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Characterization of C_{60} fullerene complexation with antibiotic Doxorubicin

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Abstract

The aim of the paper was to provide the physico-chemical characterization of key process leading to amplification of antitumor effect of antibiotic Doxorubicin (Dox) *in vivo* and *in vitro* and occurring at molecular level through complexation with C_{60} fullerene. A wide range of physico-chemical tools was used such as UV/Vis and NMR spectroscopies, atomic force microscopy, isothermal titration calorimetry and zeta-potential methods. The unusual thermodynamic behavior of the complexation process was reported, featuring unexpected and, to certain extent, contradictory experimental observations. The explanation of the obtained results was proposed resulting in creation of a general view on aromatic drug binding with C_{60} fullerene. Based on these results some important practical outcomes for anticancer therapy were formulated.

Key words: C_{60} fullerene aqueous colloid solution; doxorubicin; complexation; UV/Vis spectroscopy; NMR spectroscopy; atomic force microscopy; zeta potential; isothermal titration calorimetry;

Introduction

C_{60} fullerene has now emerged as an important component of contemporary nanotechnologies and finds extensive application in nanobiotechnology and nanomedicine.[1-3] One of the most promising properties of C_{60} fullerene is its anticancer activity, therefore C_{60} molecule is now considered as a potential drug candidate for antitumor therapy.[2,3] Apart from the anticancer properties of C_{60} fullerene itself, it has been recently shown that the administration of water soluble pristine C_{60} fullerene along with anticancer drug Doxorubicin (Dox, Fig.1) results in metastasis inhibition, increase in animal life duration[4] and protective effect towards various cell lines.[4,5] At least two key features of these effects have been noted, *viz.* (*i*) the maximum therapeutic activity is observed when Dox and C_{60} fullerene are administered together, *i.e.* present in biosystem simultaneously, and (*ii*) the correlation of the *in vitro* biological effect with equilibrium constant of complexation of C_{60} fullerene with aromatic drug molecules. Basing on results of spectrophotometric titration it was assumed that the Dox and C_{60} molecules may directly interact in solution forming stable non-covalent complex in which C_{60} fullerene acts as a carrier of the antibiotic to target cells.[6] However, no further support to this hypothesis has been provided so far.

The possible link between the complexation ability and biological synergism in C_{60} fullerene with Dox system may be correlated with the well-recognized ability of aromatic drug

molecules to form non-covalent hetero-complexes in solution and alter the biological effect of aromatic drugs, including Dox,[5-7] as well as with the ability of carbon nanotubes (CNTs) to lower the toxicity of Dox via non-covalent complexation with the antibiotic *in vivo*.[8,9] This property of pristine C_{60} fullerene appears to differ strikingly from the very popular now strategy of covalent attachment of C_{60} molecule to other drugs including Dox.[1,10] The knowledge of thermodynamic nature of non-covalent C_{60} fullerene-drug complexation may be crucial for understanding the mechanism of biological synergism on their simultaneous administration *in vivo* or *in vitro*, and may rationalize search for other antitumor drugs which should potentially exert medico-biological synegrism with C_{60} molecule.

Experimental Section

Preparation of C_{60} fullerene with Dox aqueous solution

The highly stable purified C_{60} fullerene aqueous colloid solution (C_{60}FAS; concentration 0.15 mg/ml) was prepared according to the method reviewed in[11] and based on the technology of transferring C_{60} molecules from toluene to an aqueous phase (light or heavy water) with the help of ultrasonic treatment. Full characterization of the morphology of the resultant C_{60}FAS is given in[11].

Dox (Doxorubicin hydrochloride; Sigma, Germany) was dissolved in distilled water (or D_2O for NMR) with initial concentration of 5 mg/ml. Preparation of the C_{60}+Dox mixture (C_{60}FDAS) was executed according to protocol: C_{60}FAS and Dox were mixed in 1:2 weight ratio (0.15 : 0.3 mg), and the resulting C_{60}FDAS was treated in the ultrasonic disperser for 20 min, and after that stirred magnetically for 12 h at room temperature. In each experiment the concentration of Dox was maintained constant (depending on the experimental method used) and the concentration of C_{60} fullerene varied starting from the highest concentration down to zero.

AFM characterization of the studied solutions

In order to characterize the composition of the prepared C_{60}FDAS, the state of Dox molecules and C_{60}+Dox complexes was monitored using atomic force microscopy (AFM; "Solver Pro M" system; NT-MDT, Russia) technique. For AFM study the sample was deposited by droplet precipitation from an aqueous solution onto a cleaved mica substrate (V-1 Grade, SPI Supplies). Measurements were performed after complete evaporation of the solvent. The sample visualization in the AFM experiments was conducted in a semicontact (tapping) mode using NSG10 (NT-MDT) probes.

UV/Vis measurements

UV/Vis absorption spectra of Dox and C_{60}+Dox mixture were recorded using a double-beam spectrophotometer SQ-4802 (UNICO, USA). The solution was poured into a polymethylacrylate cuvette (Spain) having an optical path length of 1 cm, which enabled to perform the measurements in the range of 200–700 nm. The temperature was maintained constant at T=298 K. In order to exclude background scattering from C_{60} fullerene clusters all experiments were carried out by recording spectra of the mixture of Dox and C_{60} molecules against the solution of pure fullerene at a similar concentration in the reference cuvette.

ζ-potential measurements

Zeta potential measurement for C_{60}FDAS was carried out on a Zetasizer Nano-ZS90 (Malvern, Worcestershire, UK) at T=298 K. The results were evaluated using the Smoluchowski approximation, which is known to be rigorously valid only for spherical-like particles.

ITC measurements.

All isothermal titration calorimetry (ITC) experiments were performed as described before[7] in distilled water at T=298 K using an AutoITC isothermal titration calorimeter (MicroCal Inc. GE Healthcare, Northampton, USA) with 1.45 ml of sample and reference cells. The cell containing distilled water was used as the reference. The data, specifically the heat normalized per mole of injectant, were processed with Origin 7 from MicroCal. An initial 2 μl injection was discarded from each data set in order to remove the effect of titrant diffusion across the syringe tip during the equilibration process. The experiment consisted of injecting 10.02 μl (29 injections, 2 μl for the first injection only) of Dox solution (213.7 μM) into the reaction cell initially containing C_{60}FAS (13.9 μM). Background titrations were performed using identical titrant with water placed in the sample cell and using water as a titrant with C_{60}FAS in the sample cell. The results of background titrations were subtracted from experimental titration to account for the heat of dilution. All the solutions were degassed before titrations were performed. The titrant was injected in 4 min intervals to ensure that the titration peak returned to the baseline prior to the next injection. Each injection lasted 20 s. In order to achieve a homogeneous mixture in the cell, the stirrer speed was kept constant at 300 rpm.

