

Dimer-promoted fluorescence quenching of coralyne by binding to anionic polysaccharides

Mónika Megyesi,^a László Biczók^{*a} and Helmut Görner^b

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The effects of anionic chondroitin or dextran sulfates on the absorption and fluorescence properties of coralyne, a cationic benzo[c]phenanthridine type alkaloid, were studied in aqueous solution. The remarkably strong binding to both polysaccharides promotes the coralyne dimer formation, which was evidenced by the changes in the absorption and fluorescence spectra and the fluorescence decay. The extent of dimerization, induced by chondroitin, shows a significant pH dependence because the competitive protonation of the carboxylate moieties of the polymer chain decreases the number of binding sites. A larger molecular weight of dextran sulfate stabilizes the coralyne dimer more efficiently.

Introduction

The binding of oppositely charged species to water soluble polyelectrolytes has been extensively studied because this type of interaction plays a pivotal role in biological systems, biomedical applications, drug delivery and controlled release of biologically active compounds.¹ Synthetic polyions act as model systems for the understanding of the often far more complex behaviour of the natural charged macromolecules such as proteins and DNA.² Polyelectrolyte–dye complexes can self-aggregate to nanoparticles leading to amplified quenching of its fluorescence, which can be exploited in the design of sensitive optical sensors.³ Some effects of counter ions, temperature and the characteristics of the components were studied in polyelectrolyte–antidepressant drug complexes.^{4,5} To the best of our knowledge, no information is available on the binding of alkaloids to polyanions other than DNA.

In the present work, we focus on coralyne, a monocationic benzo[c]phenanthridine type alkaloid (Scheme 1), which intercalates in DNA,⁶ induces DNA aggregation or precipitation,⁷ stabilizes triple helices⁸ and alters the nucleic acid secondary structure.⁹ Its biological activity and interaction with polynucleotides have been recently reviewed.¹⁰ Spectrophotometric measurements demonstrated that it forms dimers and multiple aggregates in water. The increase of the ionic strength or the presence of small amount of DNA was found to promote the association.¹¹ Garcia *et al.* revealed the effect of ethanol on the thermodynamics and kinetics of coralyne aggregation.¹² Coralyne initiates very fast photochemical DNA damage by single-strand cleavage. Although the reaction mechanism is unknown, coralyne aggregates were suggested to serve as photosensitizers in this process.¹³ The fluorescence intensity alteration of coralyne-impregnated silica gel plates allows a sensitive quantitative detection of a wide number

of analytes by high-performance thin layer chromatographic techniques.¹⁴

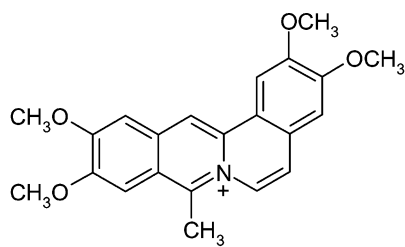
Our main goal is to explore how the photophysical characteristics of coralyne change upon self-aggregation and association with negatively charged polysaccharides, such as a sodium salt of chondroitin-6-sulfate (CS) and two dextran sulfates. The former compound is a glycosaminoglycan, which is a major component of extracellular matrix and a widely used dietary supplement for treatment of osteoarthritis. It has an unbranched chain composed of about 100 alternating *N*-acetylgalactosamine and glucuronic acid moieties. Owing to the presence of the carboxylate and sulfate groups (Scheme 1), each repetitive disaccharide unit possesses two negative charges. Dextran sulfate salts having average molecular masses of 500 000 and 5000 Da (D500k and D5k) and branched structures were also employed as polyelectrolytes since side chains can induce a coil conformation.¹⁵ The repulsion of the negatively charged sulfate groups stabilizes the fully extended molecular structure in low ionic strength solution¹⁶ but condensation of counter ions can bring about conformational change.¹⁷ Dextran sulfate salts are known for their antithrombotic and antiviral activity¹⁸ and commonly used as both experimental and clinical laboratory reagents.

