

Article

Synergistic π – π Stacking and Hydrogen Bonding of Doxorubicin Electrostatically Bound to Sodium Alginate Drive the Formation of Supramolecular Nanoparticles with Exceptional Drug Loading

Lucrezia Desiderio ¹, Paolo Di Gianvincenzo ², Desire Di Silvio ², Sunisa Thongsom ², Alessio Olivieri ¹, Anita Scipioni ¹, Donato Monti ¹, Marco D'Abramo ^{1,*}, Sergio E. Moya ^{1,2,*} and Mauro Giustini ^{1,3,*}

- ¹ Chemistry Department, Sapienza University, P.le Aldo Moro 5, 00185 Rome, Italy; lucrezia.desiderio@uniroma1.it (L.D.); alessio.olivieri@uniroma1.it (A.O.); anita.scipioni@uniroma1.it (A.S.); donato.monti@uniroma1.it (D.M.)
 - ² Centro de Investigación Cooperativa en Biomateriales—CICbiomaGUNE, Paseo Miramón 194, 20014 Donostia/San Sebastián, Gipuzkoa, Spain; pdgianvincenzo@cicbiomagune.es (P.D.G.); ddsilvio@cicbiomagune.es (D.D.S.); sthongsom@cicbiomagune.es (S.T.)
 - ³ Consorzio Interuniversitario per lo Sviluppo dei Sistemi a Grande Interfase—CSGI c/o Chemistry Department, University of Bari “A. Moro”, Via Orabona 4, 70126 Bari, Italy
- * Correspondence: marco.dabramo@uniroma1.it (M.D.); smoya@cicbiomagune.es (S.E.M.); mauro.giustini@uniroma1.it (M.G.)

Abstract

Doxorubicin hydrochloride (DX) is a widely used chemotherapeutic agent whose severe side effects can be attenuated by suitably designing drug delivery systems. The complexation between DX and the biocompatible and biodegradable sodium alginate (ALG) results in the formation of DX@ALG nanoparticles under conditions where neither the drug nor the polymer tends to form nanoscopic structures. DX@ALG complex formation leads to the quenching of DX intrinsic fluorescence, and the drug shows chiroptical properties characteristic of supramolecular gels of DX. The association of DX and ALG into supramolecular nanoparticles takes place at low DX concentration and without salt addition. More than 99% of the moles of DX complexed with ALG form a gel-like phase, and DX@ALG nanoparticles show a high DX loading efficiency. Nanoparticle formation is revealed by Dynamic Light Scattering (DLS) and Atomic Force Microscopy (AFM). Quantum Mechanics (QM) calculations support the formation of supramolecular DX-ALG aggregates by π – π stacking and hydrogen bonds. DX@ALG nanoparticles have been fully characterized by several spectroscopic techniques. Preliminary in vitro evaluation of DX@ALG toxicity revealed an antiproliferative activity against selected cancer cell lines.

Keywords: doxorubicin; sodium alginate; nanoparticles; fluorescence spectroscopy



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1. Introduction

Anthracyclines show a fascinating tendency to self-associate once they are in aqueous solution [1]. Although most of the molecules belonging to the anthracycline family can form dimers, doxorubicin hydrochloride (DX; Figure 1), a widely used chemotherapeutic agent, is the only anthracycline capable of generating supramolecular structures, in a polymerization process that can be described as an equilibrium between monomeric (DX_{mon}), dimeric (DX_{dim}), and polymerized (DX_{pol}) forms [2]. In water, at room temperature and increasing DX concentration, the equilibrium $DX_{mon} \rightleftharpoons DX_{dim}$ is progressively shifted towards the aggregated form of the drug, with an equilibrium constant of the order of 10^4 M^{-1} [3]. Only

when close to the drug solubility limit [2] or in the presence of critical amounts of NaCl and DX (the so-called Critical Gel Formation Concentration—CGFC) [4] does the drug start to self-assemble into cylindrical supramolecular aggregates of a significant length, turning the solution into self-supporting gels, made only of DX molecules stabilized by a complex interrelation of weak π – π interactions and hydrogen bonds between themselves and with the solvent [2]. Although this DX polymerized gel phase (from now on referred to as DX_{GEL}) is of great interest from a chemical-physical point of view [3], its therapeutic relevance is limited, since the concentration required for gel formation is about 1000 times higher than the therapeutic dose of DX and dilution rapidly leads to the breakdown of DX_{GEL} into a mixture of DX_{dim} and DX_{mon} [2,3].

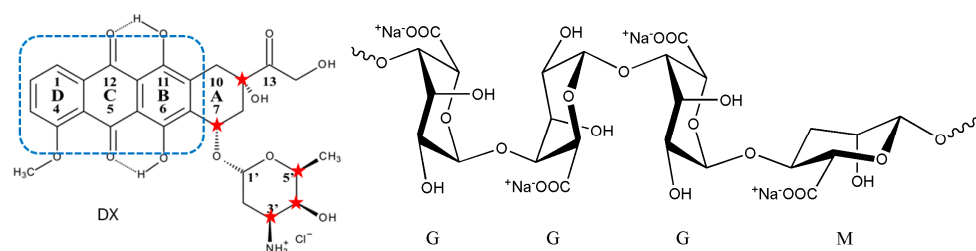


Figure 1. Chemical structure of doxorubicin hydrochloride (DX) and of sodium alginate tetramer (ALG; G, guluronic acid and M, mannuronic acid monosaccharide blocks) as representative building blocks of the ALG copolymer used for the stoichiometry calculations—see Supplementary Materials. DX chemical structure is represented with the highlighted anthraquinone moiety used in QM calculations (rounded blue rectangle) and the stereocentres (red asterisks) responsible for the CD activity shown by the drug.

DX has a characteristic planar anthraquinone moiety, a specific arrangement of hydrogen bond donor/acceptor groups, and five chiral centers (Figure 1), allowing the drug to intercalate with high affinity between adjacent base pairs of DNA [5], providing characteristic fluorescence [6] and also circular dichroism (CD) [7] spectroscopic properties. The presence of DX_{GEL} is coupled with unique spectroscopic characteristics: disappearance of any DX ¹H NMR resonances, shape and intensity of absorption bands, peculiar fluorescence and CD spectra, and a decrease of almost one order of magnitude in the fluorescence lifetime of the drug, to mention the most prominent changes in its properties [2,8,9]. Therefore, the formation of DX_{GEL} can be easily detected by non-invasive spectroscopic techniques.