NMR measurements

Nuclear magnetic resonance (NMR) spectra were acquired at a magnetic field strength of 14.1 T using a Bruker Avance III NMR spectrometer operating at a ^{1}H resonance frequency of 600.13 MHz and working under TopSpin version 2.1 (Bruker Biospin, Karlsruhe, Germany).

1D ^{1}H NMR spectra were acquired over a frequency width of 12.3 kHz (20.55 ppm) centered at a frequency offset equivalent to 6.175 ppm into 65536 data points during an acquisition time aq = 2.66 s with a relaxation delay d1 = 2 s for each of 32 transients. All measurements have been performed under the fast exchange regimen on the NMR timescale at *T*=298 K in deuterated C_{60}FDAS. Chemical shifts were measured relative to an internal reference of tetramethylammonium bromide (TMA) and recalculated with respect to (sodium 2,2 dimethyl 2-silapentane-5-sulphonate, (DSS)) according to $\delta_{DSS} = \delta_{TMA} + 3.178$ (ppm).

Diffusion measurements (Diffusion-Ordered NMR Spectroscopy, DOSY) were conducted using a bipolar gradient pulse program (Bruker pulse program ledbpgppr2s) in which presaturation was used to suppress residual solvent signal during the recycle delay. Typically 32 gradient increments were used by which the gradient strength was varying linearly in the range 2% to 95% of full gradient strength (54 G/cm with a rectangular gradient) using a sine-shaped gradient profile. Typically the gradient pulse duration was set to 1 ms and the diffusion period to 200 ms. Diffusion data were processed under TopSpin (version 2.1, Bruker Biospin) using the T1/T2 analysis module in order to fit the data to the standard expression of diffusion coefficient as a function of gradient strength.

Results

A range of various physico-chemical methods was applied in order to detect the complexation between C_{60} fullerene and antibiotic Dox. In neutral solution conditions the Dox molecule bears positive charge, whereas the C_{60} fullerene is negatively charged.[6]

AFM study of C_{60}+Dox complexation

AFM investigation of the C_{60}+Dox mixture precipitated onto mica substrate (Fig.2a) clearly indicates the existence of sphere-like particles with dimensions in the range of 1…100 nm. The observed particle distribution is qualitatively similar to that previosly noted for C_{60}FAS without Dox (for the same C_{60} molecule concentration and method of preparation), and agrees well with literature data (see[11] for review). Hence, the observed particles may be assigned to C_{60} fullerene clusters, although the binding of Dox with C_{60} fullerene cannot be explicitly confirmed at this point. However, in 20-fold dilution of the mixture new objects can be clearly seen in the AFM picture, which are the islands having 0.6…1.1 nm in height (Fig.2b). The formation of these islands and their dimensions cannot be associated solely with Dox molecules, as well as the precipitate from pure C_{60} fullerene solution does not indicate the formation of such islands.[11] One can assume that the positively-charged Dox molecules form complexes with C_{60} fullerenes

thereby lowering the electrostatic repulsion between them. Hence, the presented AFM study most likely evidences the formation of complexes between C_{60} fullerene and Dox.

UV/Vis study of C_{60}+Dox complexation

The set of Dox spectra in the visible wavelength range, where C_{60} fullerene does not absorb, is given in Fig.3. Apparent hypochromic changes of the absorption maximum with a slight bathochromic shift are observed with increasing C_{60} fullerene concentration, which evidence a complex formation between C_{60} fullerene and antibiotic molecules. An isosbestic point is also noticeable at low concentrations. These results generally agree with previous investigation of C_{60}-drug systems[6,12,13] as well as with the related carbon systems such as CNT-drug interactions, [14,15] and point out on stacking-type complexation between the Dox and C_{60} molecules in which their planes are arranged in parallel to each other.

ζ-potential study of C_{60}+Dox complexation

The results of measurement of ζ-potential for C_{60}+Dox mixture and their components in separate are given in Fig.4. The value of ζ-potential for Dox is close to zero whereas it equals to -28 mV for C_{60}FAS. The latter agrees well with literature data[13,16] and indicates the existence of negative charge on the surface of C_{60} molecules and their clusters in solution. However, the mixing of C_{60} fullerene and Dox results in a pronounced shift of ζ-potential peak up to +45 mV. This effect may be explained by complexation of positively-charged Dox with C_{60} fullerene clusters which results in shielding of the C_{60} molecule negative charge and charging of these clusters.

ITC study of C_{60}+Dox complexation

In order to check thermal effects of possible C_{60}+Dox complexation we applied ITC. A thermogram representing titrations of C_{60} fullerene with water, water with Dox (controls) and C_{60} fullerene with Dox is shown in Fig.5a. The obtained data reveal no essential differences between magnitudes of peaks recorded for titrations of water with Dox and fullerene C_{60} with Dox, with both processes being endothermic, mainly due to dissociation of Dox homoaggregates. Determined thermal effects of these titrations do not exhibit significant differences as well (Fig.5b). The value of heat difference obtained by subtracting heat effects of controls from heat effects for C_{60} fullerene titration with Dox oscillate near zero (Fig.5c).

The performed ITC analysis indicates that enthalpy changes associated with the complexation of Dox with C_{60} fullerene particles are close to zero ($\Delta H \approx 0$ kcal·mol^{-1}).

NMR study of C_{60}-Dox complexation

The measured values of proton NMR chemical shift (δ) of non-exchangeable protons and translational diffusion coefficient (D) as a function of C_{60} fullerene concentration at constant Dox concentration are given in Fig.6. The changes in δ reach 0.02 ppm at the best in the concentration range allowed by solubility of C_{60} fullerene and only for some of the protons studied (those shown in Fig.6a), whereas remaining protons feature negligible changes of δ. It indicates very weak magnetic shielding effect of Dox protons in complex with C_{60} fullerene. In contrast, the diffusion curve (see Fig.6b) displays very distinct changes at small fullerene concentrations and reaches a plateau for higher concentrations. It may be stated that the diffusion properties of Dox molecules are strongly affected by binding with C_{60} fullerene.

Discussion

The principal goal of the investigation was to detect the existence of complexation between antibiotic Dox and C_{60} fullerene in aqueous solution and to get insight into thermodynamic nature of this interaction.