Experimental

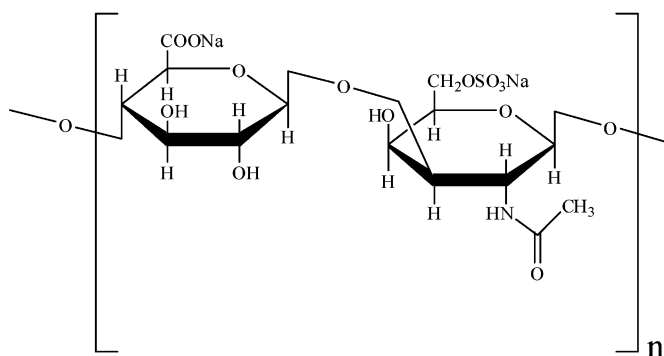
Coralyne chloride (Acros Organics) was recrystallized from ethanol. Sodium salts of chondroitin-6-sulfate (BioChemika Fluka from shark cartilage) and dextran sulfates D500k and D5k (Fluka) were used as received. According to the specification of the manufacturer, dextran sulfates contain on average 2.3 sulfate groups per glucoside unit. For the calculation of the molar concentration, 396 and 503 Da were used as the molecular mass of the sodium salt of dextran sulfate monomer and chondroitin-6-sulfate disaccharide repeating units. The UV-visible absorption spectra were recorded on a Unicam UV 500 spectrophotometer. Corrected fluorescence spectra were obtained on a Jobin-Yvon Fluoromax-P photon-counting spectrofluorometer and the fluorescence yields (Φ_F) were determined relative to that of quinine

^aChemical Research Center, Hungarian Academy of Sciences, P.O. Box 17, 1525, Budapest, Hungary. E-mail: biczok@chemres.hu

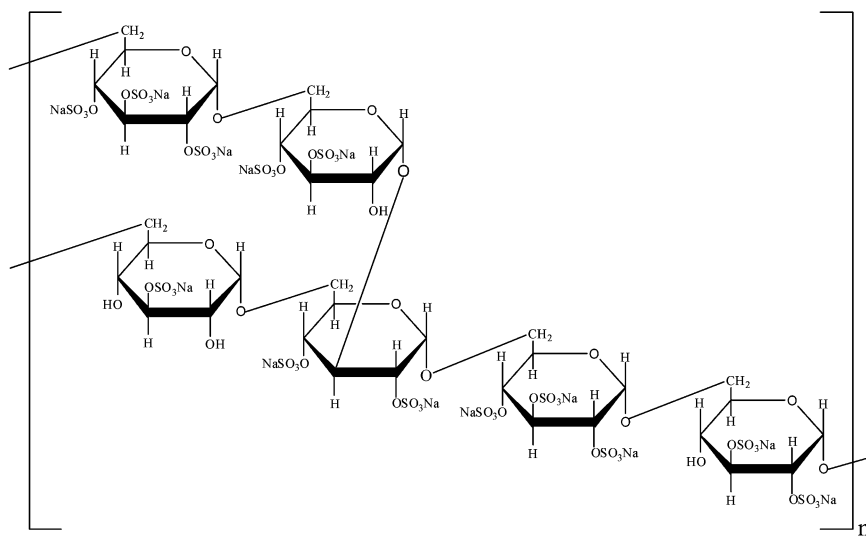
^bMax-Planck-Institute for Bioinorganic Chemistry, P.O. Box 101365, D-45413, Mülheim an der Ruhr, Germany. E-mail: goerner@mpi-muelheim.mpg.de



Coralyne cation



Chondroitin-6-sulfate sodium salt



Dextran sulfate sodium salt

Scheme 1

sulfate in 0.5 M H_2SO_4 solution, for which a reference yield of $\Phi_F = 0.546$ was taken.¹⁹ Fluorescence decays were measured with the time-correlated single-photon-counting technique. A Picoquant diode laser (pulse duration *ca.* 70 ps, wavelength 372 nm) excited the samples, and the fluorescence decays were detected with a Hamamatsu R3809U-51 microchannel plate photomultiplier, which was connected to a Picoquant Timeharp 100 electronics (36 ps/channel time resolution). All experiments were carried out at 296 K. Data were analyzed by a nonlinear

least-squares deconvolution method using Picoquant FluoFit software.

