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RESEARCH PAPER

Structural organization of C_{60} fullerene, doxorubicin, and their complex in physiological solution as promising antitumor agents

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Abstract Specific features of structural self-organization of C_{60} fullerene (1 nm size range), antitumor antibiotic doxorubicin (Dox) and their complex in physiological solution (0.9 % NaCl) have been investigated by means of atomic-force microscopy, dynamic light scattering, and small-angle X-ray scattering. Significant ordering of the mixed system, C_{60} + Dox, was observed, suggesting the complexation between these drugs, and giving insight into the mechanism of enhancement of Dox antitumor effect on simultaneous administration with C_{60} fullerene.

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Introduction

A big challenge in the development of modern biotechnology is a targeted use of biocompatible low-toxic nanomaterials for treatment of various diseases including cancer. In particular, of paramount importance now are such elements of medical treatment as early detection, determination of localization of malignant tumors, targeted delivering of drugs to tumor, development of methods of selective therapy etc.

Within the group of currently known antitumor agents the C_{60} fullerene attracts special interest of researches (Cataldo and Da Ros 2008; Montellano et al. 2011; Prylutska et al. 2011) due to the relative simplicity of synthesis, high stability, wide range of available possibilities of chemical modification, and high reduction ability (C_{60} molecule may accept up to six electrons). However the bioavailability of C_{60} fullerene is limited by its intrinsic hydrophobicity leading to poor solubility in polar solvents (Hirsch et al. 2005). The preparation of highly stable pristine C_{60} fullerene aqueous solutions (C_{60}FAS) has been reported by different authors (Deguchi et al. 2001; Andrievsky et al. 2002; Fortner et al. 2005; Prylutskyy

et al. 2014a), which has made a breakthrough in further utilization of C_{60}FAS in biomedical applications. Theoretical computations as well as microscopy and spectroscopy data (Deguchi et al. 2001; Amer et al. 2005; Avdeev et al. 2004; Brant et al. 2005; Prylutskyy et al. 2013, 2014b) have shown that the C_{60}FAS contains both single hydrated C_{60} molecules and sphere-like hydrated clusters (aggregates) with diameters up to 200 nm. Recently, a surface hydroxylation of the C_{60} fullerene nanoparticles was reported by means of FTIR spectroscopy (Prylutskyy et al. 2014a; Labille et al. 2009) which is considered to be the main reason for C_{60} fullerene stabilization in aqueous solution.

Anthracycline antibiotic doxorubicin (Dox, Fig. 1) is one of the most extensively used drug in chemotherapy of cancer.

However, the most significant drawback in practical utilization of this antibiotic is its high cardiotoxicity and relatively low selectivity of biological action which significantly reduces its overall medical effect (Carvalho et al. 2014). Recently, extended physicochemical investigation (Evstigneev et al. 2013, 2014) showed that Dox molecules may bind with C_{60} molecule suggesting that such complexation may affect the biological function of the antibiotic. Indeed, both in vivo (Panchuk et al. 2015; Prylutska et al. 2014) and in vitro (Panchuk et al. 2015; Skamrova et al. 2014) studies have proved this and have shown that immobilization of Dox on C_{60} fullerene reduces side toxic effects of this drug with respect to normal cells and increases its accumulation in target tumor cells. The proposed biological effect of C_{60} + Dox complexation is very similar to the long-known effect of Dox–xanthine complexation resulting in alteration of cytotoxic and/or mutagenic consequences of the antibiotic action in vitro (Traganos et al. 1991; Piosik et al. 2002; Evstigneev 2014). It thus follows that C_{60} fullerene–drug complexation may act as a key step in the process of their synergistic interaction in biological media and the link between C_{60} fullerene–drug complexation and the observed enhancement of antitumor effect of the drug creates a new perspective in combinational anticancer therapy.