As demonstrated by our group, polystyrene sulfonate (PSS), a strong anionic polymer, can promote stable self-assembly of DX [10]. The interaction between DX and PSS is mainly electrostatic, involving the sulphonate group of the polymer and the ammonium group of the drug, with the PSS chains also acting as a template, orienting the DX molecules to arrange into unusual parallel dimers stabilized by π – π interactions, as evidenced by CD spectroscopy and confirmed by MD calculations [10]. However, the toxicity, non-biodegradability, and synthetic nature of PSS make it unsuitable for the design of any delivery system, which is necessary to reduce the severe systemic side effects associated with treatments using DX. We have also shown that pH can be exploited to modulate the interaction between the drug and other components, influencing its charge and solubility [11].

Sodium alginate (ALG; Figure 1) is a weak natural anionic polyelectrolyte whose charge is highly dependent on pH. ALG is proposed here as a biocompatible and biodegradable alternative to PSS for DX complexation.

In a recently published study [12], it was demonstrated that ALG can promote and modulate the self-assembly of cyanidin, a molecule belonging to the anthocyanin family. Thanks to its negative charge, ALG electrostatically interacts with the positive form of

anthocyanins (flavylium cation) in an acidic environment, promoting the formation of stable complexes. Complexation with ALG not only stabilizes anthocyanins, which are otherwise unstable at neutral or basic pH, but also triggers the supramolecular arrangement of the cyanidin, which can be tuned by pH.

The anionic nature of ALG, a copolymer made of α -L-guluronic acid and β -D-mannuronic acid residues (indicated in the following as M and G, respectively), has been exploited here to facilitate the interaction with the positively charged ammonium group of DX at physiological pH. In the presence of divalent cations acting as crosslinkers, such as Ca^{2+} [13], ALG undergoes ionic gelation due to the strong interaction between the ions and the carboxylate groups, mainly of G residues, from different polymer chains. In the presence of DX, however, ALG forms discrete particles without any added divalent ion. The interaction between DX and ALG triggers the self-assembly of both species into nanoparticles with sizes in the range of 20–50 nm (making reference to the most abundant population—see Supplementary Materials) depending on R, in a synergistic way. The self-assembled DX-ALG nanoparticles display fluorescence and CD spectra that resemble those of DX in the gel phase. However, nanoparticle formation occurs at much lower DX concentrations and with the ionic strength (*I*) remaining always well below the values necessary for free DX to undergo a DX_{GEL} transition (for a 10 mM DX water solution, *I* should be at least equal to 0.2 M for NaCl addition, while for a 50 μM DX/ALG solution at $R = 1$ and $R = 80$, *I* is equal to 0.55 mM and 40 mM, respectively).

Unlike other formulations for DX delivery based on ALG [14–16], no other polymers or divalent ions are used in this work, simplifying the synthesis and reproducibility.

The use of ALG also has additional advantages in terms of eco-compatibility, biocompatibility, biodegradability, and environmental sustainability, representing a “green” approach to the future design of carrier systems, opening a wider range of possible clinical applications.

2. Materials and Methods

2.1. Chemicals and Materials

Doxorubicin hydrochloride (DX; $\geq 98\%$; MW = 580 g/mol) was a kind gift of Farmitalia-Carlo Erba Chem. Co. (Milan, Italy). Sodium alginate (ALG; low viscosity, $\overline{M}_w = 51$ kDa and $\overline{M}_n = 27$ kDa, G/M ratio: 3—data taken from ref. [17]), was purchased from Sigma-Aldrich (St. Louis, MO, USA) (Cat. No. A1112; purity $>99\%$) and used without further purification. Ultrapure water (18.2 M Ω /cm) from the Arium Pro UV system (Sartorius Stedim Italy S.r.l., Grassano (FI), Italy) was used in the preparation of the solutions.

2.2. Sample Preparation

Unless otherwise stated, samples were prepared at a fixed DX concentration ($50 \pm 2 \mu\text{M}$); such a concentration value allows fluorescence, UV-Vis absorption, and CD to be measured on the same sample by simply varying the cell pathlength. A representative tetramer (M_1 -G₃; calculated MW = 808 g/mol considering four sodium counterions; Figure 1) was arbitrarily chosen for stoichiometry calculations in accordance with the average G/M ratio of 3 for this polymer reported in the literature [17]. Samples will be hereafter defined by the molar ratio R between the ALG building block and the drug, according to:

$$R = \frac{\text{moles}_{\text{ALG}}}{\text{moles}_{\text{DX}}}$$

with $0 \leq R \leq 80$; these samples will be referred to in the following as DX@ALG ($R = X$, where X is the actual value of R).

Hereafter, a typical protocol for the sample's preparation is reported. An aliquot from a DX stock solution in methanol ($[DX]_{\text{MeOH}}^{\text{stock}} = 2.44 \times 10^{-3} \text{ mol/L}$) was transferred into a measuring flask of appropriate volume. Once the solvent was evaporated (Ar flux followed by high vacuum), water was then added to have a new DX in water stock solution ($[DX]_{\text{water}}^{\text{stock}} = 5.00 \times 10^{-4} \text{ mol/L}$) just prior to the sample preparation. The use of a stock solution in methanol was suggested by the fact that DX in this solvent does not self-associate [18,19], which allows for accurate control of its concentration via UV-Vis spectroscopy. Furthermore, the DX solution in methanol can be stored at -20°C without freezing or degrading, as would happen in water, allowing it to be used for a relatively extended period (DX stock solution in methanol was checked periodically over more than 3 months without significant changes in the drug concentration). ALG solutions were prepared by directly weighing the needed amount of polymer in water to obtain a polymer stock solution. For example, 1.0 mg of ALG dissolved in water in a 1 mL measuring flask led to an ALG stock solution of $1.24 \times 10^{-3} \text{ mol/L}$.

By mixing appropriate volumes (Eppendorf pipettes) of these stock solutions, it was possible to prepare all the water DX@ALG samples at the different values of R used throughout this work. For example, mixing 100 μL of the aqueous DX stock solution with 40 μL of the 1 mg/mL ALG solution and adding 860 μL of water allowed the preparation of 1 mL of DX@ALG ($R = 1$) sample with $[DX] = 50 \pm 2 \mu\text{M}$.

