutions of anthracyclines by addition of salts. The gels are thixotropic and thermally reversible. They are stable for several months in the refrigerator and for long times even at room temperature. The gel-solution transition (melting) temperature depends on the concentration of the anthracycline and on the concentration and nature of the added salt. The melting has been followed by ^{1}H-NMR. Only weak intermolecular interactions (stacking and hydrogen bonds) originate the drug network, within which the solvent is entrapped. ^{1}H-NMR and polarimetric data suggest a stacked helical arrangement of the anthracycline molecules. The gelation process is cooperative.

1. Introduction

Doxorubicin (**1**) is, to date, one of the most powerful and widely used antitumour antibiotics [2–5]. Administration of the product is effected by intravenous injection, thus conveying the drug to all regions of the body.

A formulation of the drug for local administration would be of great interest given the high cardiotoxicity [6–8], which is both dose- and schedule-dependent. Particularly harmful is the delayed, often fatal, cardiotoxicity, which may appear long after the completion of antitumour therapy [9].

We report here the preparation, and some physico-chemical properties, of aqueous anthracycline gels, which might prove useful for transdermal, or more generally, topical, delivery of the drug.



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The gels easily form solutions on simple dilution, and their use as a practical storage form of the antibiotic may also be envisaged.

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* M. Giomini, A.M. Giuliani, M. Giustini and E. Trotta, Italian Patent no. 19801 A/89, registered 16 March 1989.

Gel formation by doxorubicin has occasionally been observed, especially when reconstitution of the commercial formulation (freeze-dried mixture of doxorubicin · HCl and lactose) was performed with 'Sodium chloride injection' as diluent [10], but has been regarded as essentially a nuisance.

2. Materials and methods

2.1. Materials

Doxorubicin, daunomycin and epirubicin hydrochlorides were kind gifts from Farmitalia-Carlo Erba.

Either Merck or Carlo Erba reagent grade salts were used in the preparation of the gels; the products were dried in an oven at 378 K and used without further purification. The amounts of Ca^{2+} and Mg^{2+} in oven-dried $CaCl_2 \cdot 2H_2O$ and $MgCl_2 \cdot 6H_2O$ were determined by titration with EDTA [11].

Double-distilled water was employed in the gel preparation. Merck UVASOL ^2H_2O (> 98.5% deuteration) was used for NMR measurements.

2.2. Preparation of gels

The preparation procedure described in the patent [1] involves either addition of the desired amount of anthracycline to an aqueous electrolyte solution of chosen concentration, or the addition of small controlled volumes of concentrated saline solution to an aqueous solution of the antibiotic to reach the final desired concentration of both species. No buffer is employed in either preparation.

The first procedure requires that the solution be heated for the time and to the temperature necessary for the slow dissolution of the anthracycline: considerably more drug goes into solution than at room temperature; on cooling, separation of a precipitate is not observed but transformation of the whole solution into a transparent, red gel.

Gels are more easily prepared by the other procedure, which requires a shorter period of heating (only the mixing time), thus reducing the risks of thermal degradation of the anthracycline, and ensures that a true solution is obtained.

We have prepared a series of gels at constant doxorubicin concentration (10 mM) and NaCl in the range 0.15–0.8 M to study the effect of salt concentration on the formation and properties of the gel.

The influence of the nature of the electrolyte has been studied by preparing a series of gels at constant concentration of doxorubicin (10 mM) and using different electrolytes (0.25 M; LiCl, NaCl, KCl, RbCl, $CaCl_2$ and $MgCl_2$).

Gels have also been prepared at a fixed concentration (0.25 M) of NaCl and different concentrations of doxorubicin (5, 10, 15, 20 and 30 mM) in order to demonstrate the effect of the antibiotic concentration.

The gels were prepared at a temperature just above the gel-solution transition temperature (see below) and stored for 24 h at approx. 277 K before use to allow equilibration.

Dilution of the gels with water rapidly turns them into solutions on mild shaking or swirling.

2.3. NMR measurements

The ^{1}H-NMR spectra were acquired with a Bruker AC 200 instrument, operating at 4.7 T (NMR Service of the Area della Ricerca, CNR, Roma) equipped with a temperature control unit.

A coaxial capillary, containing a 20 mM aqueous solution of TSP-d_6 (sodium 3-(trimethylsilyl)-propanesulphonate) was used as reference and for relative quantitative determinations.