The results of various physico-chemical methods outlined above may be conditionally divided onto two groups, *viz*. the methods which directly evidence the fact of C_{60}+Dox complexation (NMR diffusion, UV/Vis, AFM and ζ-potential methods) and those giving no or negligible signs of complexation (ITC, ^{1}H NMR). Such distinguishment of the experimental methods is, in fact, not unusual due to the fact that different methods are more or less suited to particular experimental conditions employed and/or specificity of the system under investigation. However, the case of C_{60}+Dox complexation, from our viewpoint, appears to be quite uncommon for the following reasons:

(i) the complexation between C_{60} fullerene and Dox molecules, containing conjugated aromatic rings, should follow stacking-type complexation, in which planar aromatic surfaces of fullerene and Dox chromophore are arranged nearly in parallel (*i.e.* the π-stacked complex). This view is supported by the published material on the interaction between C_{60} fullerenes and aromatic molecules in water (*e.g.* [6,12,17]) and there is no reason to assume alternative views, for instance, the possibility of T-shaped structure of C_{60}+Dox complex. In such case the enthalpic character of complexation should be expected, accompanied by, detectable by ITC, heat effect, which is typical of aromatic-aromatic interactions,[18] and was previously observed for binding of C_{60} fullerene with calixarenes in organic solvents.[12] However, in ITC experiment heat effect detected was close to zero;

(ii) within the π-stacked complex with the typical aromatic-aromatic distance of 0.34 nm[19,20] a relatively strong magnetic shielding of non-exchangeable Dox aromatic protons should be observed in the range of 0.1…1.0 ppm due to ring current effect from C_{60} fullerene (also previously reported for calixarene binding with C_{60} molecule[12]). However, in NMR experiment very weak shielding of less than 0.02 ppm was detected for all of the studied Dox aromatic protons;

(iii) the situation when physically the same method (*i.e.* the NMR spectroscopy) applied to measure the same signal (*i.e.* magnetization decay induced by Dox protons), however, excited in different ways (standard 1D- and DOSY NMR pulse sequences), leads to qualitatively different conclusions regarding the existence of complexation, is unusual;

(iv) the dependence of Dox molecules translational diffusion coefficient on the increasing fullerene concentration exerts completely unexpected behavior. If the complexation is assumed, the decrease in diffusion coefficient should be observed, whereas an opposite trend is seen in Fig.6b.

We would suggest the following rationale to interpret these contradictory points, thereby allowing to get insight into the nature of C_{60} fullerene with Dox complexation.

Does the C_{60}+Dox complexation exist?

Although the AFM study showed the complexation, the experimental conditions of the AFM picture recording are different from the bulk water in real solution, therefore we decided to temporarily ignore the AFM data.

The UV/Vis, ζ-potential and DOSY NMR measurements evidence apparent changes in the experimental data, which, in our viewpoint, cannot be explained if the complexation does not exist. Moreover, in the cases of UV/Vis and DOSY analyses the experimental data is associated explicitly with Dox molecules (*i.e.* the Dox absorbance and the Dox ^{1}H chemical shifts) whose concentration was kept constant, whereas the concentration of experimentally invisible C_{60} molecules varied. Hence, the changes in Dox-associated experimental data with C_{60} fullerene concentration variation may only be explained by the complexation.

Why the translational diffusion coefficient exerts an unexpected behavior?

Recently[21] an extraordinary behavior of translational dissusion coefficient, *D*, of C_{60} fullerene in solution was reported and later independently confirmed.[22] Based on dynamic light scattering data[21] and molecular modeling[22] an increase in the value of *D* with increasing C_{60} fullerene concentration was observed, which is inconsistent with the concept of aggregation. It was shown that such behavior originates from the infuence of so-called 'interaction' effect, well-known in colloid chemistry,[23] *i.e.* the increase in C_{60} fullerene concentration results in increase in the fraction of large C_{60} fullerene clusters which interact with each other as microscopic objects

due to long-range forces. This interaction is being added up to Brownian motion of these clusters elevating their mean velocity and, thereby, increasing the value of *D*. Obviously, the same interaction effect governs the concentration behavior of Dox diffusion coefficient, shown in Fig.6b, *i.e.* when Dox molecules bind with fullerene clusters their diffusion properties become similar to the diffusion of these clusters. It allows one to explain the contradictory point (*iv*) and also enables to conclude that if the 'Dox diffusion' vs 'C_{60} fullerene concentration' curve originates from the interaction of large clusters, hence, the binding of Dox molecules mainly occurs with C_{60} fullerene clusters. Indirectly this conclusion is also confirmed by the existence of large-by-area islands in AFM picture (see Fig.2b and the Results section) most likely containing C_{60}+Dox complexes stabilized by Dox-induced attenuation of electrostatic repulsion between the C_{60} fullerenes.

Why ITC and ^{1}H NMR do not detect complexation?

Recently an entropic-driven aggregation of C_{60} fullerene molecules in aqueous solution was reported.[24] It was found that C_{60} fullerene aggregation features close to zero heat effect in ITC (similar to that observed here for C_{60}+Dox system), and is governed mainy by hydrophobic interactions. The origin of the observed effect was shown to be due to exceptionally strong fullerene-water interaction, overbalancing the fullerene-fullerene interactions, and emphasising the importance of hydrophobically favorable water rearrangement in the second- and higher-level hydration shells of C_{60} fullerene particles. Taking into account the similar pattern of the ITC results obtained in the present work for C_{60}+Dox system, it is reasonable to assume that the same hydrophobic-driven complexation is the case here as well, allowing explanation of the point (*i*) raised above.

However, if the nearest hydration shell is tightly attached to the C_{60} fullerene surface, the distance between the aromatic surface of C_{60} molecule and the aromatic chromophore of Dox should be higher than the distance of 0.34 nm typical of aromatic-aromatic stacking,[19,20] thus allowing one to explain why the measured induced ^{1}H NMR chemical shift of Dox protons was small on binding with C_{60} fullerene (*i.e.* point (*ii*)). Taking into account the $1/r^3$ dependence of magnetic shielding on distance, *r*, between aromatic moieties, it can be stated that an order of magnitude decrease in aromatic shielding from the typical value ~0.1 ppm down to ~0.01 ppm observed in the present work may be achieved if, *r* between the aromatic surfaces of C_{60} fullerene and Dox molecules is raised two-fold. Hence an approximate value of $r \approx 0.6$ nm should be considered as a reasonable estimate. It is also worth noting that the observed negligible changes in Dox proton chemical shifts point out on the fact that the majority of Dox molecules most likely exists in complex with C_{60} fullerenes, because, if not, the effect of self-association of

Dox[25] would contribute to the dependence of δ on C_{60} molecule concentration, which is not the case in the present work.

If the increased distance between C_{60} fullerene and Dox molecules lowers the sensitivity of the induced ^{1}H NMR chemical shift to complexation, it, however, should not influence the sensitivity of the diffusion parameter of C_{60}+Dox complex. It thus allows one to explain why the ^{1}H NMR does not sense complexation whereas DOSY NMR does (*i.e.* point (*iii*)).

