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Low-temperature ^{1}H-NMR spectroscopic study of doxorubicin influence on the hydrated properties of nanosilica modified by DNA

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Abstract The effect of the anticancer drug—doxorubicin (Dox) on hydration properties of a nanocomposite material deposited on silica and modified by small amount of DNA (0.6 wt%) was studied by means of ^{1}H NMR spectroscopy at low temperatures (in the range of 200–280 K). Signals of either weakly (WAW) or strongly (SAW) associated water, as well as water associated with electrondonor groups of the composite surface (ASW), were observed. The findings reveal that, depending on the temperature and the composition of the dispersion medium, fast molecular exchange takes place between different forms of interphase water. The presence of Dox (0.1–0.2 wt%) in the dispersion medium leads to change of the relative concentrations of different forms of water.

1 Introduction

In recent decades effective drugs against various types of cancer were developed. Among of them is doxorubicin (Dox) which belongs to the anticancer drugs of anthracyclines [1, 2]. The mechanism of Dox action is the interaction with DNA molecules through the creation of inclusion compounds, the formation of free radicals and direct effects on cell membranes, resulting in the suppression of nucleic acids synthesis. However, the wide use of Dox is largely limited due to a highly toxic drug-related, mainly myocardial damage [3, 4]. Therefore, the creation of targeted delivery of this drug to the area of the tumor is an important problem. Recently liposomal forms of Dox as well as nanocomposites based on ferromagnetic materials have been developed and used [5–7]. A promising direction could be immobilized anticancer drugs on the surface of nanodispersed particles, which are specific to this type of substances. As anticancer carriers nanosilica obtained by hydrolysis of silicon tetrachloride in a flame of hydrogen burner can be used [8]. Although they interact weakly with small and medium molecular weight materials their affinity to Dox can be significantly improved by adsorption, so that the polynucleotide molecules interact with Dox by forming supramolecular complexes with complementary pairs of nucleotides. DNA, like many other poorly water-soluble polymers, can be almost irreversibly adsorbed on silica. The adsorption can be achieved by mechanical activation of mixtures of silica powders with DNA in the presence of water [9]. Thus, the investigation of the characteristics of the hydration of nanocomposites and the effect of presence of different types of organic molecules, which may greatly determine the Dox desorption and its introduction into the tissues of the body, is important.

As it was shown previously [10–12], the method of low-temperature ^{1}H NMR spectroscopy with freezing out of the liquid phase allows determine the amount of bound water and its thermodynamic characteristics as well as the size distribution of nanodimension water-filled cavities in

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macromolecules. The magnitude of the chemical shift of hydrogen atoms (δ_H) in the bound water offers the possibility to determine the average number of H-bonds per water molecule. As for the un-associated water (either in gas phase or in solution in nonpolar solvents) $\delta_H =$ 1–1.5 ppm, and for ice $\delta_H = 7$ ppm [10, 13], one can conclude that the formation of H-bond for H_2O as a proton donor leads to an increase in δ_H at 2.7 ppm. Investigations of various systems [10–12] have shown that at the certain hydrophobic-hydrophilic balance of functional groups on the surface, the water can change into weakly associated state, characterized by the appearance of signals of protons at $\delta_H = 2.1$ ppm. In this state either the average number of H-bonds per each water molecule is less than two (out of four possible) or the structure of H-bonds is strongly distorted compared to bulk water, since the decrease of O–H…O angle leads to an increase magnetic shielding of protons. It turned out that this water is widely distributed in nature: it is recorded in partially dehydrated yeast cells, bone marrow cells, seeds, plants, spongy part of bone tissue and other biological objects [10].

In this work the effect of Dox on the hydration properties of the nanocomposite material, deposited on silica, and modified by small amount of DNA, was studied by means of ^{1}H NMR spectroscopy at low temperatures in the range of 200–280 K.

2 Experimental

2.1 Material

Highly dispersed silica (HDS, Aerosil A-300, with specific surface of 300 m^2/g (Kalush Experimental Plant of the O.O. Chuiko Institute of Surface Chemistry of NAS of Ukraine) has been used as the silica component of the nanocomposite. Lyophilized DNA, extracted from the sperm of salmon fishes (Sigma), was mixed with HDS powder and an appropriate amount of water was added. The mixture was activated mechanochemical for 20 min and afterwards the nanocomposite was dried at 60°C for 48 h. The data of optical microscopy powder and water suspension of nanocomposite show that their particles are agglomerates of micron size. As organic solvent medium the deuterosolvents, $CDCl_3$, C_6D_6, CD_3CN and $CDCl_3$ mixture, C_6D_6 and CD_3CN mixture, DMSO-d6, and pyridine-d5 were used. For immobilization on the nanocomposite's surface, fixed amount of Dox were dissolved in DMSO-d6 to obtain a 5% solutions. These solutions were added to the dispersion medium containing a SiO_2-DNA suspension. This dispersion medium discolored, and nanocomposite had a uniformly red colour due to the Dox adsorption on its surface.