The gelling process was followed by variable temperature spectra: the temperature was varied between 278 and 333 K and at least 45 min were allowed for equilibration every time the temperature was changed. Longer times are required for equilibration when the sample is cooled down from above the transition temperature; therefore, the desired temperature was always reached by heating from below.

Suppression of the water signal was effected by a presaturating selective pulse.

3. Results

3.1. Properties of the gels

Addition of small volumes of concentrated electrolyte solution under stirring to a doxorubicin solution above room temperature may result in the formation of a clear red gel upon cooling. Formation of successive liquid-crystalline phases with increasing salinity (NaCl) has been reported for dilute aqueous solutions of anionic surfactants in the presence of short-chain alcohols as co-surfactants [12]. Also, progressive addition of salts (NaBr) to aqueous ionic surfactant/co-surfactant solutions has been shown to cause disperse micellar aggregates to assemble into higher-order structures with the formation of a three-dimensional interconnected network, with the brine enclosed within the mesh of the net [13].

The doxorubicin gels are thermally reversible.

The gelation temperature depends on the concentration of both the salt and antibiotic and, to a lesser extent, on the nature of the electrolyte. The 'melting' of the gels has been followed and the transition temperature T_m visually determined for several doxorubicin-electrolyte systems (table 1).

Table 1

Apparent melting intervals, ΔT_m (K), of anthracycline aqueous gels

Composition of the gel	ΔT_m
10 mM doxorubicin + 0.15 M NaCl	278–283
+ 0.25 M NaCl	293–298
+ 0.35 M NaCl	308–313
+ 0.25 M LiCl	303–308
+ 0.25 M NaCl	293–298
+ 0.25 M KCl	313–315
+ 0.25 M RbCl	298–303
+ 0.25 M CsCl	293–298
+ 0.25 M $MgCl_2$	314–318
+ 0.25 M $CaCl_2$	308–313
+ 0.5 M KCl	314–318
+ 0.083 M $CaCl_2$ ($\mu = 0.25$)	293–298
5 mM doxorubicin + 0.25 M NaCl	290–292
10 mM doxorubicin + 0.25 M NaCl	293–298
15 mM doxorubicin + 0.25 M NaCl	308–311
20 mM doxorubicin + 0.25 M NaCl	313–316
30 mM doxorubicin + 0.25 M NaCl	320–322

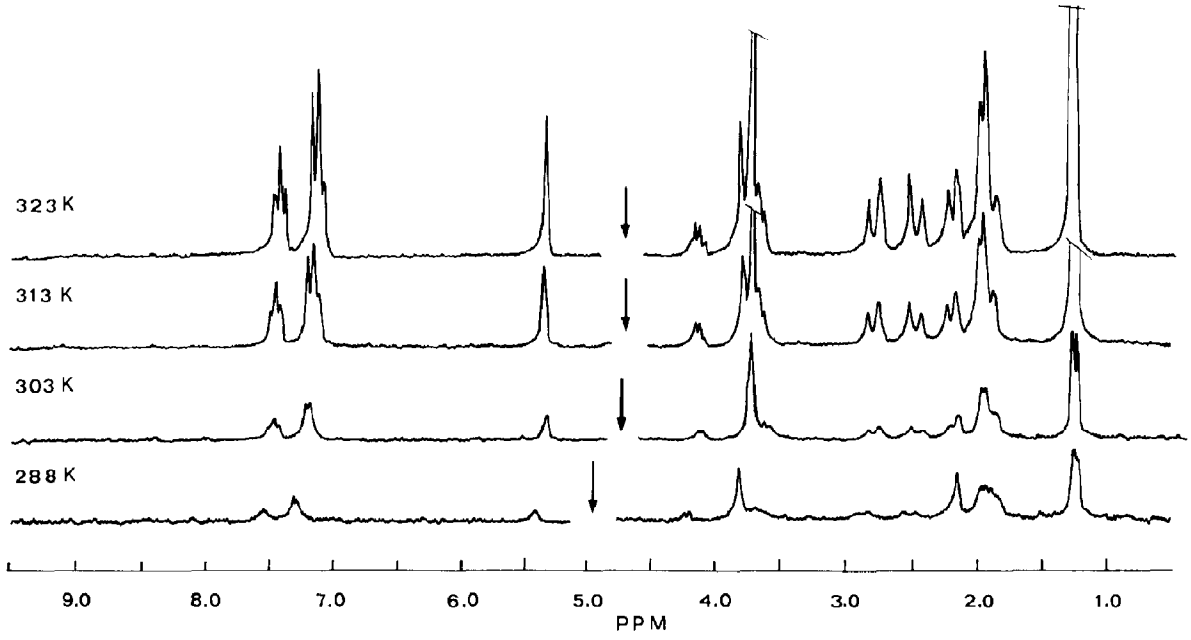

Fig. 1. Variable temperature ^{1}H-NMR spectra of a 10 mM doxorubicin-0.25 M NaCl solution. $B_0 = 4.7$ T. Arrows indicate the suppressed water peak.