What is the the structural specificity of C_{60}+Dox complex?

As evidenced from UV/Vis (see Fig.3), the hypochromic and bathochromic shifts indicate the stacking-type complexation in which the aromatic surfaces of C_{60} fullerene and Dox molecules are arranged in parallel to each other with the distance of approximately 0.6 nm between them. Alternative structures of the complex, *e.g.* T-shaped, or with non-parellel aromatic planes, would not be hydrophobically favorable and therefore are less probable. The aminosugar attached to the chromophore of the drug (see Fig.1) also enhances the hydrophobic contribution as it moulds to the convex shape of the C_{60} molecule and facilitates water release from the space between the surfaces of C_{60} and Dox molecules in the complex.[6] The distance of ca.0.6 nm is spectroscopically 'relevant' and for aromatic-aromatic stacking interactions may be deduced from application of exciton theory to UV/Vis data.[26] It is also worth noting that currently available theoretical[6,27] and X-ray experimental[28] determinations of the structures of aromatic molecules complexes with C_{60} fullerenes or related carbon systems such as CNTs, commonly use the typical aromatic-aromatic distance of r=0.3…0.35 nm. In view of the results obtained in the present work, this value should be re-considered and probably corrected in all future studies for C_{60} fullerene-aromatic ligand systems in aqueous solutions. Taking into account also the results of ^{1}H and DOSY NMR study discussed above, the majority of Dox molecules likely exists in complex with large C_{60} fullerene clusters.

What is the thermodynamic nature of C_{60}+Dox complexation?

As discussed above, the complexation of Dox with C_{60} fullerene is driven by hydrophobic interactions, which is the principal contributor to the net Gibbs free energy of the complexation process. However, the ζ-potential measurements (see Fig.4) also demonstrate drastic shift of the ζ-value from deep negative up to high positive values. This result may be interpreted by incorporation of positively-charged Dox molecules into the C_{60} fullerene clusters, which is also confirmed by the AFM study (see Fig.2b), *i.e.* the formation of large-by-area islands containing Dox and C_{60} molecules. It thus may be concluded that electrostatic forces should also contribute to binding energy and, being enthalpic in nature, should lead to negative enthalpy change. However, the enthalpic contribution to Dox-fullerene complexation has appeared to oscillate around zero. Recent results of energy decomposition for C_{60}+drug complexations[6] predicted that

the favorable C_{60} fullerene-Dox electrostatic interactions should be compensated by unfavorable loss of C_{60} fullerene-water and Dox-water electrostatic interactions, thus leading to close to zero net effect of electrostatic contribution to the enthalpy of complexation. Therefore, it may be considered the reason why the experimentally-measured heat effect of the C_{60}+Dox complexation approaches zero (see Fig.5c) under the condition that electrostatic interactions provide important contribution into the formation of complexes.

What might be the practical outcome of knowing the mechanism of C_{60}+Dox complexation?

Although the above-discussed results were obtained in water solution not containing any other components than Dox or fullerene, the pilot study reported in [4] had confirmed pronounced C_{60}-Dox complexation in physiological solution. It enables projecting the obtained results to biological system.

Predominant binding of Dox molecules with C_{60} fullerene clusters, reported in the present work, provides scientific grounding and confirmation to the hypothesis initially suggested in[6] and discussed in the introductory section, *viz*. the C_{60} fullerene clusters absorb Dox molecules thereby acting as nanocarriers of the antibiotic to target cells. This process may explain at the molecular level the initial stage of the whole mechanism of the pronounced antitumor effect exerted by administration of the mixture of C_{60} fullerene and Dox both *in vitro* and *in vivo*.

However, the most important outcome is the understanding that the mechanism of such C_{60}+Dox cluster binding is single, *i.e.* relatively unspecific to the structure of Dox molecule, except, probably, the requirement for mandatory existence of aromatic moiety as a major part of the structure. It follows that other very important antitumor drugs such as actinomycin D, mitoxantrone, topotecan, *etc*. may also bind with C_{60} fullerenes in the same manner, which creates an opportunity for improvement of their antitumor properties by means of simultaneous administration with C_{60} fullerene. An indirect evidence of this has been recently reported on *in vitro* level.[5] Hence the results obtained in the present work point out on the possibility of rational search of C_{60} fullerene-aromatic ligand pairs as potential drugs with improved medico-biological effect.

Conclusions

We performed extended investigation of C_{60} fullerene and anticancer antibiotic Doxorubicin interactions in water solution using various physico-chemical methods with an aim of understanding the specificity of complexation of these two compounds. A rather unusual situation was observed, *viz*. some of the experimental methods (*i.e.* NMR diffusion, UV/Vis,

AFM and ζ-potential) evidence the complexation, whereas the others (*i.e.* ITC and ^{1}H NMR) do not. The most unexpected result was the contradictory data obtained from NMR and ITC which did not match the initially expected views on aromatic-aromatic complexations grounded on literature review.

Comprehensive analysis of the obtained data has enabled us to formulate a single view on thermodynamics of C_{60}+Dox complexation removing the contradictory points and allowing to address the questions raised by experiment. It was suggested that the binding of Dox occurs with large C_{60} fullerene clusters. This process is purely entropic in origin, *i.e.* features close to zero enthalpic effect and is governed by hydrophobic interactions utterly.

The main structural specificity of C_{60}+Dox complexation is the stacking-type binding of Dox, occuring mainly with large C_{60} fullerene clusters and featuring presumably the unusually big distance of ca. 0.6 nm between the aromatic planes of Dox and C_{60} molecules. As a consequence of this specificity the common methods for probing aromatic-aromatic stacking, such as ^{1}H NMR and ITC, have appeared to be less informative, whereas the diffusion NMR, UV/Vis and ζ-potential have appeared as the most relevant for C_{60} fullerene-drug investigations.

The principal practical outcome of the current study is the prediction of medico-biological synergism for using C_{60} fullerene together with other aromatic drugs, which may bind with C_{60} fullerene clusters in the same manner as Dox does, creating a challenge for further studies.