2.3. Spectroscopic Analysis

Absorption spectra were recorded with a UV-Vis-NIR Cary 5E (Agilent, Santa Clara, CA, USA) spectrophotometer using a 0.3 cm optical path quartz cuvette (unless otherwise stated). CD spectra were acquired with a JASCO J-750 CD (Jasco Europe S.r.l., Milan, Italy) spectrometer, using a 1.0 cm quartz cuvette; spectra (average of three scans) were recorded in the 250–650 nm interval, 1 nm step, with a bandwidth of 1 nm. Steady-state fluorescence measurements were performed with a Fluoromax-2 (Horiba, Rome, Italy) spectrofluorometer. Emission spectra were acquired using a $0.3 \times 0.3 \text{ cm}$ quartz cuvette (unless otherwise stated) in the 500–800 nm wavelength range. The excitation wavelength was set at 410 nm (unless otherwise stated) to avoid the Raman scattering interfering with the recorded interval ($\lambda_{\text{Raman}}^{410 \text{ nm}} = 476 \text{ nm}$). Excitation and emission slits, filters if used, varied among different experiments, and the relevant information will be specified at due time.

Absorption, CD and fluorescence spectra were converted into ASCII files with the help of the software Spectragryph (Ver. 1.2; <https://www.effemm2.de/spectragryph/%E2%80%94downloaded> on 17 April 2025) and then imported into Sigmaplot software (Ver. 12.5, Systat Software Inc., San Jose, CA, USA) for the experimental baseline subtraction (for CD and fluorescence measurements) and for graph construction.

First derivatives of the absorption spectra and their smoothing (kernel smoothing) were performed making use of the algorithms built in the Sigmaplot Transform environment (first derivative: code written by the authors; kernel smoothing: SMOOTH.XFM). The smoothing percentage applied never exceeded 2%.

Fluorescence lifetime measurements have been performed with a home-built time-correlated single-photon counting (TCSPC) instrumentation described in a previous work [2], with the only notable exception being the use of a Becker & Hickl SPC-130-EMN PCI acquisition board (Becker & Hickl GmbH, Berlin, Germany) with a time resolution below 7 ps. TCSPC data were analyzed by means of iterative deconvolution of the Instrument Response Function (IRF) with a sum of exponentials to the experimentally recorded decays using the FluoFit software package (version 4.6.6.0 Pro; PicoQuant GmbH, Berlin, Germany). In the case of samples containing only DX ($R = 0$), due to the relatively long lifetime, only the portion of the signal starting at the end of the laser pulses was fitted to

a single exponential (tailfit); this strategy allowed more randomly distributed residuals and better χ^2 values to be obtained. For the recording of the IRF, the intrinsic scattering of the samples was used. All spectroscopic analyses were performed at 25.0 ± 0.1 °C. All the quartz cuvettes used for the spectroscopic analysis were from Hellma.

2.4. Dynamic Light Scattering (DLS)

DLS measurements were performed with a Malvern Zeta Sizer Ultra (Malvern Panalytical S.r.l., Lissone (MB), Italy) equipped with a 10 mW He-Ne gas laser ($\lambda = 632.8$ nm) in backscattering mode (173°). All measurements were performed at 25 ± 0.1 °C using a quartz cuvette with 1.0 cm optical pathlength (Hellma GmbH, Müllheim, Germany).

2.5. Atomic Force Microscopy (AFM)

AFM images in wet were collected by Multimode 8HR (Bruker, Santa Barbara, CA, USA) in Peak Force (PF) tapping mode, using SCANASYST FLUID probes (Bruker) on MgCl_2 -functionalized mica. The probes were calibrated by thermal tuning prior to the measurements. PF frequency used was 2 kHz with a scan rate of 0.5 Hz, while the PF setpoint was adjusted in the range of 250 pN $\div$ 1 nN to apply as little force as possible to avoid deforming the nanoparticles. Images were treated with Gwyddion software Ver. 2.68 (<https://gwyddion.net/>, accessed on 3 September 2026), and a flatten filter of first order was applied. Statistical analysis was performed on $n = 146$ ($R = 1$), $n = 1830$ ($R = 10$), and $n = 4023$ ($R = 80$) individually identified particles.

2.6. Quantum Mechanics (QM) Calculations

Due to the size and conformational flexibility of DX, the 1,4-dihydroxy-5-methoxy-9,10-anthraquinone was used as a model for the drug (DX_{mod}), in accordance with previous studies on the excited states of the drug [20]. To model ALG, the hydroxyacetate ion was used (ALG_{mod} , Figure S1). QM calculations of the ground and the first 10 and 20 excited states for the $(\text{DX}_{\text{mod}})_2$ and $(\text{DX}_{\text{mod}})_2@ \text{ALG}_{\text{mod}}$, respectively, were performed using Gaussian 16 [21]. For the $(\text{DX}_{\text{mod}})_2$, starting from an ideal antiparallel stacked structure, a QM optimization at the B3LYP/6-31+G(d) level of theory was performed. The effect of the solvent was considered using the PCM model [22], while Grimme's dispersion (GD3 method [23]) was adopted to model the long-range interactions. On the optimized structure, excited-state calculations were performed at the B3LYP/6-311++G(2d,2p) level of theory using the GD3 method [23]. This choice was supported by previous studies on similar systems [20,24]. The gas-phase electronic transitions were calculated using the Franck–Condon–Herzberg–Teller (FCHT) method, as implemented in Gaussian 16 [25]. Accordingly, the $(\text{DX}_{\text{mod}})_2@ \text{ALG}_{\text{mod}}$ system was treated using the same procedure. Starting from a slightly different relative orientation between the $(\text{DX}_{\text{mod}})_2$ and the ALG_{mod} , two different optimized structures were obtained. For both geometries, excitation energies and transition dipole moments were calculated.

2.7. Cytotoxicity Tests

Cytotoxicity tests were performed on selected cancer cell lines (A549 and HeLa) for DX in comparison with $\text{DX}@ \text{ALG}$ ($R = 1$, $R = 80$). Cells were seeded at a density of 5000 cells/well in 96-well plates, incubated overnight, and then treated with DX or $\text{DX}@ \text{ALG}$ ($R = 1$, $R = 80$) at various concentrations (covering the 0–5 μM range of DX concentration) for 24 and 48 h. All cytotoxicity tests were carried out in phosphate-free culture medium (DMEM, Cat. No. 11971025; Gibco-Thermo Fisher, Madrid, Spain.), as phosphate ions may interfere with the electrostatic interactions stabilizing the $\text{DX}@ \text{ALG}$ complexes.

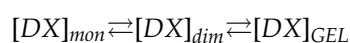
Cell viability was determined with the Sulforhodamine B (SRB) assay, as described in the literature [11]. For the dose-response curves and IC_{50} values calculation, GraphPad

Prism (version 8.0) was used. The results in the graph are presented as mean $\pm$ SD from triplicate independent experiments.