Results and discussion

Association in water

As seen in Fig. 1, the spectral characteristics of coralyne depend significantly on the concentration. Wilson and coworkers have

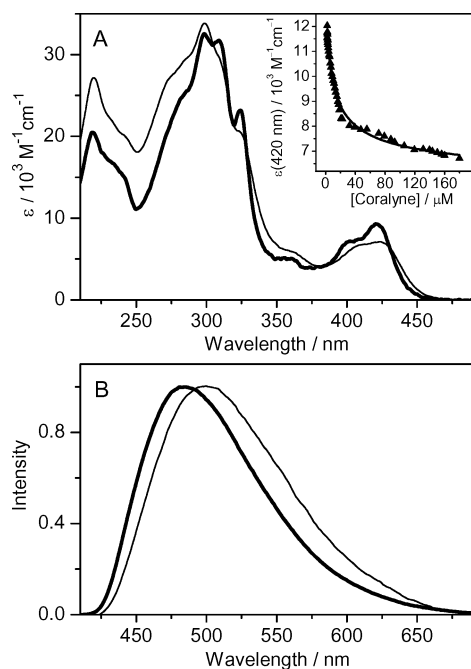


Fig. 1 Absorption (A) and fluorescence emission (B, $\lambda_{\text{exc}} = 350$ nm) spectra of 3.1 (thick line) and 40.2 μM (thin line) coralyne in aqueous solution at pH 6. The inset shows the apparent molar absorption coefficient at 420 nm as a function of the coralyne concentration. The line represents the best non-linear least-squares fit.

studied the coralyne absorption above *ca.* 350 nm. They observed hypochromicity and a small red-shift of the lowest energy absorption band with increasing concentration. The spectral changes were attributed to dimerization for which $1.1 \times 10^5 \text{ M}^{-1}$ equilibrium constant was calculated in neat water.¹¹ Our results are in accordance with these findings, and an examination in a wider spectral domain showed that significant alterations also occur in the higher energy absorption bands. A recent paper, published during the preparation of our revised manuscript, also demonstrates the concentration dependence of the coralyne absorption.¹² As a representative example, the inset to Fig. 1 displays the variation of the apparent molar absorption coefficient at 420 nm with increasing total coralyne concentration ($[C]_{\text{T}}$). On the basis of the equilibrium constant of dimer (D) formation, $K = [D]/[M]^2$, the fraction (α) of $[C]_{\text{T}}$ present as monomer (M) is given by the following equation:

$$\alpha = \frac{-1 + \sqrt{1 + 8K[C]_{\text{T}}}}{4K[C]_{\text{T}}} \quad (1)$$

The measured apparent molar absorption coefficient (ε) is:

$$\varepsilon = \varepsilon(\text{M})\alpha + \varepsilon(\text{D})(1 - \alpha)/2 \quad (2)$$

where $\varepsilon(\text{M})$ and $\varepsilon(\text{D})$ represent the molar absorption coefficients for the monomer and dimer, respectively. The computed function, displayed in the inset to Fig. 1 as solid line, matches the experimental data well, and the parameters corresponding to the best fit are: $K = 1.1 \times 10^5 \text{ M}^{-1}$, $\varepsilon(\text{M}) = 14100 \text{ M}^{-1} \text{ cm}^{-1}$ and $\varepsilon(\text{D}) = 11200 \text{ M}^{-1} \text{ cm}^{-1}$ at 420 nm. NMR measurements at high (5 mM) coralyne concentration by Garcia *et al.* provided a somewhat lower, $8693 \pm 102 \text{ M}^{-1}$ value for the self-aggregation constant.¹²

Under their experimental conditions not only dimer but also a significant amount of larger aggregates were formed. The results of NMR measurements implied that the monomer aggregation came about by stacking interactions, which involve mainly hydrophobic forces.¹²

The normalized fluorescence spectra (Fig. 1B) demonstrate a bathochromic displacement and a slight broadening of the emission band in concentrated solution. The fluorescence maximum is plotted as a function of the concentration in Fig. 2A. The approximately 16 nm red-shift of the peak is accompanied by the diminution of the fluorescence quantum yield from 0.34 to 0.20 on going from 3 to 40 μM coralyne solution (Fig. 2B). Using the $K = 1.1 \times 10^5 \text{ M}^{-1}$ value for the equilibrium constant of dimerization, we can calculate that from 69 to 28% of coralyne is present as monomer in this concentration range. Thus, the decline of Φ_{F} is due to the weaker emission of the dimer.