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FIGURE LEGENDS

Figure 1 Structure of Doxorubicin

Figure 2 AFM images of C_{60}+Dox mixture precipitated onto mica surface at initial state (a) and at 20-fold dilution (b)

Figure 3 Absorption spectra of doxorubicin at concentration $4 \cdot 10^{-5}$ M measured for different fullerene concentrations

Figure 4 Zeta potential of C_{60}FAS (a), Dox solution (b) and C_{60}+Dox mixture (c)

Figure 5 (a) Microcalorimetric titrations of C_{60}FAS (initial concentration 13.9 μM) with Dox (Dox, concentration range 1.8 – 37.6 μM), C_{60}FAS (initial concentration 13.9 μM) with water and water with DOX (concentration range 1.8 – 37.6 μM); solid, dashed and dotted lines, respectively
(b) Thermal effects of titrations: C_{60}FAS with water (circles), water with Dox (triangles) and C_{60}FAS with Dox (squares). Crosses represent differences between the heat of titration of C_{60}FAS with Dox and sum of heats of C_{60}FAS with water and water with Dox titrations
(c) Heat of C_{60} fullerene-Dox interaction (corrected for background thermal effects), calculated as kcal·mol^{-1} of injected Dox. The enthalpy change (ΔH) of the C_{60} fullerene-Dox interaction was calculated by the linear regression of experimental points to the infinite dilution of Dox ([Dox]→0) and is equal to 0.0134 ± 0.0257 kcal·mol^{-1}

Figure 6 Dependence of chemical shifts of non-exchangeable protons (a) and translational diffusion coefficient (b) on fullerene concentration, measured at fixed concentration of Dox 1 mM

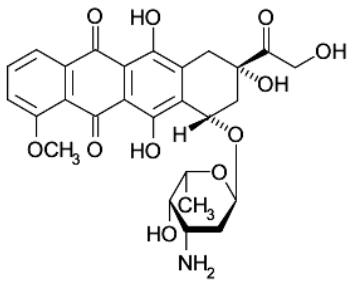
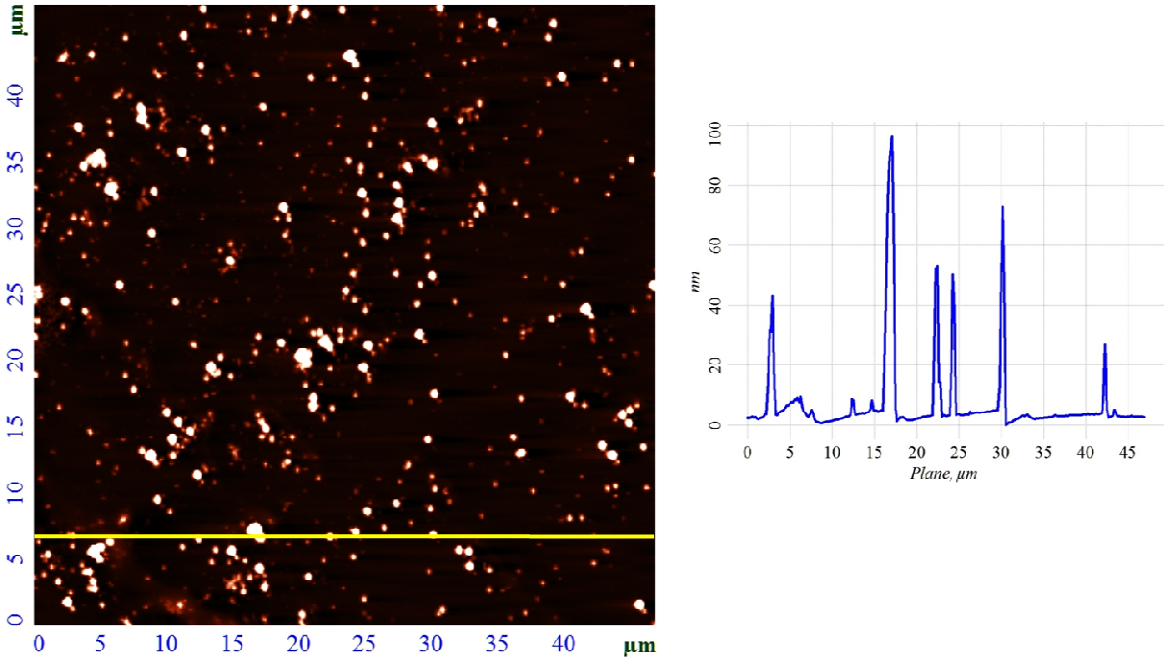
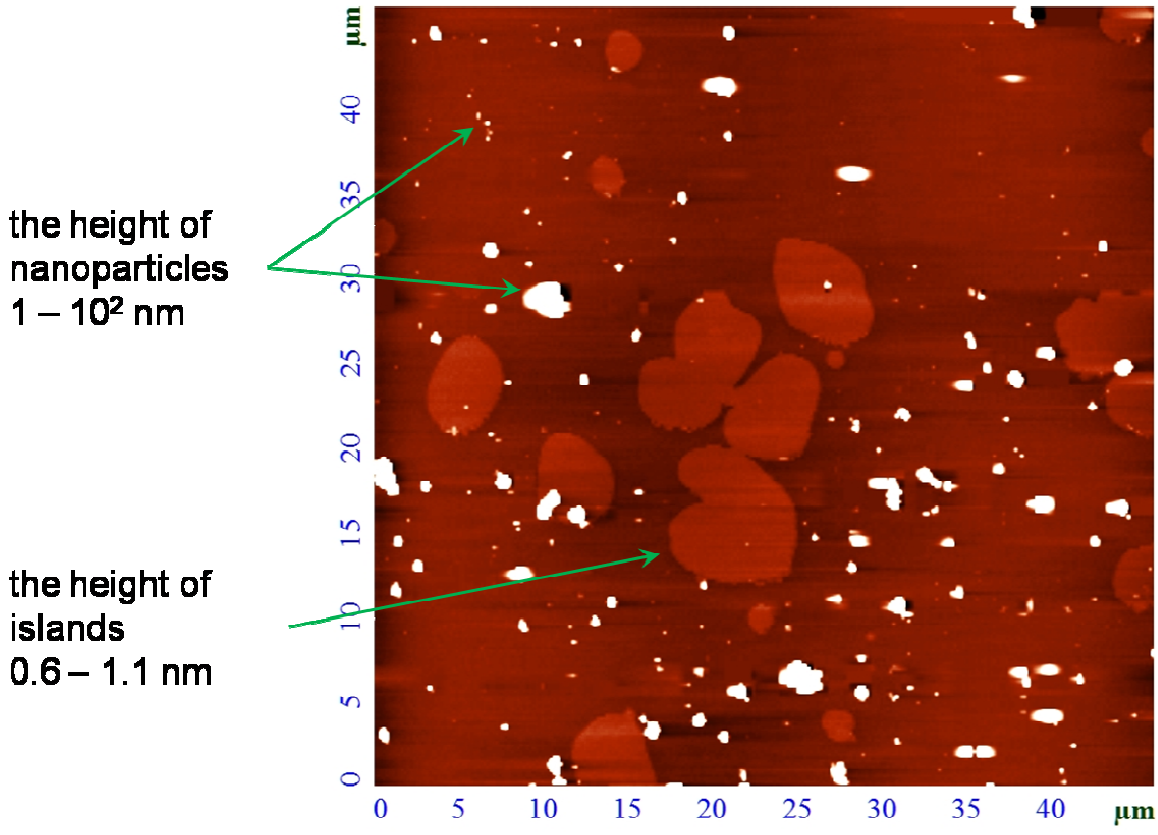