3. Results and Discussion

Each monosaccharide unit of ALG bears a carboxylate group; at pH values higher than its pK_a (~ 3.6 [12]), the polymer fully dissolves in water in its polyanionic form. Different pK_a values for the ammonium group of DX can be found in the literature [26–31]; by assuming an average value of 8 [11], at pHs ≤ 7 , more than 90% of DX molecules are in the protonated form. Since DX and ALG water stock solutions have pH values of 5.5 ± 0.2 ($n = 4$) and 6.8 ± 0.2 ($n = 3$), respectively, the electrostatic interaction between the positively charged DX and the negatively charged ALG could be exploited as the initial driving force for the formation of DX-ALG adducts [after mixing, the pHs are 6.6 ± 0.2 ($n = 9$)].

In previous works, it has been demonstrated that the DX self-association equilibria leading to the DX_{GEL} formation [2,3] can be represented as



To assess the impact of ALG on these equilibria, the absorption and emission characteristics of the drug were measured for the DX@ALG ($R = 1$) samples prepared within the DX concentration range of 1 to 50 μ M, and these were compared to the spectra recorded for DX solutions within the same concentration range. In water, when reaching a DX concentration of 50 μ M, the drug absorption spectrum revealed the presence of DX_{dim} (slight decrease of ϵ at 495 nm; Figure 2). For DX@ALG ($R = 1$), the DX self-association is shifted toward DX_{GEL} rather than DX_{dim}, already at a DX concentration of 5 μ M, as can be deduced by absorption (Figure 2) and fluorescence (steady-state and time-resolved) spectroscopy (Figure S2 and Figure S3, respectively). Therefore, even under conditions far from the CGFC [4], when the DX_{dim} concentration is not appreciable in the matching drug-water solutions, ALG induces the DX_{GEL} formation. In the absence of ALG, DX_{dim} only evolves towards the formation of DX_{GEL} upon increasing the ionic strength. The peculiarity of DX-ALG interaction can also be observed in the trend of the emission intensity ratio (I_{558}/I_{max} —inset of Figure S2; see Supplementary Materials for details) upon increasing the drug concentration. For DX alone, this quantity assumes the typical value of the drug in water (ca. 0.8) [6], while in the presence of ALG, the I_{558}/I_{max} progressively decreases, indicating the exposure of the fluorophore to a less polar environment [6,32]. This is more evidence for the formation of DX_{GEL}, where the anthraquinone moieties of numerous DX molecules are engaged in the formation of the π - π and H-bond network holding together the DX_{GEL}, secluding the drug fluorophore from direct contact with the solvent.

The above-discussed experimental data suggest that at the DX analytical concentration of $50 \pm 2 \mu$ M, the effects of the DX-ALG interactions are more evident. In the following, the DX@ALG system was studied at this drug concentration, evaluating the effect of increasing R , in the range $0 \leq R \leq 80$. The chosen drug concentration had the advantage of allowing all the spectroscopic analyses to be performed without diluting or concentrating the formulations.

The fluorescence spectra acquired on these samples are reported in Figure 3—left panel—where not only is it possible to appreciate the strong quenching of the DX fluorescence upon the addition of ALG (around 85% for $R = 1$), but also the ~ 15 nm red shift of the drug fluorescence emission for the same R value (Figure 3—left panel, inset).

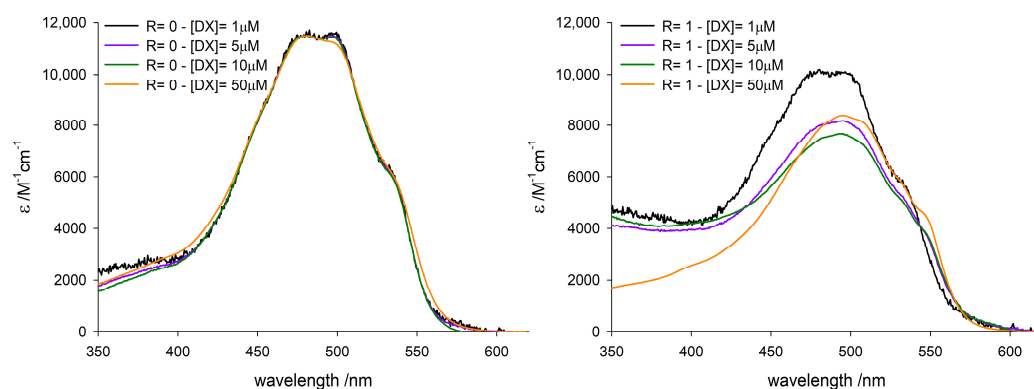


Figure 2. Absorption spectra of DX (**left panel**) and of DX@ALG ($R = 1$) matching samples (**right panel**) recorded at different values of the analytical DX concentration (see legend). The spectra are plotted as apparent molar extinction coefficients to easily compare the spectral shapes at the different concentrations, i.e., the experimental absorbances have been divided by the analytical DX concentration and cell pathlength. For all the tested DX concentrations, the apparent molar extinction coefficient at 480 nm matched the value reported in the literature for the monomeric form of DX, $11,500 \text{ M}^{-1} \text{ cm}^{-1}$ [7]. For the construction of the graph reported on the right panel, the analytical DX concentrations were therefore used for the calculation of the apparent molar extinction coefficient of DX@ALG ($R = 1$) system as a function of the wavelength. Cell pathlength: 0.3 cm; $T = 25^\circ \text{C}$.