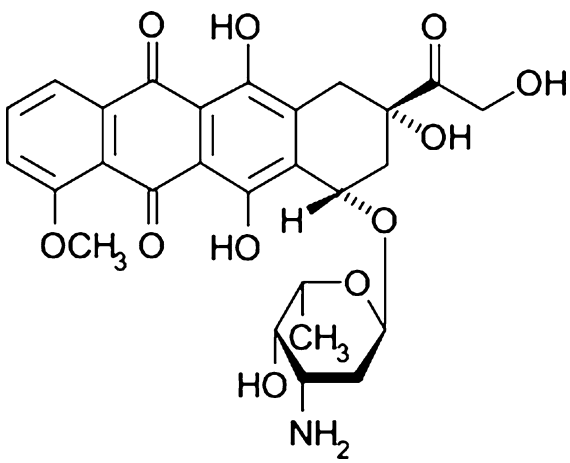

Fig. 1 Chemical structure of doxorubicin

It is important to note that previous physicochemical investigations of the complexation between C_{60} fullerene and Dox cited above have been accomplished in non-salted aqueous environment, thus making ambiguous the projection of the proposed mechanism of complexation onto biological system. Further utilization of C_{60} fullerene in medical nanotechnologies, in particular, evaluation of the in vivo antitumor activity in combination with Dox, requires reliable data on structural state of C_{60} fullerene, Dox, and their complex in solution close by its properties to physiological solution as a common media suitable for direct administration of these drugs in vivo. Such information is also needed for getting insight into the mechanism of specific biological action of these drugs, viz. membranotropic, immunomodulating, radioprotector etc. So, the aim of the present work was the investigation of structural self-organization of C_{60} fullerene, Dox, and their complex in physiological solution (0.9 % NaCl) as effective therapeutic agents against cancer.

Experimental

C_{60}FAS in concentration 0.15 mg/ml was prepared according to the method described in (Prylutskyy et al. 2014a). Homogeneity of the media containing C_{60} fullerene dissolved in physiological solution was achieved by mixing C_{60}FAS and physiological solution in equal volumes (1:1) with further treatment of that mixture in ultrasonic bath (BK-9050, Germany; power 50 W, frequency 40 kHz, mixing time 3 h).

In all experiments the Dox solution ('Doxorubicin-TEVA', Pharmachemie B.V., lyophilized powder, 10 mg) was prepared by means of dissolving the

powder in physiological solution at initial concentration of 0.15 mg/ml.

Immobilization of Dox on C_{60} fullerene was made as follows. Initial stocks of C_{60}FAS and Dox were dissolved in physiological solution and then mixed together under volume ratio 1:2, which was considered to be the most optimal for loading of C_{60} fullerene clusters with Dox (Evstigneev et al. 2013). The resulting mixture was treated in ultrasonic dispergator during 30 min with further mixing within 12 h in magnetic stirrer at room temperature.

Structural state of C_{60} fullerene, Dox, and their complex was monitored with an aid of atomic-force microscopy (AFM). The droplets of physiological solution were deposited onto a cleaved mica substrate (V-1 Grade, SPI Supplies) and the measurements were carried out in dry layers after complete evaporation of the solvent. The sample visualization was carried out in a semi-contact (tapping) mode using NSG10 AFM probes in the 'Solver Pro M' AFM microscope (NT-MDT, Russia).

The size distribution of the particles in physiological solution was estimated by dynamic light scattering (DLS) and small-angle X-ray scattering (SAXS) methods. DLS instrument (Zetasizer Nano ZS90, Malvern, Worcestershire, UK), equipped with a He–Ne laser (max. 5 mW) operating at the wavelength of 633 nm, was used. SAXS experiments were carried out on a Rigaku X-ray instrument with a high-speed Cu rotating anode SMAXS-3000 Point SAXS system (Moscow Institute of Physics and Technology, Dolgoprudniy, Russia) using a standard transmission configuration. An X-ray wavelength of $\lambda = 1.54$ Å was used, resulting in a momentum transfer, Q in the range of 0.007–1 $Å^{-1}$, where $Q = (4\pi/\lambda)\sin(\theta/2)$ and θ is the scattering angle. The samples studied were placed in borosilicon capillaries having 1.5 mm diameter and 0.01 mm wall thickness (W. Muller, Berlin, Germany).

Results and discussion

Preliminary investigation of the possibility of complexation between Dox and C_{60} fullerene in physiological solution was carried out by means of UV–Vis spectroscopy. The absorption spectra of native Dox and C_{60} fullerene with Dox mixture were measured in the range of wavelengths $\lambda = 400$–600 nm at room temperature. The pronounced hypochromic effect observed as a result of such mixing (data not shown) indicates the formation of a stable complex between Dox and C_{60} fullerene similar to that reported before in non-salted aqueous solution (Evstigneev et al. 2013, 2014). This result enabled further detailed investigation of the C_{60} + Dox solution using probe microscopy and light scattering techniques.

The process of water evaporation from the droplet of physiological solution containing C_{60} fullerenes without Dox was monitored by means of optical microscopy. The formation of salt crystals spread over the substrate initially covered by the solution was observed on the mica surface. AFM investigation was performed on the smooth regions of the surface not containing the salt crystals. The islands with characteristic lateral dimension ~ 1 μm were observed in these regions (Fig. 2).