Figure 1



(a)



(b)

Figure 2

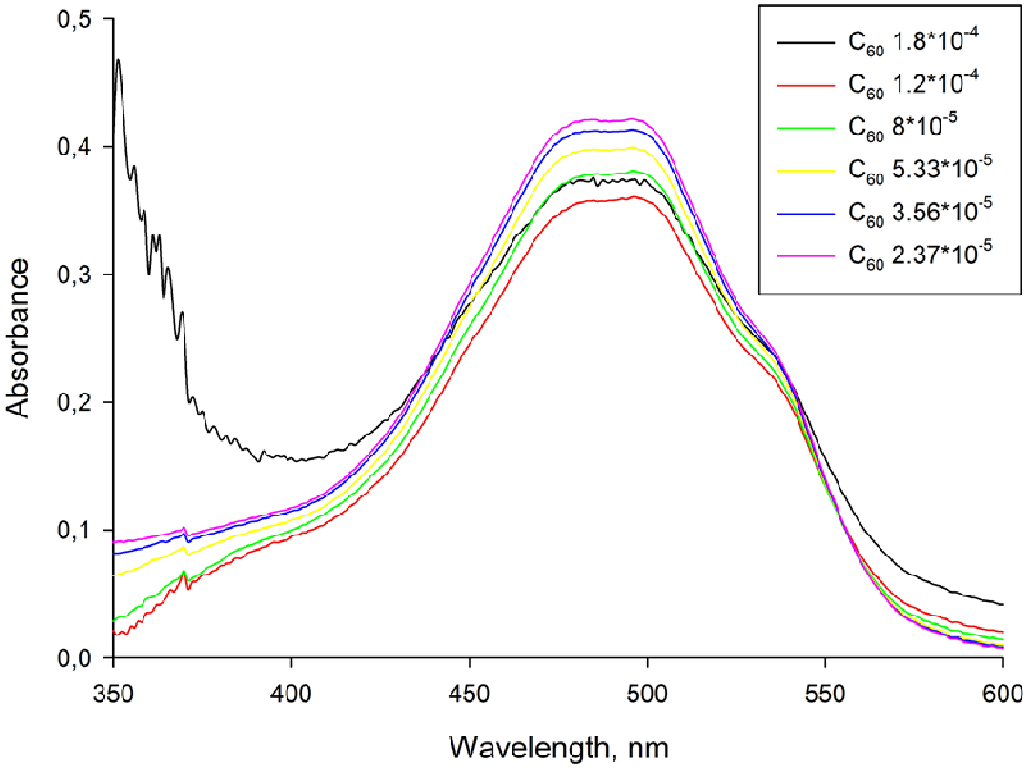
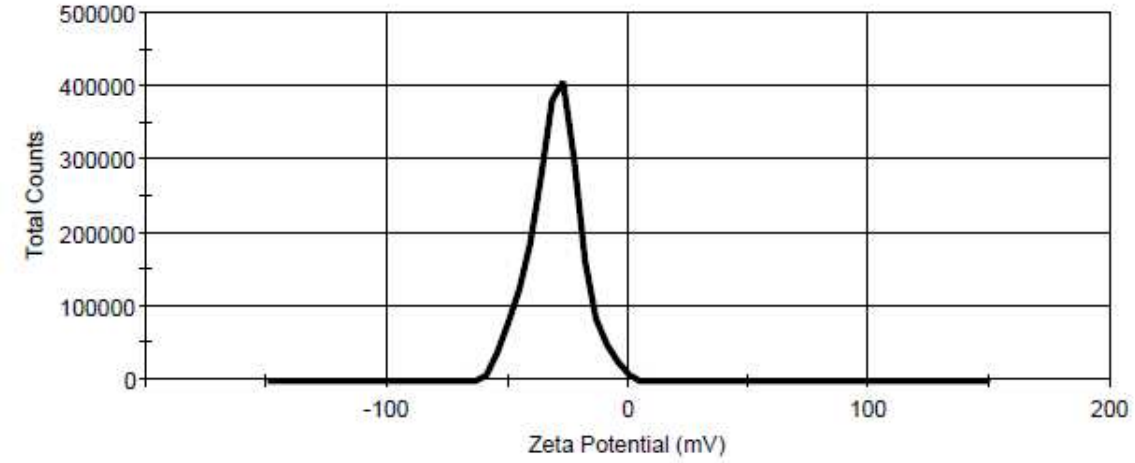
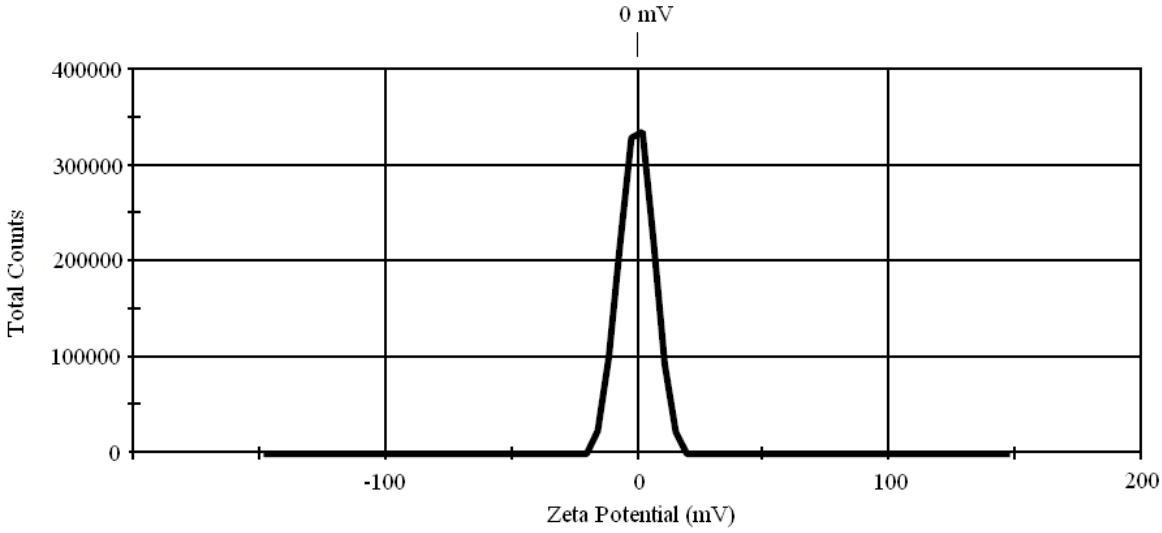