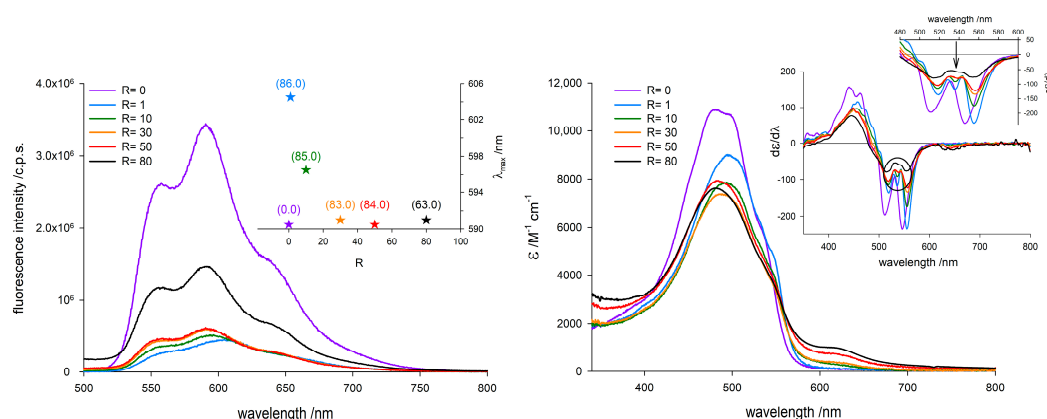


Figure 3. DX@ALG emission (**left panel**) and absorption (**right panel**) spectra at different values of R (see the relevant legends). The inset in the left panel shows the position of the drug emission maximum and the extent of quenching (figures in parentheses) as a function of R (same colors as in the main graph). The inset in the right panel shows the first derivative of the spectra of the main graph (same colors as in the main graph); the circled area highlights the portion further enlarged in the same plot, with the arrow pointing to the band typical of the DX_{GEL} [2]. Experimental conditions for fluorescence measurements: $[\text{DX}] = 50 \text{ } \mu\text{M}$; $\lambda_{\text{exc}} = 410 \text{ nm}$; slits: 2.5/2.5 nm; $0.3 \times 0.3 \text{ cm}$ cell pathlength. Experimental conditions for absorption measurements: $[\text{DX}] = 48 \text{ } \mu\text{M}$; 0.3 cm cell pathlength. In both cases, $T = 25.0^\circ \text{C}$.

Interestingly, both these effects are more pronounced when an equal number of moles of ALG and DX were mixed ($R = 1$), with the DX emission maximum recovering the initial peak position for $R = 50$ but with no recovery of the initial DX fluorescence intensity (quenching still higher than 60% for all the tested R). The recovery of the initial peak position is not due, however, to the release of free drug in solution, as the quenching is still above 60% in the whole composition range explored, but is due to a sort of redistribution of the DX molecules along the ALG chains upon increasing R .

The corresponding UV-Vis spectra shown in Figure 3—right panel—highlight this relationship with the aggregation state of the drug induced by ALG. The DX@ALG ($R = 1$) absorption spectrum is characterized by a decrease in the molar absorptivity, a red shift

higher than 10 nm in the peak position (in this instance, 20 nm, from 475 nm to 495 nm) and two shoulders at 530 nm and 545 nm, and is more evident in the relevant first-derivative spectra (Figure 3—right panel, insets), in agreement with precedent literature data on DX_{GEL} [2]. Moreover, for $R > 1$, the appearance of a new broad absorption band located around 610–630 nm is evident. This band is very prominent for the DX@ALG ($R = 80$) sample and is not associated with any detectable fluorescence emission (Figure S4). Since the absence of fluorescence is a typical signature of DX_{dim} [33], this new band suggests the hypothesis that an excess of ALG forces the drug molecules to organize into a distinct form of dimers responsible for this new absorption band. As a confirmation of this hypothesis, the dilution of a DX@ALG ($R = 1$) sample with a concentrated ALG solution to reach a final R value of 80 rapidly led to the disappearance of the DX_{GEL} absorption signatures and to the evolution, in a matter of hours, toward the same spectral shape as a DX@ALG ($R = 80$) directly prepared sample (Figure S5). This highly associated form of the drug, which to our knowledge has never been described in the literature, will be further addressed later in the manuscript.

As mentioned, the formation of DX_{GEL} is also coupled to a peculiar CD spectrum. The DX molar ellipticity in different aggregation states (namely, DX_{mon}, DX_{dim}, DX_{GEL}) is compared in Figure 4 to those of the DX@ALG samples at $R = 1$ and $R = 80$. The DX@ALG ($R = 1$) CD spectrum closely resembles that of DX_{GEL}, although the spectrum of DX_{GEL} was acquired at a drug concentration 200 times higher than for DX@ALG ($R = 1$) and in the presence of a high NaCl concentration [2]. The slight difference in the molar ellipticity is plausibly ascribable to the different molecular environment provided by the ALG chains. The CD spectrum of DX@ALG ($R = 80$) is instead dominated by the strong positive bands at ≈ 495 nm and 625 nm, and is clearly associated with the modified absorption spectrum of DX@ALG ($R = 80$) shown in Figure 2, and related to a distinct form of DX dimers and also to the chiral nature of the templating ALG substrate [34].

Fluorescence lifetime measurements can help to understand the DX partitioning in the different aggregation states since DX_{dim} does not fluoresce [33], DX_{mon} fluorescence lifetime is around 1 ns [35], and that of DX_{GEL} is below 0.20 ns [2]. The DX@ALG ($R = 1$) fluorescence decay curves as a function of DX concentration shown in Figure S2 are all markedly biexponential except for $[DX] = 1 \mu\text{M}$. The decays were globally fitted to a reconvolution function of the IRF with two exponentials (Figure S3) whose lifetimes were imposed as equal to $\tau_1 = 0.16$ ns and $\tau_2 = 1.00$ ns, according to literature data [2]. The fastest component reached a contribution of 89% to the total decay for $[DX] = 50 \mu\text{M}$, progressively decreasing to zero for $[DX] = 1 \mu\text{M}$ (Figure S3), a condition at which DX is likely to be distributed in the ALG chains without associating. To compare these percentages to the true amount of the drug present as DX_{GEL}, the difference in the fluorescence quantum yield of DX_{mon} (4.4×10^{-2}) [35] and DX_{GEL} (3.0×10^{-3}) [2] must be considered. Bearing this in mind and making reference to the 50 μM sample, the effective contribution in moles of the DX in the two phases to the fluorescence decay curve shown in Figure S2 is then 99.2% as DX_{GEL} and 0.8% as DX_{mon}; this means that in the 50 μM DX@ALG ($R = 1$) sample of Figure S3, the concentration of DX interacting with ALG is around 49.6 μM (see Supplementary Materials for further details). These figures allow the calculation of the DX-to-ALG mass percentage that, for the 50 μM DX@ALG ($R = 1$) system, is $ML_{\%} = 41.5\%$ (w/w) (see Supplementary Materials). On passing, such a value is sensibly higher than those reported for the FDA-approved liposomal formulations of DX, Myocet[®] and Doxil[®], 25% (w/w) and 30% (w/w), respectively [36,37]. Taking into consideration the amount of DX in the DX@ALG ($R = 1$) nanoparticles with respect to the total mass of DX and ALG, the mass loading percentage ($L_{\%}$) is $\approx 72\%$ (see Supplementary Materials).