2.2 ^{1}H NMR spectroscopy

NMR spectra were recorded on an NMR spectrometer of high resolution (Varian Mercury 400 with an operating frequency of 400 MHz). The temperature was controlled within ±1 K using a thermodevice Bruker VT-1000. To prevent the supercooling of suspensions the ^{1}H NMR spectra of non-freezing water were recorded under the heating of suspensions, previously cooled at the temperature of 200 K.

The method for determining the physical characteristics of interphase water layers by ^{1}H NMR spectroscopy is based on the influence of the interphase boundary on temperature of water–ice phase transition [10–12]. Due to the adsorption interactions, the freezing temperature of water is reduced at the interface. The free energy of ice varies linearly with the temperature according to the following relation [14]:

$$\Delta G = -0.036(273 - T), \tag{1}$$

The area, bounded by the $\Delta G(C_{uw})$ curve (where C_{uw}(T) is the temperature dependence of the concentration of unfrozen water), determines the magnitude of interphase energy (γS), which is equal to the magnitude of the total decrease in free energy of water, caused by the presence of the interphase boundary

$$\gamma_S = -K \int_0^{C_{uw}^{max}} \Delta G(C_{uw}) dC_{uw}, \tag{2}$$

where $C_{uw}^{max}$is the concentration of non-freezing water at T = 273 K and K represents a complex dimensional coefficient.

With respect to their ability to form hydrogen bonds with their neighbors, the bound water molecules can be characterized either as strongly (SAW at $\delta_H > 3$ ppm for the 3D structures) or weakly (WAW at $\delta_H = 1$–2 ppm for low-dimensional 1D and 2D structures of water) associated water.

The $\Delta G(C_{uw})$ dependencies usually contain areas relating to WAW and SAW types of water. WAW is that part of the non-freezing water where the free energy is only slightly reduced ($-\Delta G \leq 0.5$–0.8 J/mol) as a result of adsorption interactions and it freezes at T > 250–260 K. SAW ($-\Delta G > 0.8$ kJ/mol) cannot freeze even with a cooling of the suspension [9, 10]. Quantitative ($C_{uw}{}^{s}$ and $C_{uw}{}^{w}$ for SAW and WAW, respectively) and energy (ΔG^s and ΔG^w) characteristics of the layers of bound water can be obtained by extrapolating the relevant regions of the above curves to the abscissa and ordinate axes.

The process of water freezing in either intracellular cavities or in reservoirs of macromolecules can be described by the Gibbs–Thomson equation [10, 15]

$$\Delta T_m = T_m(R) - T_{m,\infty} = \frac{2\sigma_{sl} T_{m,\infty}}{\Delta H_f \rho R}, \tag{3}$$

where $T_m(R)$ represents the melting temperature of ice, localized in the pores (voids) of radius R, $T_{m,\infty}$ the melting temperature of bulk ice, ρ the density of the solid phase, σ_{s1} the interaction energy of a solid body with fluid (via hydrogen bonds), and ΔH_f the bulk melting enthalpy. The Eq. 3 can be used for the calculation of the pores (voids filled with bound water) distributions on the size basing on C_{uw}(T) dependencies.