The height of all islands is similar and amounts to 0.8 ± 0.2 nm which well agrees with the diameter of single C_{60} fullerene. It means that the C_{60} molecules are grouped on the mica surface into densely packed monolayer clusters. Besides the C_{60} fullerene islands, the acicular nano-objects having ~ 10 nm height and lateral length up to ~ 1 μm were also seen which may be assigned to NaCl crystals, formed after evaporation of water. It is important to note that in previous AFM investigations of the C_{60}FAS structure (Prylutskyy et al. 2013, 2014a), the formation of the monolayer islands was not observed. Hence, it may be concluded that the formation of the densely packed C_{60} fullerene islands is due to the presence of Na and Cl ions in the studied solution.

A set of papers is currently available in literature dealing with the investigation of C_{60} fullerene aggregation in electrolytes. In particular, in Deguchi et al. (2001) the shift of the C_{60} fullerene absorption peak toward red range of UV–Vis spectrum on the addition of sodium chloride was reported. This process was accompanied by the formation of yellow sediment which indicated coagulation of the C_{60} molecules. Along with that it has long been known that the C_{60} fullerene clusters are negatively charged in aqueous solution (the typical values of ζ-potential are −20 to −50 mV) (Brant et al. 2005). Thus the electrostatic repulsion between C_{60} molecules is thought to be one of the reasons responsible for stabilization of the disperse system as a whole. In contrast, diluted solutions of electrolytes (<0.001 M) induce destabilization of the

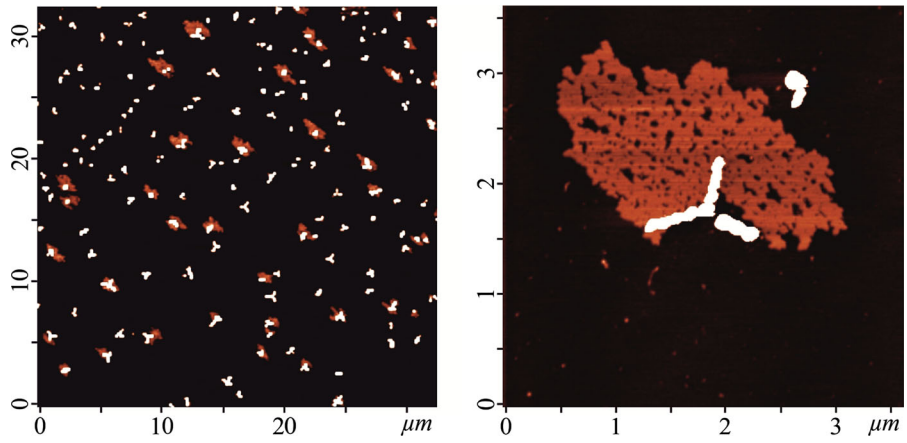

Fig. 2 AFM picture of monolayer clusters of C_{60} fullerene (*brown islands*) and NaCl crystals (*white acicular nanostructures*) which are formed on the mica surface from the mixture of C_{60}FAS (0.15 mg/ml) with physiological solution (0.9 % NaCl) (volume ratio as 1:1). (Color figure online)

C_{60} fullerene suspensions (Deguchi et al. 2001). The observed gathering of C_{60} molecules into islands in the presence of the ions is obviously a consequence of the decreasing of electrostatic repulsion due to shielding effect, also confirmed by the increase in the negative value of ζ-potential on lowering the ionic strength of C_{60}FAS (Chen et al. 2009). As a result the attractive non-electrostatic forces (van der Waals and hydrophobic) facilitate the formation of the densely packed monolayer islands of C_{60} molecules during the process of solvent evaporation.

The obtained results also suggest that C_{60} molecules influence the growth of the crystals of salt. In particular, in certain regions of the surface the NaCl crystals, looking like the three-arm 'stars', are seen (Fig. 2). The height of such crystals equals approximately to 8–30 nm and the arm lengths reach ~1 μm. This observation points out the changing of the surface free energy of the NaCl crystals in solution under the influence of the closely located C_{60} molecules. This is confirmed by the fact that the NaCl nanocrystals are either attached to the C_{60} fullerene islands or fully surrounded by the C_{60} molecules. It may be assumed that the negatively charged C_{60} molecules or their clusters in solution are bound or surrounded by Na^+ ions. Reassociation of the Na^+ and Cl^- ions on water evaporation is accompanied by liberation of the C_{60} fullerenes which can then form islands located in close proximity to the salt crystals.