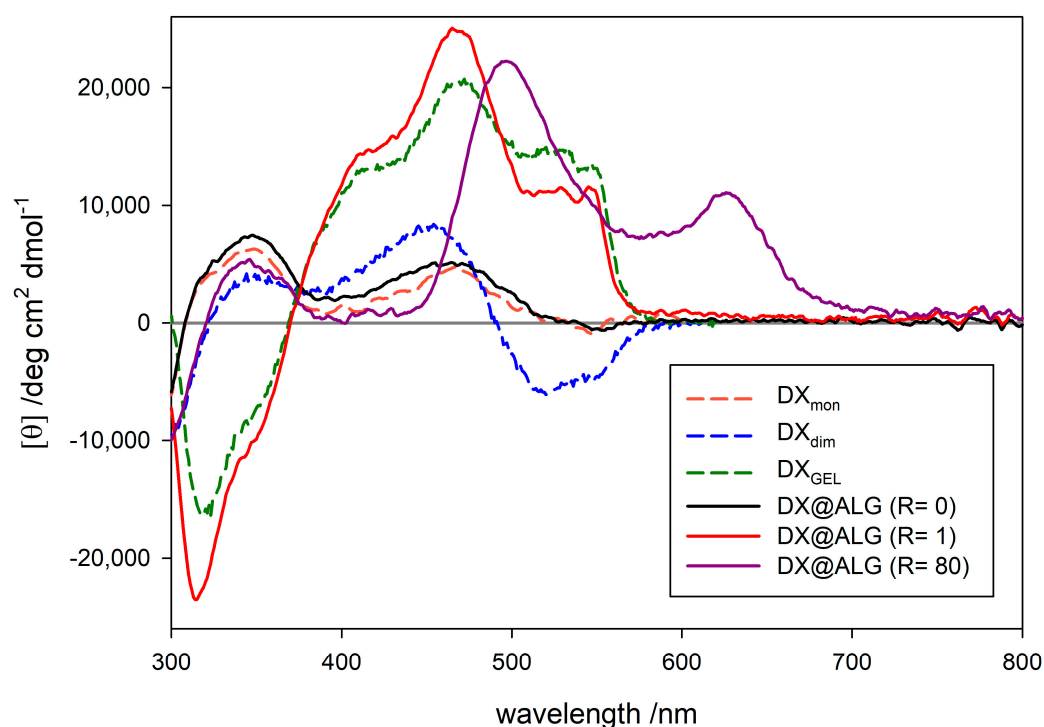


Figure 4. Molar ellipticity of DX@ALG (black continuous line: $R = 1$; red continuous line: $R = 80$) in comparison with DX molar ellipticity in different aggregation states (dashed lines, see legend). Continuous lines: $[DX] = 40 \mu\text{M}$; $l = 1.00 \text{ cm}$. Orange dashed line: $[DX] = 23 \mu\text{M}$, $l = 1.00 \text{ cm}$. Blue dashed line: $[DX] = 10 \text{ mM}$, $l = 0.01 \text{ cm}$. Green dashed line: $[DX] = 10 \text{ mM}$, $[\text{NaCl}] = 0.75 \text{ M}$; $l = 0.01 \text{ cm}$. In all cases, $T = 25^\circ\text{C}$. Continuous lines: this work; dashed lines from ref. [2].

Dynamic Light Scattering (DLS, Figure S6) measurements revealed that the interaction between DX and ALG leads to the formation of discrete nanoparticles. The intensity distributions gave Z-average values in the range 200–300 nm, which would suggest the presence of large aggregates. However, since the intensity of scattered light increases with approximately the sixth power of the diameter, this value is strongly influenced by larger objects. The number and volume distributions show that the samples are mainly made up of nanoparticles of 20–50 nm. For $R = 1$ and $R = 10$, these represent about 90% of the sample volume, while the larger aggregates constitute only 10%. For $R = 80$, however, the fraction of larger aggregates increases (about 60% of the volume). The presence of these aggregates, although a minority for $R = 1$ and $R = 10$, is at the origin of the overall high values of the polydispersity index observed (PDI 0.40–0.50; Table S1).

As expected, pure ALG exhibits the formation of poorly defined aggregates. All samples exhibit highly negative ζ -potential values in water, indicating a strong electrostatic contribution to their colloidal stabilization and suggesting good colloidal dispersibility under these conditions, although no clear dependence of the ζ -potential on R can be established from the present measurements (Table S1).

The morphology of the DX@ALG ($R = 1$, $R = 10$ and $R = 80$) nanoparticles was then assessed by Atomic Force Microscopy (AFM). The AFM images (Figure 5; Table S2) show indeed well-defined spherical particles deposited on the substrate for all the tested R s, with sizes between 50 and 90 nm in diameter comparable to those obtained by DLS, considering the different nature of the particle's dimensions obtained with the two techniques. It is important to bear in mind that DLS measures hydrodynamic diameter, which includes the nanoparticle solvation shell that is affected by dynamic fluctuations, while in AFM, the negative nanoparticles are adsorbed on mica treated with a solution of MgCl_2 (Mg^{2+} ions behave as bridges with the particles). After removing the excess of unbound NPs by gently

washing the mica substrate with ultrapure water, a selection of the nanoparticles with higher surface charge density over the bigger, looser aggregates likely to be less efficiently adsorbed on the substrate probably occurred. In the case of AFM measurements in wet mode (Figure 5), both the objects' height and diameter do not take into consideration the solvation shell, and the particles may be flattened because of both the adhesion to the substrate and the force applied through the tip to probe the sample. Indeed, even in PF tapping mode at the very low probing force (250 pN – 1 nN) used in this work, compression and lateral spreading cannot be ruled out. The AFM data reported in Table S2 clearly indicate that upon increasing R, the particle's height increases, indicating a progressive stiffening of the internal network of the nanoparticles.