3 Results and discussion

Figure 1a shows the ^{1}H NMR spectra recorded of DNA containing 10% water in weakly polar medium $CDCl_3$. In addition to the signals of water, the spectra reveal signals corresponding to the non-deuterated chloroform ($\delta_H = 7.3$ ppm) and to tetramethylsilan ($\delta_H = 0$ ppm). The chemical shifts of $\delta_H > 4$ ppm and $\delta_H < 2$ ppm correspond to SAW and WAW, respectively, while the chemical shift of $\delta_H = 3$–4 ppm is related to water, forming H-bonds with electron centers of DNA molecules (ASW). SAW is observed as a broad single signal having nearly Gaussian shape, whose intensity progressively decreases with decreasing temperature. At the temperature of about 220 K, it has been observed that the SAW-signal almost disappears. The large signal width is due to both low-mobility bound water molecules, as well as inhomogeneous broadening of NMR spectra, resulting from significant differences in diamagnetic susceptibilities of DNA and air [16]. This type of bound water has a part from WAW (which freezes at T > 250 K) and SAW. Other types of water-related DNA, should be uniquely attributed to SAW. It should be noted that in the cluster 2D-cyclic structures of water half of the H atoms are not involved in hydrogen bonds. Therefore, such structures are characterized by chemical shifts, averaged between the signals corresponding to WAW (H atoms that do participate in H-bonds) and SAW (H atoms involved in H-bonds).

The observation of several water-signals reveals slow (in the NMR time scale) molecular exchanges [16] between the water molecules involved in the formation of structures (clusters) of different types. Since SAW freezes at a relatively high temperature, it can not be attributed to the crystallization water. As it can be seen in Fig. 1a, two forms of WAW are observed in the spectra. The intensity and chemical shift of the signal at $\delta_H = 1.2$ ppm (WAW1) practically does not change with temperature in the studied temperature range. At the same time the δH value of the signal WAW2, lying in a weak field ($\delta_H = 1.6$ ppm at 280 K), significantly increases with decreasing temperature.

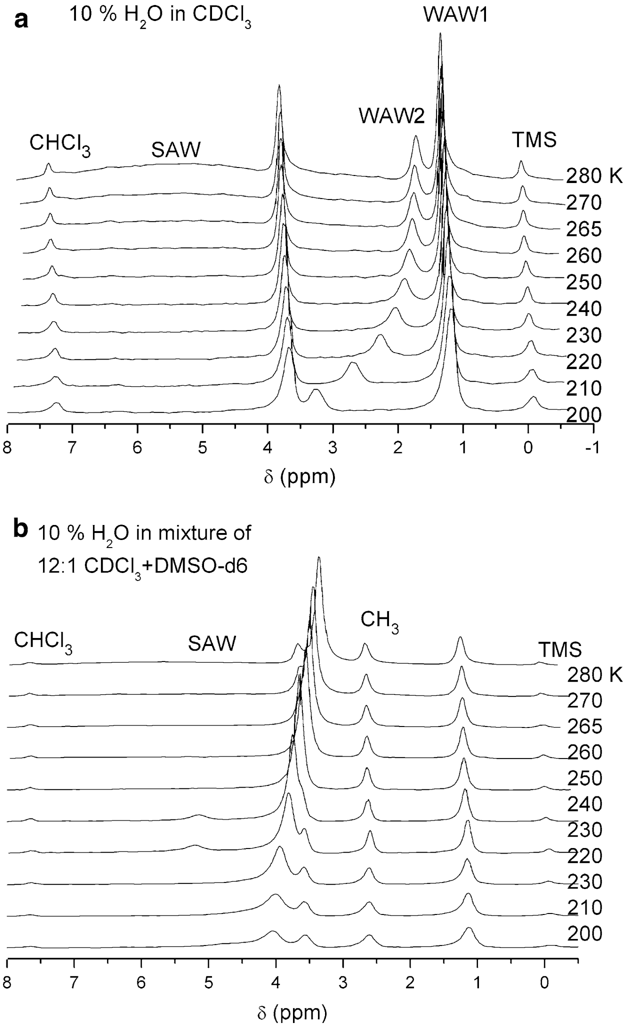


Fig. 1 ^{1}H NMR spectra recorded at different temperatures (200–280 K) of water-related powder DNA ($C_{H_2O} = 10\%$) in $CDCl_3$ (**a**) and $CDCl_3$ with the addition of 8 wt% DMSO-d6 (**b**)

As a consequence of these changes, the water molecules can increase their coordination number with decreasing temperature (probably due to the transition WAW2 → ASW). In a chloroform medium, both forms of WAW practically do not freeze. This is probably due to the fact that the weakly polar chloroform prevents the diffusion of WAW molecules to the SAW structures, and also reduces the probability of formation of low-dimensional nanocrystallites of ice from WAW.