On water evaporation from the Dox solution and from the mixture of Dox with C_{60} fullerene containing NaCl, a non-homogeneous distribution of the precipitated material on the mica surface was observed, which is similar to that in the case of investigation of the physiological solution containing C_{60} molecules. NaCl crystals are localized in the 'salt' spot which was clearly seen in optical microscope. The area of the spot occupies ~50 % of the whole surface area initially covered by the solution. It was established that the main fraction of NaCl and the studied compounds, i.e., Dox and C_{60} fullerene, is localized within the 'salt' spot (the region of high concentration).

In the region of high concentration, Dox molecules form ordered long-chain branched nanostructures having the height of 6–20 nm (Fig. 3, region I). In the vicinity of Dox, one can notice the nanocrystals of salt from physiological solution which are seen on the picture as white points (Fig. 3, region II). The height of these nanocrystals is approximately ~35 nm.

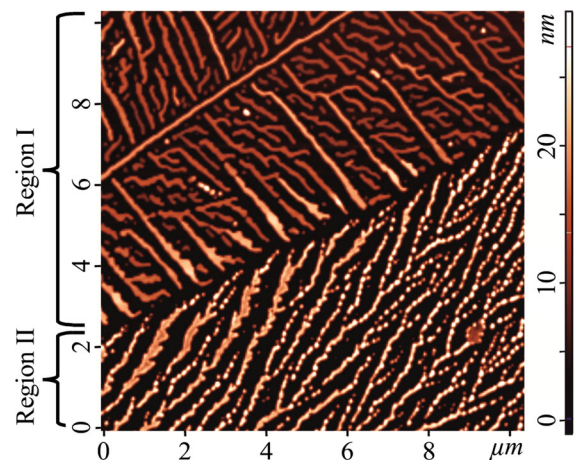

Fig. 3 AFM picture of the Dox layer (0.15 mg/ml) containing NaCl salt crystals on the mica surface (the range of high concentration)

Investigations of the layer of the C_{60} fullerene with Dox mixture showed that in the range of high concentration, its structure is similar to that of the Dox layer (see Figs. 3, 4). It thus may be concluded that the principal influence on the process of formation of the C_{60} fullerene–Dox-containing system is due to the Dox molecules or salt ions which are always present in all studied solutions.

In the region of the surface away of the 'salt' spot (the range of low concentration) the structure of the layer of C_{60} fullerene–Dox-containing system is seen as an island-like structure (Fig. 5). It qualitatively differs from the Dox layer structure (Fig. 3), as well as quantitatively differs from the C_{60} fullerene structure (Fig. 2), which were precipitated from physiological solution in separate. The height of the observed islands is more than 1 nm, indicating the presence of molecular complexes of C_{60} fullerene with Dox in their structure, which well agrees with the available predictions (Evstigneev et al. 2013, 2014; Skamrova et al. 2014).

Once the formation of molecular complexes has been visualized by AFM under condition of solvent evaporation, further confirmation of complex formation directly in physiological solution may be obtained from light scattering techniques.

A typical result of DLS experiment shown in Fig. 6 gives the distribution of the number of light scattering particles according to their mean hydrodynamic diameters in the studied systems. The main fraction of light scattering particles had diameters in the range of 132–33 nm for the mixture of C_{60} fullerene with Dox and for C_{60} fullerene without Dox, respectively, in physiological solution. It is important to note that in the previous DLS investigations of the C_{60}FAS structure in non-salted solution the particles having diameters around 100 nm were reported (Prylutskyy et al. 2013, 2014a). Hence the observed decrease of average dimensions of fullerene clusters may be due to the presence of Na and Cl ions. In contrast, the addition of Dox to C_{60}FAS significantly increases the average cluster size ($\sim$132 nm) which evidences the C_{60}-Dox complexation. Importantly, this result was obtained in physiological solution and fully agrees with the same conclusion reported before in non-salted

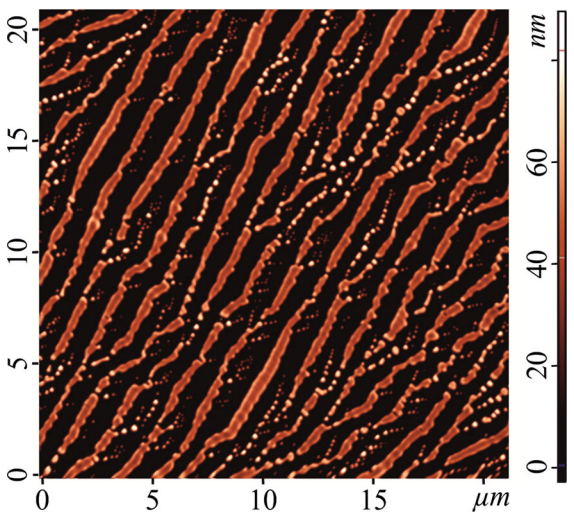

Fig. 4 AFM picture of the layer of the C_{60} fullerene–Dox-containing system (volume ratio as 1:2), containing NaCl salt crystals on the mica surface (the range of high concentration)