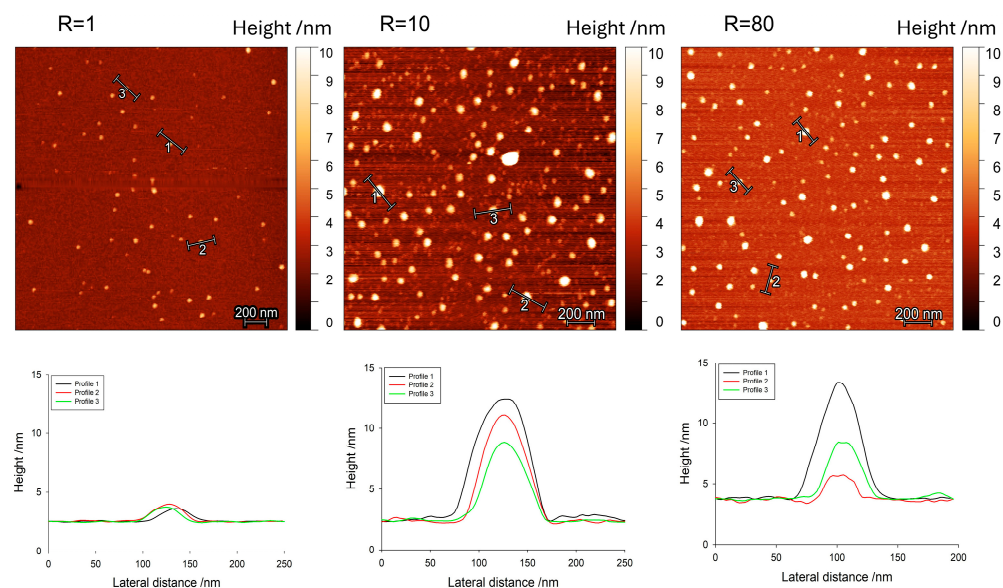


Figure 5. Wet mode AFM images of DX@ALG nanoparticles at different R (see legend) with their corresponding height profiles extracted from three representative particles for each sample (indicated by numbered segments in the panels).

Regarding the new DX absorption band rising at around 620 nm for $R > 1$ (Figure 2), its non-fluorescent nature (Figure S4) coupled with the strong positive CD signal (Figure 4) suggests that it could arise from some sort of antiparallel DX dimers formed on the ALG chains, a consequence of a templating effect exerted by ALG in analogy with what was already observed in the case of DX interacting with PSS [10].

To gain insight into this hypothesis, QM calculations were performed. Two previously optimized DX_{mod} molecules (Figure 1) were stacked to obtain a (DX_{mod})₂ dimer, and a QM optimization was performed (Figure 6a), followed by the calculation of the excitation energies and transition dipole moments.

A comparison between the calculated transitions and the experimental data supports that the model well reproduces the DX@ALG ($R = 0$) spectrum, without showing the transition centered at 620 nm characteristic of the DX@ALG ($R = 80$) sample (Figure 6a,b). Due to the computational costs of treating an entire polymer chain at the QM level, to simulate the interaction between (DX_{mod})₂ and ALG, the hydroxyacetate ion was used (ALG_{mod}, Figure S1). Starting from the (DX_{mod})₂ optimized geometry, a hydroxyacetate ion was added to obtain the (DX_{mod})₂@ALG_{mod} system. Then, QM calculations were performed at the same level of theory used for the (DX_{mod})₂ system; two optimized geometries were obtained. As shown in Figure 6c, the first geometry shows the formation of two H-bonds between the hydroxyl and the carboxylate oxygen of ALG_{mod} and the anthraquinone moiety of (DX_{mod})₂ at 2.02 Å and 2.21 Å, respectively. This arrangement supports the

hypothesis that ALG might form H-bonds with DX_{dim} , not only with carboxylate oxygens, but also with the endocyclic oxygen (O_5) of ALG (Figure S1). The other optimized geometry obtained is shown in Figure 6e; it is characterized by the formation of two H-bonds with the two carboxyl oxygens of the ALG_{mod} ion at 2.29 Å and 2.50 Å, respectively.

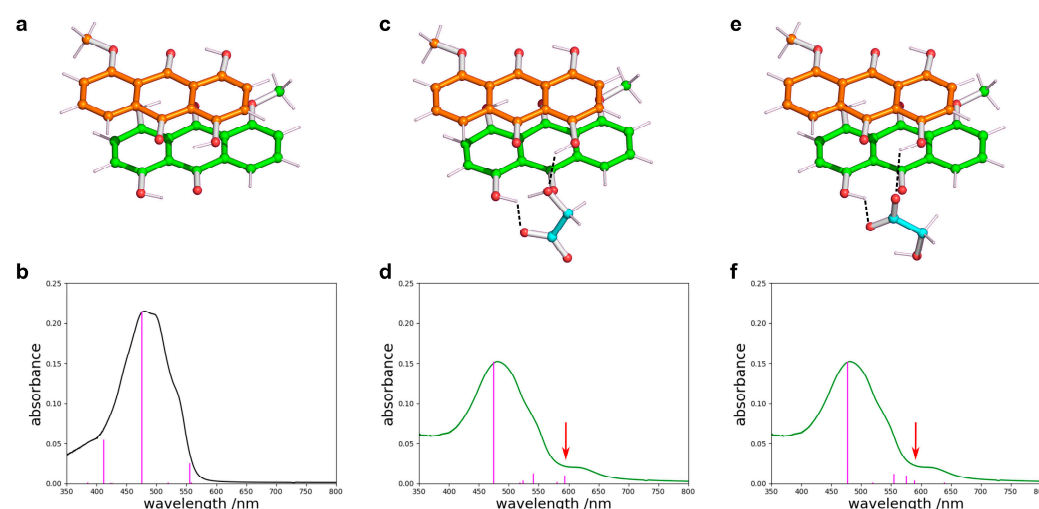


Figure 6. Optimized geometries and comparison of the experimental absorption spectra of $DX@ALG$ ($R = 0$ —black line—and $R = 80$ —green line) with the calculated electronic transitions (magenta vertical lines, rescaled to match the experimental data) of the $(DX_{mod})_2$ (a,b) and $(DX_{mod})_2@ALG_{mod}$ (c–e). Arrows in panels (d,f) indicate the position of the calculated electronic transition at the longer wavelength.

Therefore, both the optimized geometries (coordinates are presented in the Supplementary Materials) feature electronic transitions compatible with that at $\lambda \approx 620$ nm experimentally found for the $DX@ALG$ system at $R > 1$ (Figure 6d,f; Table S3). Although these results do not provide details regarding the exact binding between ALG and DX due to the models used (see Figure 1 and Figure S1), they lend support to the hypothesis that ALG may promote the self-association of DX into non-fluorescent dimers. These dimers are stabilized by both π – π stacking and hydrogen bonding, through the formation of an anionic pocket capable of accommodating and stabilizing the DX_{dim} .