Upon addition of small amounts (≈ 8 wt%) of DMSO in CDCl3 the spectra shape changes (Fig. 1b). Namely, the WAW2 signal lying in the weaker magnetic fields disappears and the ASW signal at $\delta_H = 3.5$–4 ppm, that is attributed to the formation of H-complexes between water molecules and DMSO (HO–H…O=S(CH_3)$_2$) dissolved in the organic phase, appears. Furthermore, the SAW signal almost disappears. In addition, the spectra revealed a signal corresponding to the CHD_2 groups of DMSO at $\delta_H = 2{,}8$ ppm. Thus, in clustered water associated with DNA and organic phase the following equilibrium can be considered:

$$\overset{\text{I}}{nH_2O(SAW_{DNA})} \leftrightarrow \overset{\text{II}}{nH_2O(SAW_{Solv})} \leftrightarrow \overset{\text{III}}{HO{-}H\ldots A(ASW)}, \qquad (4)$$

where A represents electron-donor. It has been observed that the equilibrium is shifted toward the formation of complexes III in the investigated temperature range.

With immobilization of a small amount of DNA (6 wt%) on the nanosilica surface, all the basic signals, characteristic for the hydrated DNA powder (Fig. 2a), can be observed in the spectra of water, adsorbed on the nanocomposite surface. SAW is observed as a broad intense signal with a chemical shift of $\delta_H = 5$ ppm and WAW1 reveals two signals with different intensities ($\delta_H = 0.9$–1.3 ppm), that may be associated with a higher heterogeneity of the nanocomposite surface compared with the DNA powder. The signal WAW2 is shifted to weak magnetic fields with decreasing temperature, probably due to the existence of equilibrium between the WAW and ASW. At $T > 260$ K the chemical shift of this signal overlap with the chemical shift of methyl groups of non-deuterated DMSO. In contrast to the case considered in Fig. 1b, the intensity of signal WAW1 significantly decreases with decreasing temperature, indicating a weaker strength of binding of this type of water to the surface. With increasing DMSO concentration in the dispersion medium (Fig. 2c) the redistribution of intensities of signals SAW and ASW + WAW2 is observed. It should be noted that for the SiO_2-DNA nanocomposite, in contrast to the hydrated DNA powder, the two separate signals of ASW water, associated with DMSO molecules in the dispersion medium and the electron-donor groups of DNA, are not observed. This is due to the rapid molecular exchange of different types of ASW in a relatively small amount of DNA, which is uniformly distributed over the surface of silica.

Figure 3 shows the temperature dependence of the WAW2 + ASW (a, b) and SAW (c, d) for nanocomposite containing different amounts of DMSO and Dox. In the case, where the DMSO concentration in the dispersion medium is 2 wt% (Figs. 3a, c), the dependence of $C_{WAW2+ASW}(T)$ and $C_{SAW}(T)$ are represented in the curves where the values decrease monotonically with decreasing temperature. Reducing the concentration of bound water occurs due to its freezing. In the presence of Dox, C_{SAW} is reduced by a corresponding increase of $C_{WAW2+ASW}$. By doubling the concentration of DMSO (Figs. 3b, d), $C_{WAW2+ASW}(T)$ curves contain an area in which the rise temperature decreases the concentration of interphase water. This can be attributed to the transition of interphase water in the volume of the dispersion medium. As it can be seen in Fig. 3, the presence a small amount of Dox in DMSO leads to a substantial redistribution of the intensities