Figure 3



(a)



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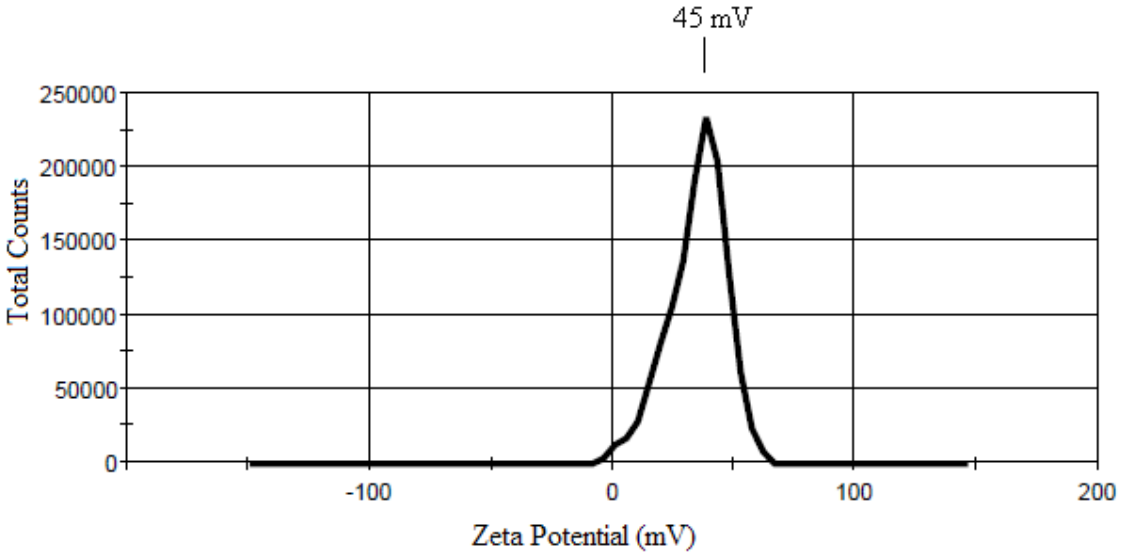
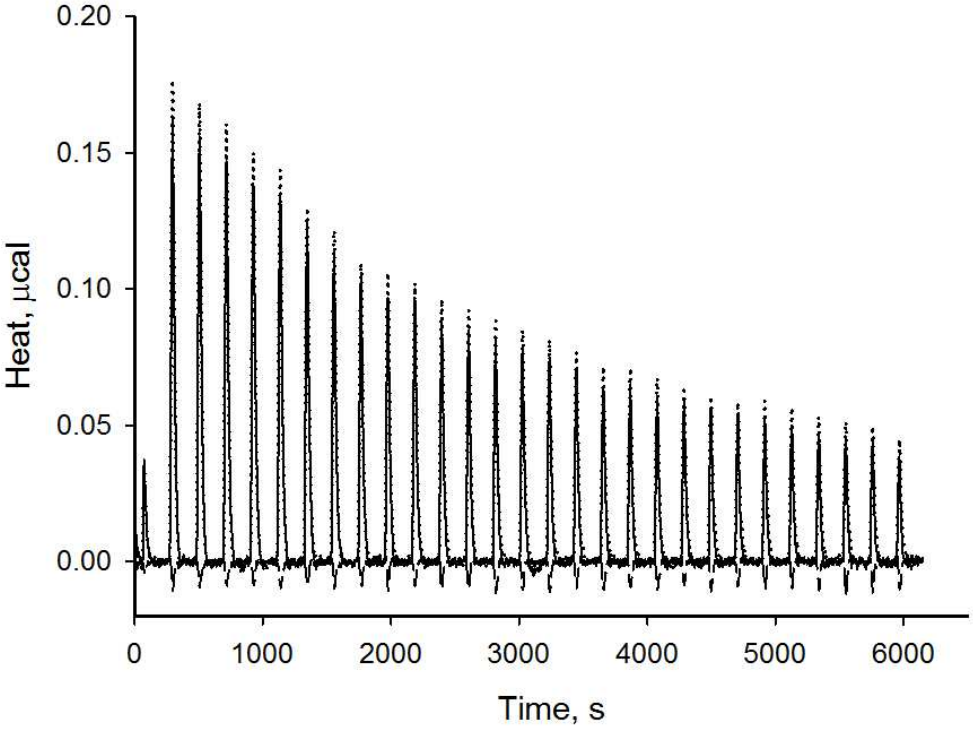


(c)

Figure 4



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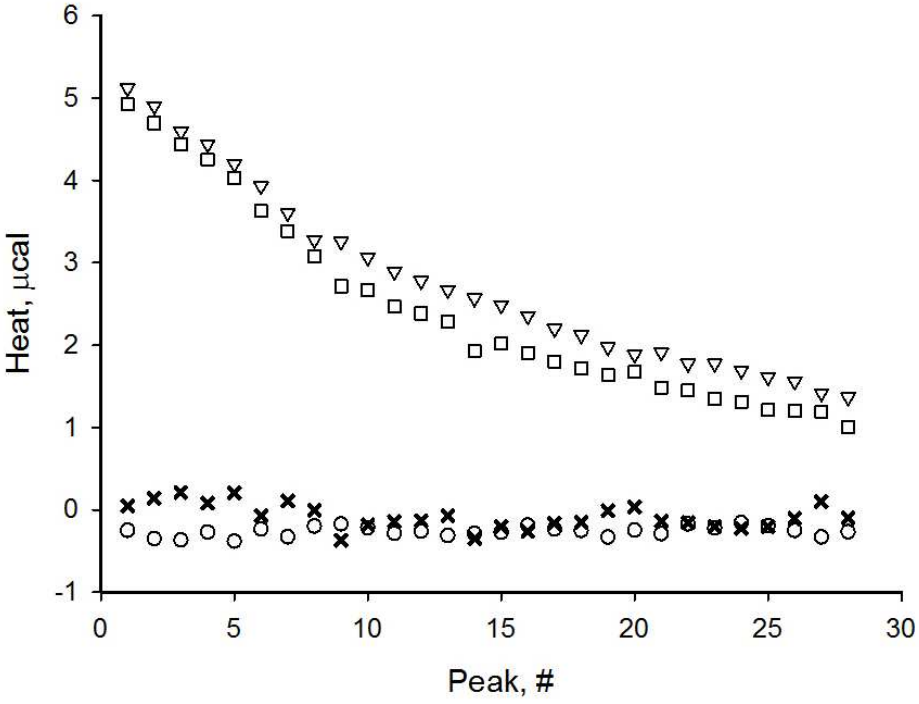

(b)