Some preliminary in vitro cell viability tests have then been attempted using two cancer cell lines with a well-known and different sensitivity to DX, by freeze-drying the $DX@ALG$ water solutions and then resuspending the lyophilized samples (Figures S7 and S8) in ultrapure water; the reconstituted sample was then diluted with the medium suitable for the cells' growth. The toxicity of all the tested $DX@ALG$ samples ($R = 1$ and $R = 80$) was comparable, within the experimental uncertainty, to that of the free drug (Figure 7). On the one hand, these results could indicate that DX is released from $DX@ALG$ in cell media or eventually in endosomes, reaching the nucleus as free drug; on the other hand, they are a confirmation that the amount of DX trapped in the $DX@ALG$ nanoparticles corresponds to the analytical DX concentration, as evidenced by fluorescence lifetime measurements (Figures S3 and S4). The self-assembled nature of $DX@ALG$ systems means that their dilution inevitably not only results in the loss of the DX_{GEL} 's spectroscopic signature, but also reasonably results in the drug being released from the nanoparticles as DX_{mon} (Figure S9). Ongoing studies are focused on optimizing the formulation to minimize premature release of the entrapped drug. Although the $DX@ALG$ nanoparticles do not show a decrease in DX toxicity compared to the free drug, due to the instability of the system upon dilution, the complexation with alginate results in a very high drug loading per

nanoparticle, which could translate into improved formulation of DX for in vivo settings, once the DX@ALG stability issue is resolved.

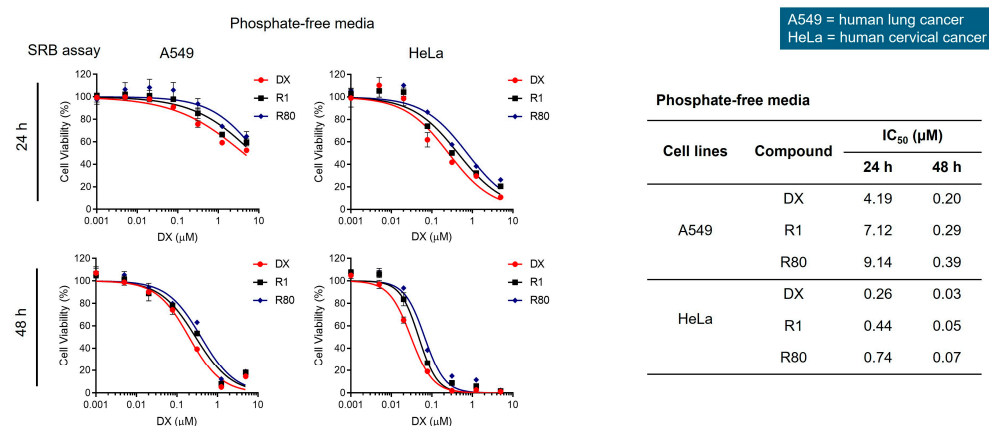


Figure 7. Cell viability of selected cancer cell lines (A549 and HeLa) as a function of DX analytical concentration for DX and DX@ALG (R = 1, R = 80) nanoparticles.

4. Conclusions

As shown, DX@ALG nanoparticles easily and reproducibly form by mixing appropriate volumes of the DX and ALG aqueous solutions based on the desired R value. DX complexation with ALG results in the formation of supramolecular nanoparticles with diameters in the range of 20–50 nm. These nanoparticles exhibit optical and chiroptical properties that closely resemble those of DX supramolecular gels. Notably, the formation of DX@ALG nanoparticles led to the aggregation of DX molecules into a condensed phase (DX_{GEL}) at significantly lower DX concentrations than those required in water and does not require the addition of NaCl. Fluorescence spectroscopy indicates that the association of DX within the nanoparticles screens the fluorophore from contact with the solvent, creating a hydrophobic environment, as evidenced by changes in the emission bands arising from variations in the local polarity surrounding the fluorophores. Furthermore, fluorescence lifetime measurements suggest that DX is predominantly present in an aggregated, gel-like phase within the nanoparticles. These measurements also indicate that the DX loading achieved in the DX@ALG nanoparticles exceeds that reported for the clinically approved formulations Myocet[®] and Doxil[®] [36,37].

The synergy between DX and ALG, leading to the formation of nanometric aggregates in conditions where neither the drug nor the polymer tends to aggregate into nanoscopic structures, and the ease of preparation combined with the high drug loading efficiency shown by the DX@ALG system, are very encouraging results and justify further studies on these supramolecular nanoparticles with a view to developing more efficient and environmentally friendly DX carriers. Future work will focus on the stabilization of the nanoparticles in physiological conditions and their integration in pharmaceutical formulations.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/colloids10050073/s1>, Figure S1: Schematic representation of ALG_{mod}; Figure S2: Steady-state and time resolved fluorescence for the same sample as in Figure 2; Figure S3: Results of the fits of the data of Figure S2; Figure S4: Absorption and fluorescence spectra (λ_{exc} = 410 nm and 620 nm) of DX@ALG (R = 80) sample; Figure S5: Dilution of DX@ALG (R = 1) sample with ALG; Figure S6: DLS size distribution of DX@ALG samples; Figure S7: Snapshot of freeze-dried DX@ALG samples; Figure S8: Absorption spectra of DX@ALG before and after freeze-drying; Figure S9: Emission spectra of DX@ALG (R = 1) sample upon dilution; Table S1: DLS data of DX@ALG samples; Table S2: AFM parameters of DX@ALG samples; Table S3: Experimental and

calculated absorbance data for DX@ALG and $(DX_{mod})_2@ALG_{mod}$; Further computational details, including the cartesian coordinates (Å) of the optimized structures of Figure 6a,c,e.

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Abbreviations

The following abbreviations are used in this manuscript:

ALG	Sodium alginate
DX	Doxorubicin hydrochloride
DX_{mon}	Doxorubicin hydrochloride in monomeric form
DX_{dim}	Doxorubicin hydrochloride in dimeric form
DX_{GEL}	Doxorubicin hydrochloride in gel phase (polymerized form)
DX@ALG	Doxorubicin hydrochloride-sodium alginate nanoparticles
CGFC	Critical Gel Formation Concentration
CD	Circular dichroism
UV-Vis	Absorption spectroscopy
TCSPC	Time-Correlated Single-Photon Counting
IRF	Instrument Response Function
DLS	Dynamic Light Scattering
AFM	Atomic Force Microscopy
QM	Quantum Mechanics
DX_{mod}	1,4-dihydroxy-5-methoxy-9,10-anthraquinone
$(DX_{mod})_2$	1,4-dihydroxy-5-methoxy-9,10-anthraquinone dimer
ALG_{mod}	Hydroxyacetate ion
$(DX_{mod})_2@ALG_{mod}$	Complex mimicking the DX@ALG aggregates in QM calculations

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