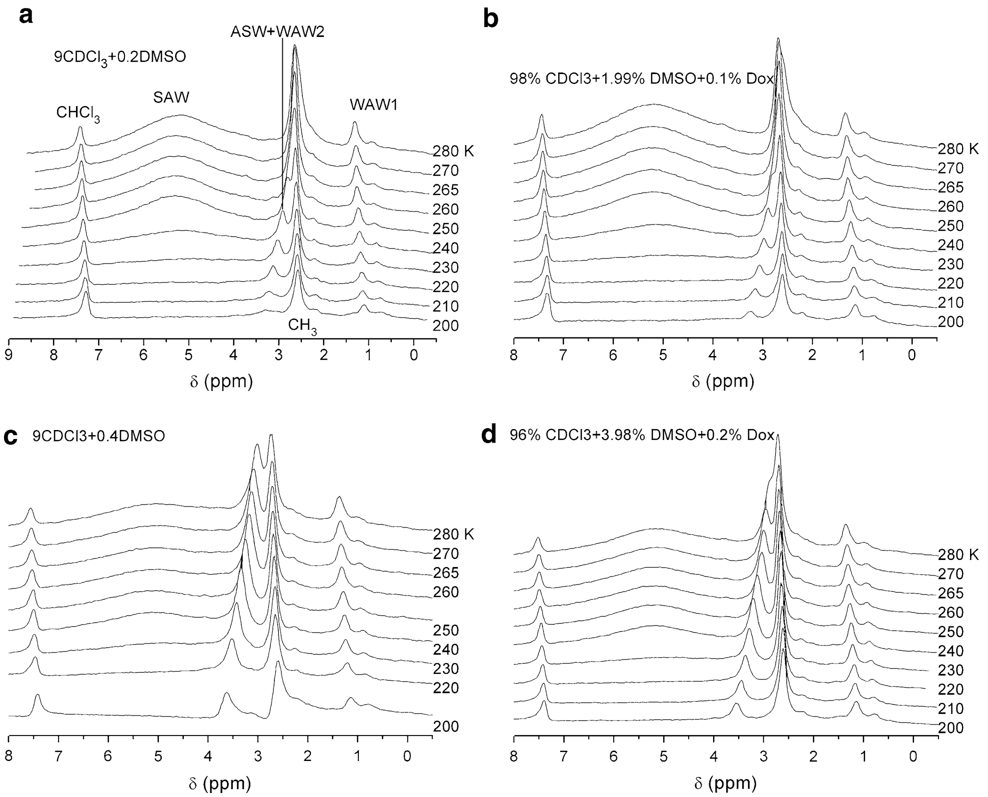
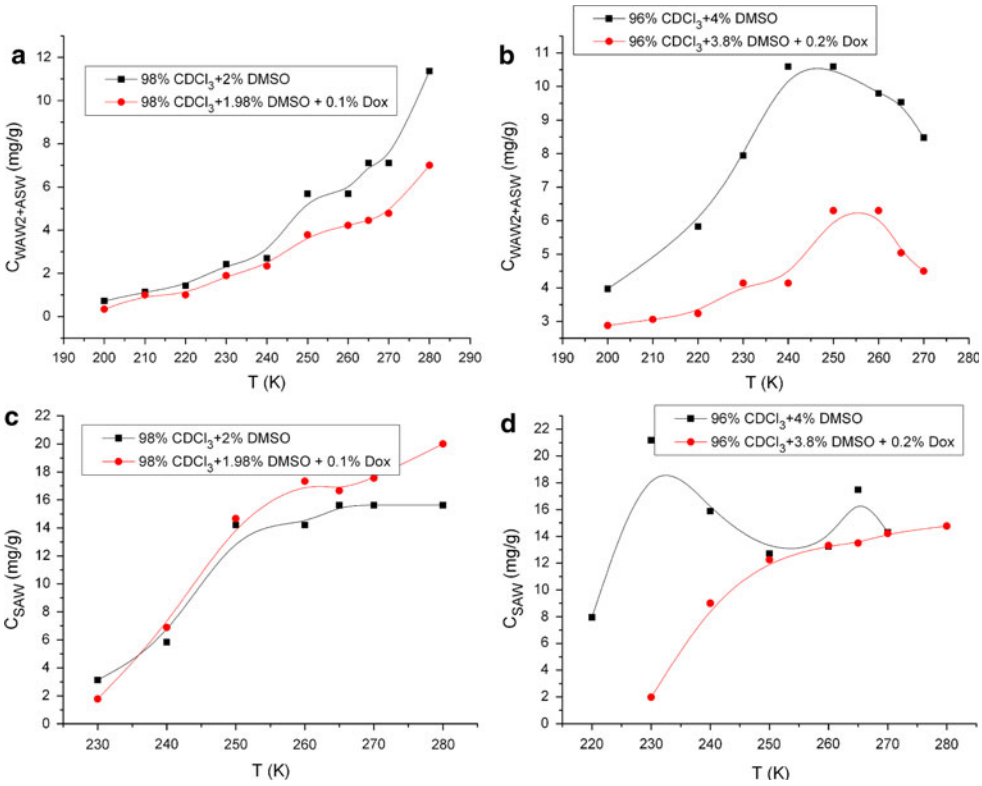


Fig. 2 ^1H NMR spectra recorded at different temperatures (200–280 K) of water-related SiO_2-DNA nanocomposite ($C_{H_2O} = 10\%$) in $CDCl_3$ with addition of DMSO: initial samples (**a**, **b**) and samples containing different amounts of Dox (**c**, **d**)