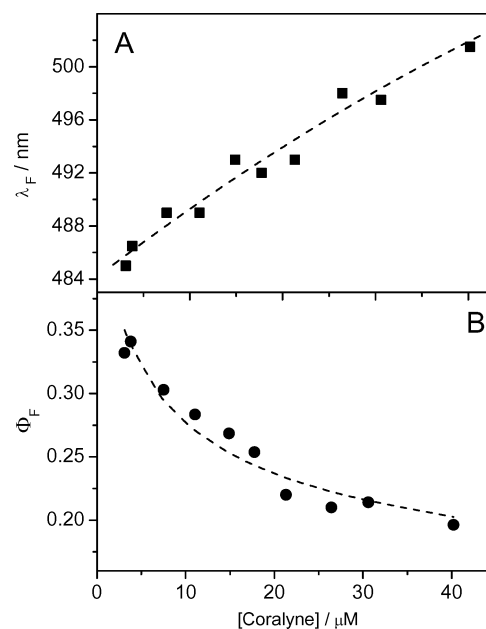


Fig. 2 Coralyne concentration dependence of the fluorescence maximum (A) and the fluorescence quantum yield (B) in aqueous solution at pH 6.

Time-resolved fluorescence measurements showed an exponential decay with a lifetime of $\tau_1 = 9.7$ ns below 0.04 μM coralyne concentration. When the concentration was raised, a long-lived fluorescence component with a lifetime of $\tau_2 = 40.7$ ns appeared with gradually increasing amplitude due to dimerization. Both decay parameters were constant within the limits of experimental errors in the whole concentration domain indicating that no higher aggregates were produced. The longer lifetime and lower Φ_{F} of the dimer compared to the monomer suggest that association slows down both the radiative and the radiationless deactivations of the singlet-excited state.

Effects of CS in neutral solution

The effect of polysaccharides in aqueous solution was examined for 2.7 μM coralyne, where the majority of the alkaloid molecules are present as monomers. In order to eliminate the effect of polydispersity, the amount of polyelectrolyte is given as the concentration of the disaccharide repeat unit. Fig. 3 depicts the change of

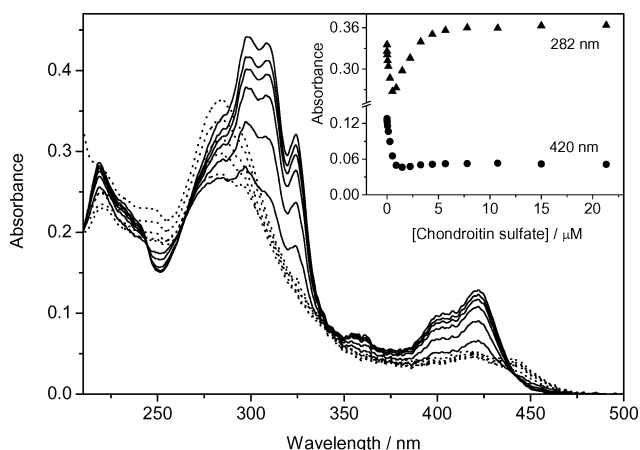


Fig. 3 Absorption spectra of 2.7 μM coralyne in aqueous solution at pH 6 and in the presence of 0, 0.028, 0.066, 0.14, 0.29, 0.54 μM (solid line) and 0.90, 1.5, 2.2, 3.3, 21 μM (dotted line) CS (optical path is 5 cm). Inset presents the absorbance change at 282 (\blacktriangle) and 420 nm (\bullet) as a function of the CS concentration.

the absorption spectrum upon addition of gradually increasing amounts of CS and the inset illustrates the absorbance variation at 282 and 420 nm. In the 0–0.54 μM CS concentration domain marked hypochromicity is observed and the isosbestic points appearing at 245, 265 and 442 nm indicate 1:1 complexation of coralyne to the CS repeating sequence. Further augmentation of CS concentration brings about a bathochromic displacement of the first absorption band and a concomitant rise of a new absorption band with a maximum at 284 nm. This suggests that the binding of a second coralyne molecule to the disaccharide segment facilitates dimer formation. The absorption spectrum of CS-bound and free dimers are displayed in Fig. 4. For the sake of better comparison, the two spectra are normalized at the maximum of the most intense band. On the basis of eqn (1) and $K = 1.1 \times 10^5 \text{ M}^{-1}$, 81.4% of coralyne is dimerized in 107 μM aqueous solution. When CS induces aggregation, a somewhat different spectrum is obtained indicating that the association with CS probably modifies the structure of the dimer and the association of more than two coralyne to the disaccharide unit of CS cannot be excluded. The charge recombination upon association with the negatively charged CS decreases the electrostatic repulsion