The samples have been equilibrated as gels in a thermostatic bath (Haake F3 cryostat) and the temperature gradually increased in small steps allowing time for thermal equilibration at each change and observing the corresponding change in fluidity. Generally, within a range of approx. 5 K, a change is observed from a mass not flowing on inverting the test tube, to an apparently viscous fluid, to solution.

The gels exhibit thixotropic behaviour: attempts to measure the apparent viscosity with a cone-and-plate viscometer cause disruption of the gels and solutions with viscosities similar to that of water are eventually obtained.

The gels, especially those with higher transition temperature, appear to be chemically more inert than doxorubicin solutions of the same concentration. Sodium hypochlorite solutions bleach the anthracycline solutions almost at once, while the gels turn violet on the surface, due to a change in pH, but the bulk remains red and clear even under shaking. The bleaching, which implies destruction of the gel, occurs only slowly.

The gels, kept in the dark, can be stored for several months in a refrigerator, and at room temperature. However, at room temperature, after several weeks, some degradation of the drug takes place, as shown by TLC on silica gel.

3.2. NMR results

The gel-solution transition may readily be followed by ^{1}H-NMR: gelation causes a generalized broadening and intensity decrease of the resonances. A typical set of variable temperature proton spectra is shown in fig. 1 for a 10 mM doxorubicin-0.25 M NaCl system.

To allow the possibility of comparing the signal intensities obtained at different temperatures (the 90° pulse width changes with the temperature), we have added to our samples a concentric capillary containing a 20 mM solution of TSP-d_6, whose chemical shift and signal area are not expected to change with temperature. The TSP chemical shift was set to zero, its integrated intensity to 100 and the chemical shifts and intensities of our resonances were measured relative to this external reference.

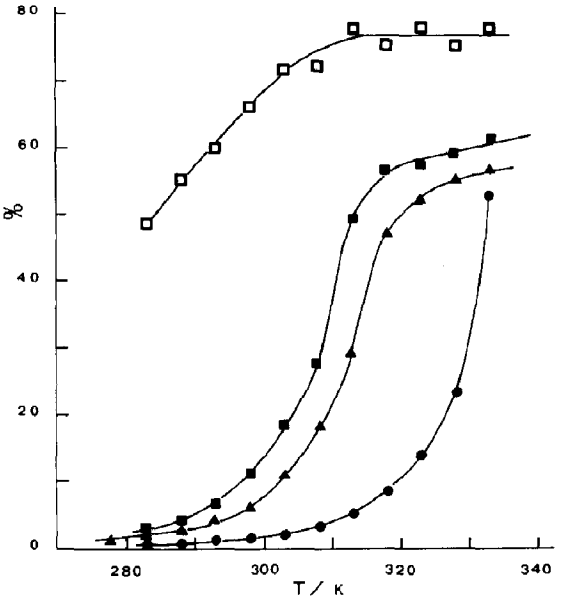

Fig. 2. Relative integrated intensity of the aromatic proton resonances as a function of temperature ([doxorubicin] = 10 mM, [NaCl] = 0.8 M). The area of the external TSP-d_6 reference is taken as 100.

The area of the aromatic protons of doxorubicin, which can be measured with satisfactory accuracy because the resonances are well separated from the rest of the spectrum, has been determined for our systems throughout the entire range of temperatures explored (278–333 K). The results, given as percentage of the external TSP resonance intensity, are shown in fig. 2 for systems comprising 10 mM doxorubicin and 0.15–0.8 M NaCl. The curves are S-shaped: this behaviour is indicative of a co-operative association process connected with the solution-gel transition.

The temperature at the foot of the rising portion of the plots, T_m, corresponds to the beginning of the 'melting' of the gel, while that at which the curve begins to level off (T_s) corresponds to complete transition to the solution state. Thus, the gelation point is not precisely defined and is somewhat subjective: the values of T_m and T_s determined for various doxorubicin-electrolyte solutions are collected in table 2.