Fig. 3 Effect of Dox on the temperature variation of the concentration of different types of bound water for hydrated powder of SiO_2-DNA nanocomposite ($C_{H_2O} = 10$ wt%) in the $CDCl_3$ with addition of DMSO



of signals of different types of water, associated with the nanocomposite surface, which becomes more significant upon increasing Dox concentration. This indicates the effective interaction of Dox molecules with DNA. The greatest changes are observed in the intensity of the total signal, relating to ASW + WAW2, for which the decrease in $C_{ASW+WAW2}$ is almost doubled in the presence of Dox. If we consider that the DNA concentration on the surface is only 0.6 wt%, it can be concluded that the modification of nanosilica by DNA improves its specific adsorbent with respect to Dox. The binding of the main part of Dox by the nanocomposite surface is also confirmed from the fact that the originally brightly colored Dox solution is completely decolorized with the addition of nanocomposite.

Using a dispersion medium containing three components ($CDCl_3 + CD_3CN$ + DMSO, Fig. 4a, b), the spectra exhibit signals which corresponds to SAW, ASW + WAW2 and WAW1, as well as signals due to methyl groups of DMSO ($\delta_H = 2.5$ ppm) and acetonitrile ($\delta_H = 2$ ppm). Introduction to the dispersion medium of acetonitrile an even greater extent shifts the equilibrium (4) to the right, that due to the overall increase in the concentration of polar molecules in chloroform. However, as follows from Fig. 5a, b, addition of Dox into solution did not lead to a substantial redistribution of the intensities of signals of different types of interphase water. The most probable reason is the transfer of significant amounts of water in the dispersion medium. The observed ASW signal is the average between the water-related electron-donor centers of the nanocomposite surface and water, forming H-bound complexes with DMSO molecules and acetonitrile in the liquid medium.

Figs. 4c, d show the spectra of water for the SiO_2-DNA nanocomposite, taken at different temperatures, by using as dispersion medium deuterobenzene instead of deuterochloroform. Since benzene freezes at a sufficiently high temperature (280 K), it would be interesting to study how the ratio of the concentrations of different forms of bound water affects the phase state of the dispersion medium. It can be seen in Fig. 4c, d, that the shape of the spectra changes significantly at $T < 265$ K, which is the freezing temperature of the mixture of organic solvents. In the spectra of liquid dispersion medium the signals of protons of CH_3 groups of DMSO and CD_3CN, C_6H_6, TMS, as well as a broad SAW, the average ASW + WAW2 and WAW1 signals are observed. When organic solvents frozen, all signals are significantly broadened, and ASW + WAW2 signal transformed into averaged SAW + ASW + WAW2 signal.

The presence of Dox at the nanocomposite surface affects insignificantly the SAW concentration in the solid dispersion medium (Fig. 5). Significant differences are observed only in the liquid dispersion medium to the total signal-freezing water WAW1, SAW and ASW + WAW2 (Fig. 5d).