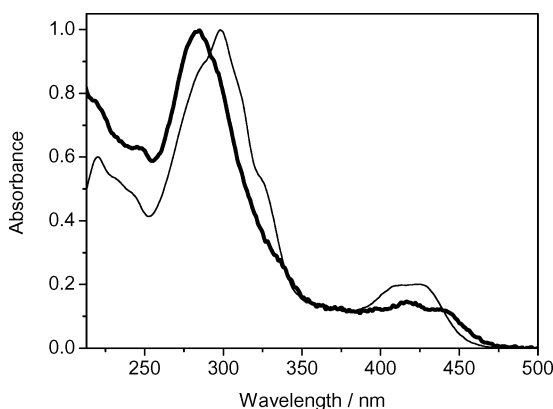


Fig. 4 Normalized absorption spectra of 107 μM coralyne (thin line) and 2.7 μM coralyne + 21 μM CS (thick line) in water at pH 6.

between the bound coralyne cations and the conformation change of the polymer may bring them close enough to interact with each other. Probably hydrophobic forces and the π – π interaction between the flat moieties facilitate the dimerization.

As shown in Fig. 5, addition of even a small amount ($<0.7 \mu\text{M}$) of CS brings about a significant fluorescence quenching but only a negligible shift of the fluorescence maximum is observed in such diluted solutions. In contrast, a moderate fluorescence intensity diminution and a marked red-shift of the fluorescence maximum is seen in the 0.7–1.6 μM CS concentration range but these changes level off and the spectral characteristics do not alter up to a CS concentration of about 0.1 mM. In more concentrated polymer solutions, fluorescence enhancement and a blue-shift of the emission peak were detected.

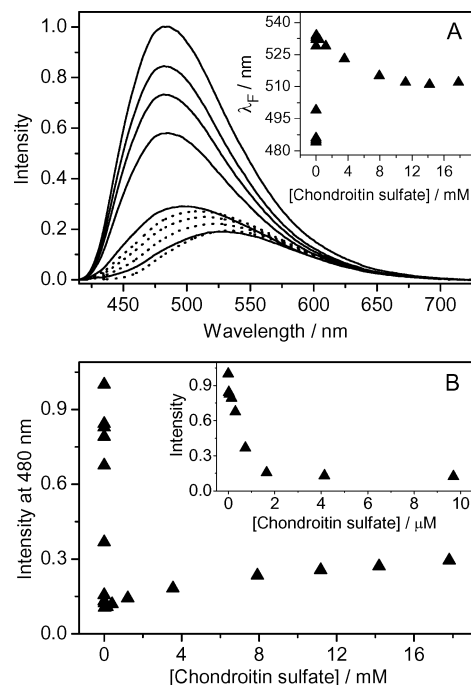


Fig. 5 (A) Fluorescence spectra of 3.16 μM coralyne aqueous solution at pH 6 on addition of 0, 0.022, 0.14, 0.31, 0.74, 1.6 μM (solid line), 0.14, 3.6, 7.9, 14 mM (dotted line) CS, excitation at 372 nm. Inset shows the fluorescence maximum vs. chondroitin sulfate concentration. (B) The fluorescence intensity at 480 nm corrected by the change of absorbance at the excitation wavelength (372 nm) plotted as a function of CS concentration at pH 6. Inset represents the low concentration domain.

In order to gain deeper insight into the origin of the significant alteration of the fluorescent behaviour, time-resolved measurements were performed in 3.16 μM coralyne solution. Below 0.7 μM CS concentration, exponential fluorescence decays with an invariable lifetime were observed. This implies that the 1:1 complexation of coralyne to the disaccharide unit of CS does not alter the fluorescence decay kinetics. However, the considerable reduction of the fluorescence intensity (Fig. 5) indicates that the radiative rate constant decreases upon 1:1 binding. The slowdown of the radiative deactivation from the singlet excited state upon 1:1 complexation is probably compensated by the acceleration of the radiationless energy dissipation processes.