An increase in electrolyte concentration markedly shifts the gel-solution interconversion to

higher temperatures. The effect of adding salt may be two-fold. First, it may screen out the electrostatic repulsive interactions between the charged groups of the anthracycline molecules; second, it might abstract water from the surroundings of the drug molecules, allowing their closer approach and favouring their mutual interactions. Thus, progressive addition of salt may encourage the formation of larger aggregates.

Support for this interpretation of the experimental findings comes from the variable temperature spectra at constant salt (0.25 M NaCl) and increasing antibiotic concentrations (from 5 to 30 mM): progressively higher concentrations of the drug, which allow closer contact and easier mutual interactions of the doxorubicin molecules, lead to higher transition temperatures (tables 1 and 2).

Smaller, and not easily rationalized, effects are connected with the nature of the cation (tables 1 and 2). This is particularly evident when the effect of Ca^{2+} or Mg^{2+}, and Na^{+} or K^{+} concentrations corresponding to equal ionic strength values are compared (tables 1 and 2).

The dramatic decrease in intensity of the resonances due to gelation is also observed in the ^{13}C-NMR spectra: the solution spectrum turns to a flat baseline showing only noise at temperatures below the transition, in spite of the large number of FIDs accumulated before Fourier transformation.

These experimental findings show that the doxorubicin molecule is immobilized in the gel structure. On the other hand, no sign of restricted mobility is shown by the water: half-height bandwidth ($\Delta\nu_{1/2} = 3$–4 Hz in both the gel and solution) and T_1 values (~ 4 s) remain practically unchanged throughout the transition. Moreover, gelation has a considerable effect on the chemical shifts of doxorubicin resonances, especially for the aromatic protons: a typical δ vs T plot is presented in fig. 3.

Above the transition temperature, the resonances shift upfield as the temperature decreases. The behaviour corresponds to the well-known phenomenon of self-association of the drug molecules [14]. In general, stacking interactions of

Table 2

Melting temperatures (K) of anthracycline aqueous gels from ^{1}H-NMR data. The uncertainty in the temperatures is ± 1 K, unless otherwise indicated.

Composition of the gel	T_m [a]	T_s [a]	T_s [b]
10 mM doxorubicin + 0.15 M NaCl	< 283	309	< 283
+ 0.25 M NaCl	298–301	315	313
+ 0.35 M NaCl	305–306	321	320–323
+ 0.50 M NaCl	313–314	328	> 323
+ 0.80 M NaCl	326	> 333	> 333
+ 0.25 M LiCl	302–303	314	313
+ 0.25 M NaCl	298–301	315	313
+ 0.25 M KCl	302–303	318	318
+ 0.25 M RbCl	–	–	323
+ 0.25 M CsCl	298–301	315	313
+ 0.25 M $MgCl_2$	307	323–328	323
+ 0.25 M $CaCl_2$	306	323–328	323
+ 0.50 M KCl	313–318	328	328
+ 0.083 M $MgCl_2$ ($\mu = 0.25$)	293–294	305–308	308
+ 0.083 M $CaCl_2$ ($\mu = 0.25$)	288	305–307	303
10 mM doxorubicin + 0.25 M NaCl	298–301	315	313
15 mM doxorubicin + 0.25 M NaCl	309	319	318
20 mM doxorubicin + 0.25 M NaCl	311	323	323
30 mM doxorubicin + 0.25 M NaCl	314	324	323

[a] From integrated intensities of aromatic resonances; see text.
[b] From chemical shifts of aromatic protons; see text.

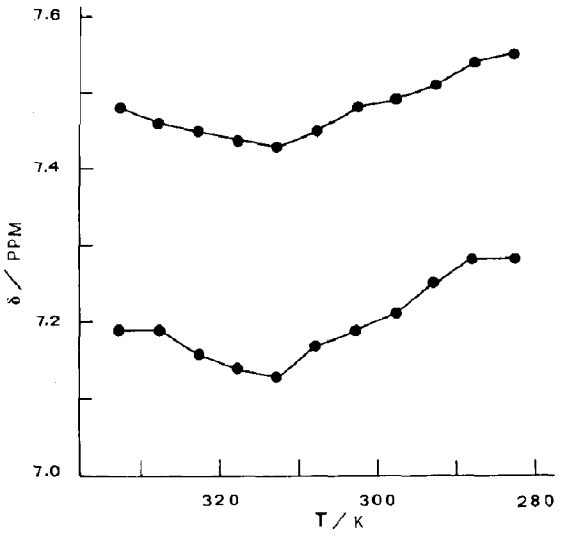

Fig. 3. Temperature dependence of the aromatic proton chemical shifts for a 10 mM doxorubicin-0.25 M NaCl system ($B_0 = 4.7$ T; TSP-d_6 reference $\delta = 0.0$ ppm).

anthracyclines or other chemical species with planar aromatic systems lead to upfield shifts of the resonances [14,15], due to ring-current shielding. However, in our systems, below the transition temperature, a downfield shift of several resonances, particularly those of the aromatic protons, is observed on cooling (fig. 3). This finding indicates the onset, at gelation, of intermolecular interactions whereby the protons of one drug molecule are located in the deshielding region of the adjacent molecules.