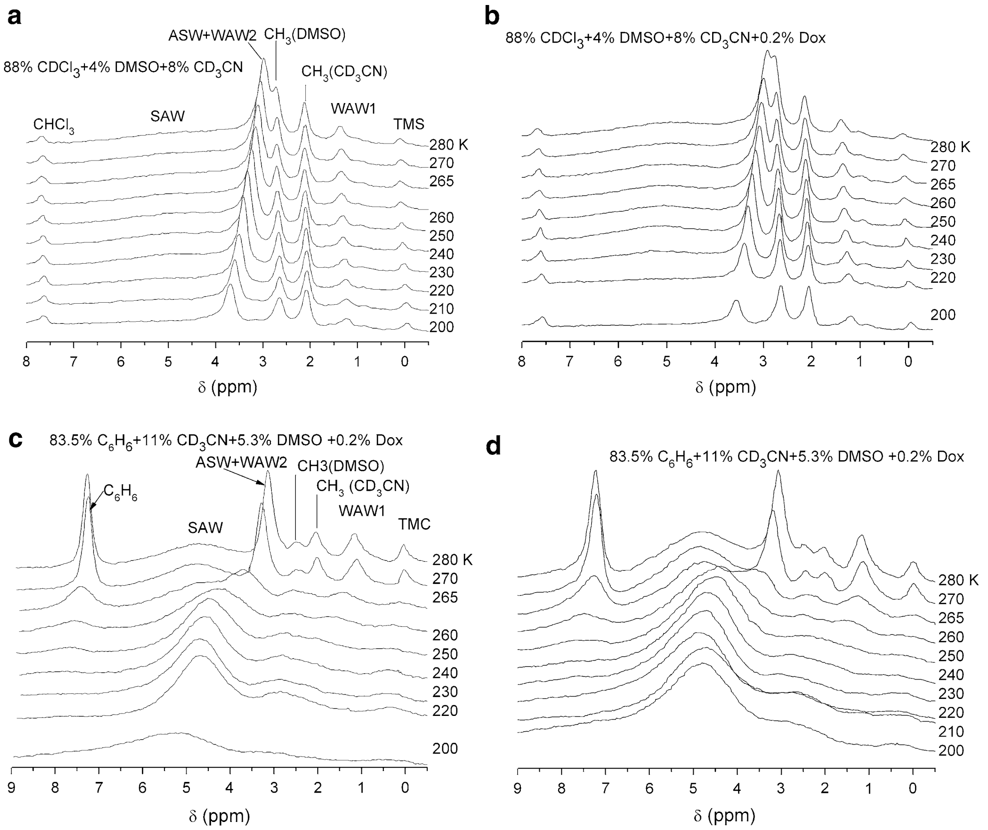


Fig. 4 ^{1}H NMR spectra recorded at different temperatures (200–280 K) of water-related SiO_2-DNA nanocomposite (C_{H_2O} = 10%) in $CDCl_3$ (**a**, **b**) and benzene (**c**, **d**) with addition of DMSO and CD_3CN: initial samples (**a**, **c**) and samples containing Dox (**b**, **d**)

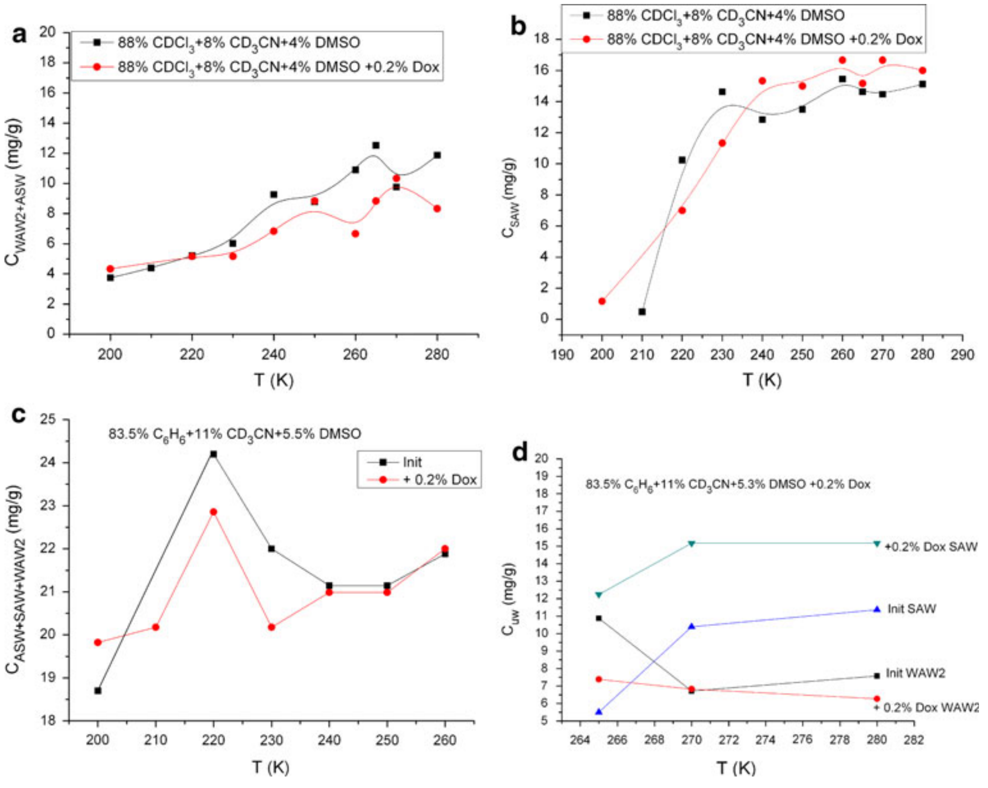


Fig. 5 Effect of Dox on the temperature variation of the concentration of different types of bound water for hydrated powder of SiO_2-DNA nanocomposite (C_{H_2O} = 10 wt%) in $CDCl_3$ (**a**, **b**) and C_6H_6 (**c**, **d**) with addition of DMSO and CD_3CN