In the presence of 0.7 μM CS, a long-lived fluorescence component with a constant 61 ns lifetime emerged, whose fractional

amplitude ($a_2/(a_1 + a_2)$) grew up to 54 μM CS concentration, and thereafter declined (Fig. 6). This emission is attributed to interacting polymer-bound coralyne. Each disaccharide unit has two anionic substituents, a carboxylate and a sulfate groups, providing links to coralyne cations. The saturation of the anionic binding sites by the oppositely charged alkaloid eliminates the electrostatic repulsion among the polyelectrolyte segments thereby allowing larger flexibility in the polymer chain. The more facile conformational movement of the polysaccharide backbone promotes the interaction between the bound alkaloid molecules. The diminution of the $a_2/(a_1 + a_2)$ values at CS concentration above 54 μM reflects the reduced mobility of the polymer chain and the lesser chance for binding to two neighboring sites, where guests can interact. The fractional amplitude of the long-lived fluorescence does not vanish completely at high CS concentration in water, indicating that the polyelectrolyte-bound coralyne can dimerize more efficiently than the free coralyne. The fluorescence enhancement and a blue-shift of the emission at high CS concentration are due to the decreasing extent of dimerization.

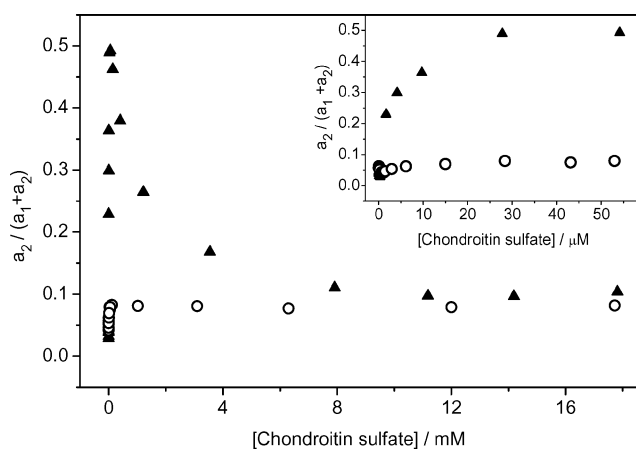


Fig. 6 Fractional amplitude of the long-lived fluorescence component detected at 490 nm as a function of the CS concentration in 3.16 μM coralyne solution. Inset represents the zoomed view of the initial part. Triangles and open circles represent the results at pH 6 and 2, respectively.

Effect of CS at pH 2

In acidic solution, CS has a different effect on the fluorescence of coralyne than in neutral conditions. As seen in the inset to Fig. 7B, the intensity diminution at pH 2 levels off at about 3 μM CS, which corresponds to 1 : 1 complexation stoichiometry. The 2 : 1 association with a repeating unit is hindered because at low pH the carboxylate groups of the polymer are blocked by protonation. Above 3 μM CS concentration, a weak fluorescence component with $\tau_2 = 55$ ns appears but its fractional amplitude ($a_2/(a_1 + a_2)$) does not exceed 8%. This indicates the small extent of coralyne dimer formation when only a single binding site is available in the repeat unit of the polymer. The remarkable difference in the CS concentration dependence of $a_2/(a_1 + a_2)$ at pH 2 and 6 is displayed in Fig. 6. The $a_2/(a_1 + a_2)$ fractional amplitudes at pH 6 approach the value obtained in acidic solution when the large excess of CS provides little chance for binding two coralyne molecules to the neighboring sites of the polysaccharide. The shorter-lived fluorescence components have the same lifetime (9.7 ns) irrespective of the pH and CS concentration.