Figure 5

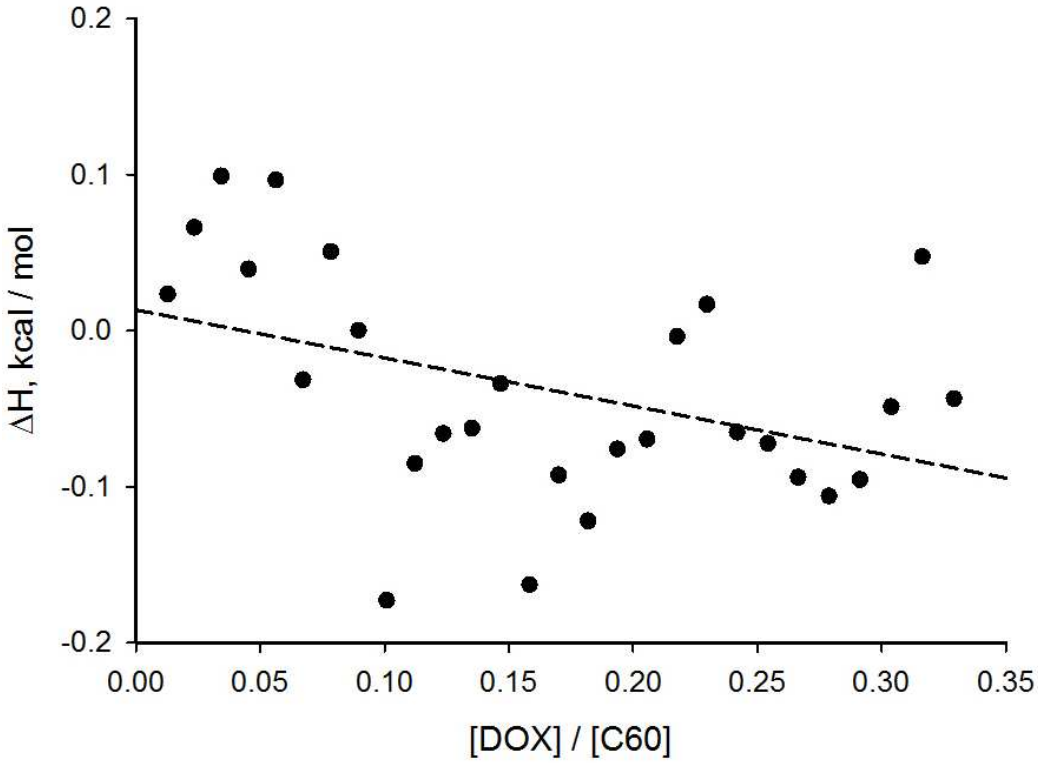

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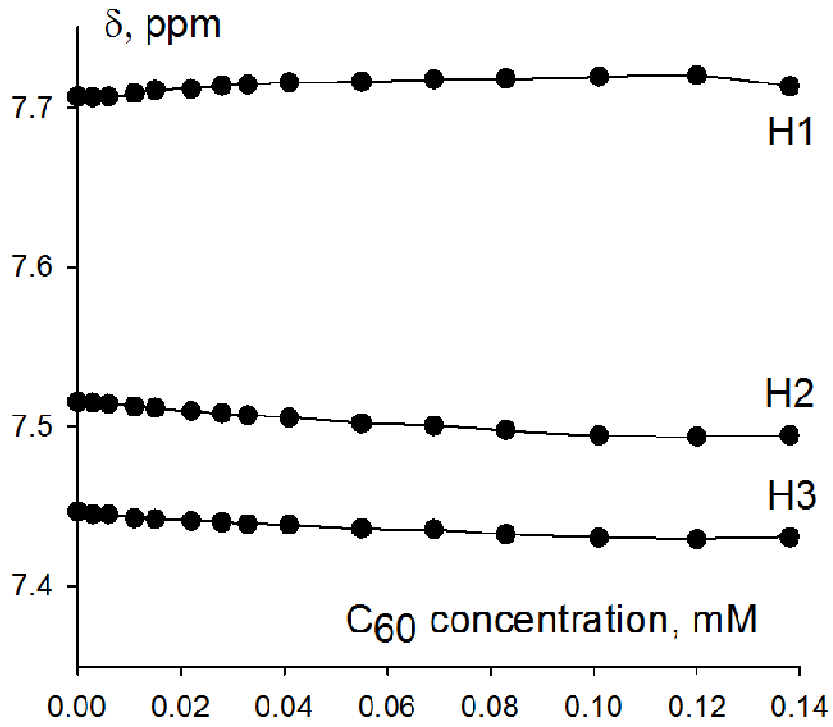
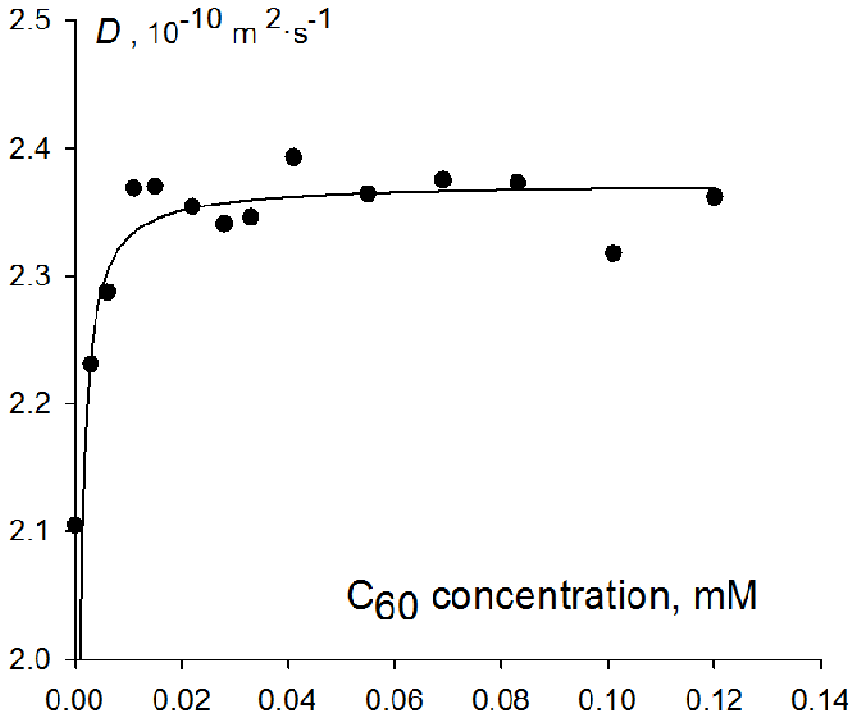
Figure 5



(a)



(b)

Figure 6