To determine the effect of Dox, adsorbed on SiO_2-DNA nanocomposite surface, on the SAW cluster size, a calculation in accordance with Eq. 3 is required using deuterochloroform as dispersion medium by addition of small amounts of DMSO (Fig. 6). In the presence of Dox the size of SAW clusters significantly increases. Thus, if in the original sample the clusters, whose size do not exceed 4 nm, were observed, SAW clusters with a radius up to 16.6 nm were observed in the nanocomposite containing Dox. The same trend persists in the case of increase of the concentration of DMSO and Dox. Since there is a sharp decrease in ASW + WAW2 concentration (Fig. 3), one can assume that large SAW clusters are formed due to binding of Dox with the centers of the surface, which in the original sample participate in the ASW formation. It is known that the interaction of Dox with DNA leads to the formation of inclusion compounds (intercalates) with the system of complementary pairs of nucleotides [17]. Then one can conclude that a significant portion of ASW and WAW2 formed by the interaction of water molecules with nitrogen atoms of nucleotides.

The interphase energy (γS) of SAW, which gives the total decrease in free energy in SAW clusters, due to the interaction with the boundaries of the phase separation, can be calculated according to Eqs. 1 and 2 on the basis of CSAW(T) dependence (Figs. 3c, d). The results show that in the dispersion medium 2 wt% DMSO in the presence of Dox the value γS increases from 1.0 to 1.1 J/g, that is close to γS accuracy [10]. With increasing DMSO concentration up to 4 wt% ($C_{Dox} = 0.2$ wt%) the value γS (in the presence of Dox) decreases from 1.4 to 0.9 J/g.

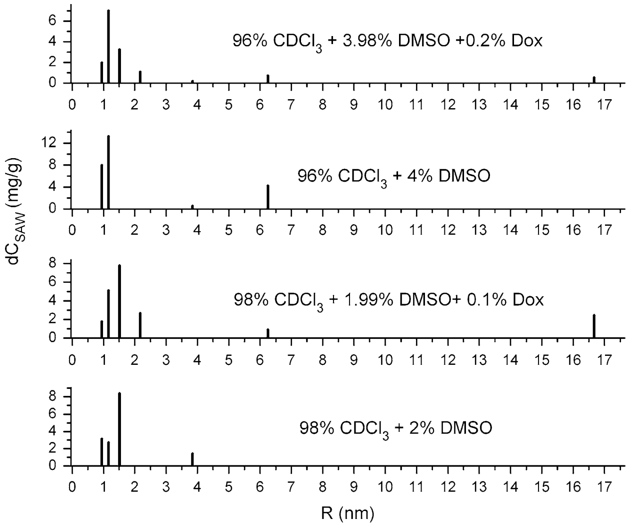


Fig. 6 Effect of Dox on the radius of the SAW clusters, related SiO_2-DNA nanocomposite

4 Conclusion

The composite material prepared on the nanosilica basis (Aerosil A-300), adsorption—modified by a small amount of DNA (0.6 wt%), can be used as an adsorbent, specific to the molecules of anticancer drug—Dox. Hydrated properties of this composite are similar in many respects to the properties of powdered DNA. In the ^{1}H NMR spectra of the nanocomposite, hydrated by a small amount of water (10 wt%), in chloroform (with the addition of 2–4 wt% DMSO), different types of water signals were observed. Namely strongly associated water signals (SAW, $\delta_H = 4.5$–5 ppm) as well as two types of weakly associated water signals (WAW1 and WAW2 with chemical shifts of $\delta_H = 1.2$ and 2.5 ppm, respectively) were observed. Additionally, a signal of water associated with electron-donor centers of the surface (probably with the nitrogen atoms of polynucleotide) and with molecules of the electron-donor solvent chloroform (ASW) was also observed. Depending on temperature and composition of the dispersion medium, fast molecular exchanges between the different forms of interphase water take place. The presence of Dox (0.1–0.2 wt%) in a dispersion medium leads to a change in the relative concentrations of different forms of water (ASW and WAW2 reduce while SAW increases). At that excess SAW exists in the form of clusters with a radius in the range 6–16.6 nm, which are not observed in the absence of Dox. The magnitude of the differentiating effect decreases with increasing concentrations of the electron-donor in the dispersion medium.

In summary our results clearly showed that SiO_2-DNA nanocomposites can be used as a basis for the development of targeted delivery of Dox to the site of the tumor process.

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