The temperature at which the chemical shift trend is inverted is fairly well defined and may be used to evaluate a 'melting' temperature for the gels instead of a melting interval as obtained from peak area measurements.

The chemical shift temperature dependence is reminiscent of the anomalous dilution shift observed for the H(1) proton of ellipticine [16], regarding as being equivalent dilution and increasing temperature effects. In that case, two types of intermolecular interactions were suggested to explain the experimental data: stacking and edge-to-edge associations of the chromophores.

For our gels similar competing phenomena, formation of stacks with π interactions between aromatic ring systems and aggregation to large molecular assemblies via attractive weak interactions (e.g., dipolar or hydrogen bonding), may be envisaged. These interactions may involve specific groups of the anthracycline. Indeed, the closely related anthracycline daunomycin, where the OH group in C(14) is replaced by an H atom, cannot be induced to form gels, suggesting that hydrogen bonding interactions involving that particular hydroxyl group are essential for the gelling of the solution. Epirubicin (i.e., 4′-epidoxorubicin) solutions, on the other hand, are easily turned into gels on addition of salts, and the gels have properties very similar to those of doxorubicin.

In the formation of the gel, the drug molecules must provide a continuous three-dimensional network within which microcompartments of the electrolyte solvent solution are encapsulated. In view of the chemical structure of the anthracycline, the data suggest helical stacking of the drug molecules, with the plane of the fused rings of one molecule slightly twisted with respect to the next and the sugar moieties and functional groups projecting out of the molecular plane, as in a cholesteric liquid crystal.

Such a type of structure, formed by a chiral species like doxorubicin, should exhibit greatly enhanced optical activity by virtue of the supramolecular helical order. We have measured the specific rotation of doxorubicin in water and in saline solutions below the gelation temperature (Perkin Elmer 241 polarimeter).

A dramatic increase in specific rotation is measured for the gels relative to the drug-alone solution (e.g., the $[\alpha]^{15}_{578}$ values are -16.4 and $-1639.0°$ for 10 mM doxorubicin in water and in 0.35 M aqueous NaCl, respectively).

Such a considerable increase in specific rotation is in support of the suggested structure of the gel.

Preliminary circular dichroism measurements are also in agreement with the presence of a helical arrangement of the molecules in the gel.

Attempts to obtain information about the order to doxorubicin molecules in the gels from X-ray measurements have been unsuccessful.

The gels that we describe here markedly resemble other gels recently reported by Weiss and

co-workers [17,18]. They have presented a new family of gelators, called ALS, whose chemical structure has several features in common with the anthracycline antibiotics. The ALS possess an anthracenyl group connected through an atom or a chain to a steroidal group; they form stable thermally reversible gels of organic fluids where only weak intermolecular interactions are responsible for the gelator network. The anthracenyl groups of ALS are partially overlapping but non-parallel, giving rise to a cholesteric liquid-crystalline phase, in which the isotropic organic fluid is entrapped. Gelling causes a slight downfield shift of some ^{1}H-NMR resonances, and dramatic changes in the CD and fluorescence spectra.

4. Conclusions

Doxorubicin can act as a 'gelator' of water in the presence of salts.

The gels are stable and thermally reversible. The NMR, polarimetric and preliminary CD data suggest that anthracycline molecules are helically stacked, giving rise to a cholesteric liquid-crystalline structure, with immobilization of the drug molecules, while the solvent micromobility remains unaltered.

In the process of formation of the three-dimensional network by the doxorubicin molecules, these interact not only through π bonding between neighbouring aromatic ring systems, but also through more specific mechanisms, e.g., hydrogen bonding. These specific interactions are made possible by the twisted arrangement of the molecules in the gel. Increasing the temperature disrupts the helically stacked order, and the gels eventually melt; addition of water causing an increase in the average intermolecular distances also destroys the gels.

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