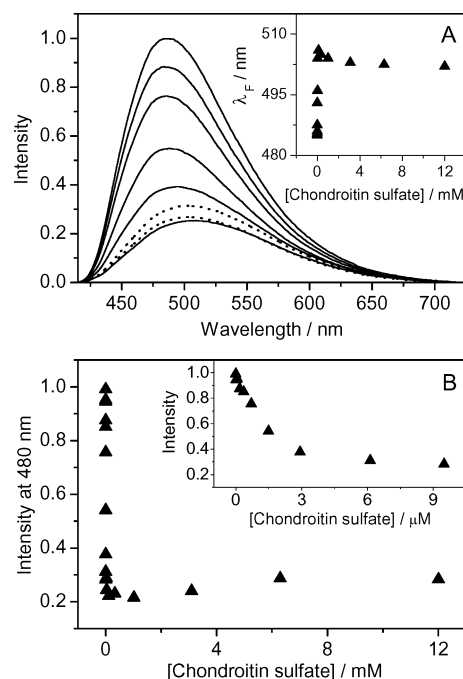


Fig. 7 (A) Fluorescence spectra of 3.13 μM coralyne in aqueous solution at pH 2 in the presence of 0, 0.18, 0.71, 1.5, 2.9, 120 μM (solid line), 3.1 and 12 mM (dotted line) CS, excitation at 372 nm. Inset shows the variation of the fluorescence maximum. (B) Concentration dependence of the fluorescence intensity at 480 nm at pH 2. Inset represents the low concentration region.

Effect of dextran sulfate salts in neutral solution

In order to reveal how the replacement of carboxylates by sulfate moieties on the polysaccharide chain influences the binding of coralyne, dextran sodium sulfate salts were used as additives. The molecular weight of these substances was changed from 5000 to 500 000 Da in order to unravel the effect of the chain length on the association. Addition of dextran sulfate leads to analogous alteration in the absorption and fluorescence properties as described for CS (*vide supra*) but the extent of change depends on the degree of polymerization. The inset to Fig. 8 illustrates that D500k induces coralyne dimerization more efficiently than D5k. The repeat unit concentration, where the fractional amplitude of coralyne dimer fluorescence ($a_2/(a_1 + a_2)$) reaches a limiting value, is lower for D500k than for D5k. In the presence of the former polymer, the $a_2/(a_1 + a_2)$ values diminish slower above 0.5 mM repeat unit concentration. A similar behavior appears for the fluorescence maximum (Fig. 8B). It is known that the degree of branching of dextran sulfates increases with the molecular weight.¹⁷ When the polymer has more side chains and the main chain is longer, encounters of the bound coralyne molecules are more likely and a less polar microenvironment can be developed. Both effects may contribute to the larger stability of the D500k bound dimer.

Conclusions

Coralyne in neutral aqueous solution exhibits a large affinity to anionic polysaccharides due to electrostatic and hydrophobic interactions. The binding promotes coralyne dimerization even in

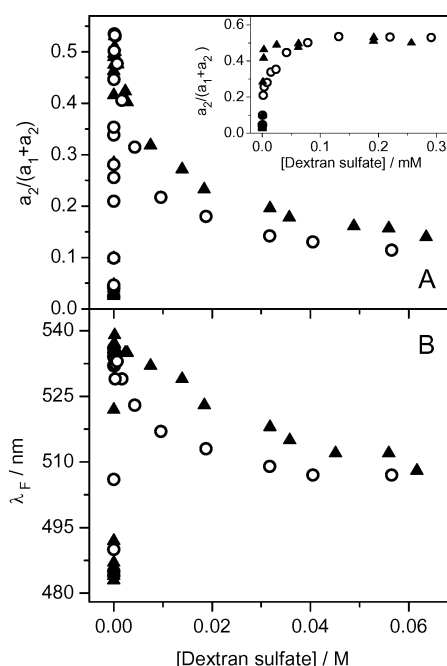


Fig. 8 Dextran sulfate sodium salt concentration dependence of the fractional amplitude of the long-lived fluorescence decay component (A) and the fluorescence maximum (B) of 3.2 μ M coralyne in aqueous solution at pH 6. Inset shows a zoomed view. Open circles and filled triangles represent the results obtained with a molecular weight of 5 and 500 kDa, respectively.

the presence of a polymeric repeat unit in large concentration. Chondroitin and dextran sulfates induce similar changes in the absorption and fluorescence characteristics of coralyne. The stability of the polymer-bound coralyne dimer is significantly diminished by the protonation of the carboxylate groups of chondroitin, whereas the change is smaller when the molecular weight of dextran is increased by a factor 100.

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