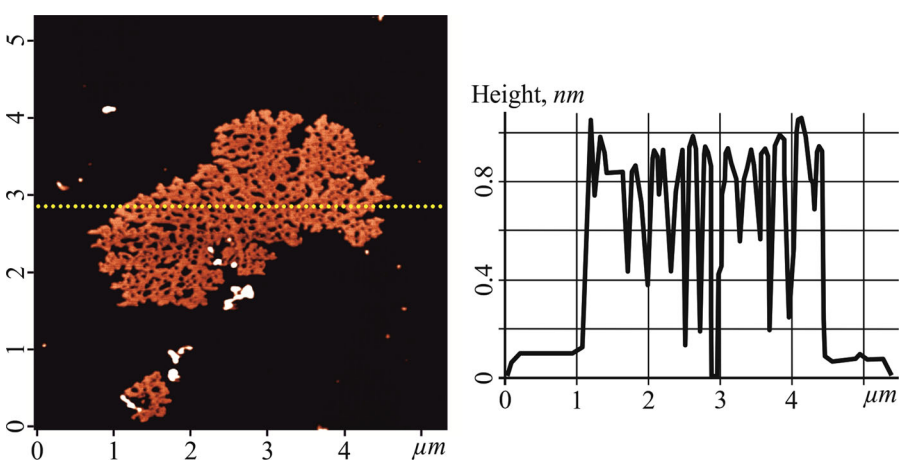

Fig. 5 AFM picture of the C_{60} fullerene–Dox-containing system (volume ratio as 1:2) on the mica surface (the range of low concentration). The figure to the *right* is the Z-cross section associated with the *dashed line* on the figure to the *left*

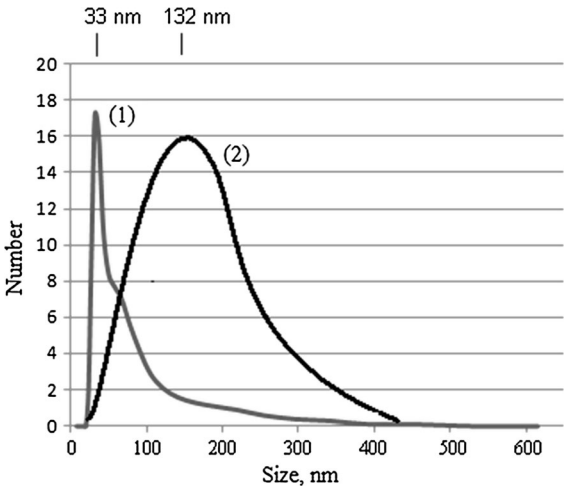

Fig. 6 Distribution of the number of light scattering particles according to their mean diameters: (*1*) C_{60} fullerene in physiological solution (volume ratio as 1:1); (*2*) C_{60} fullerene with Dox in physiological solution (volume ratio as 1:2)

media (Evstigneev et al. 2013, 2014). The complexation most likely occurs via π-stacking of the aromatic moieties of fullerene and Dox molecules (Evstigneev et al. 2013) in a manner similar to well-investigated C_{60} fullerene binding with calixarenes, porphyrins, and other aromatic molecules (Balch et al. 1999; Boyd and Reed 2005).

The AFM and DLS results described above have additionally been tested by means of SAXS technique. The SAXS data (Fig. 7) clearly indicate the existence of particles with a characteristic size <100 nm in the studied

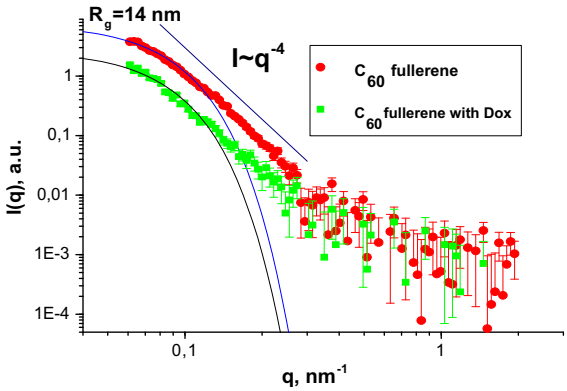

Fig. 7 Experimental SAXS curves for C_{60} fullerene in physiological solution (volume ratio as 1:1) and C_{60} fullerene with Dox in physiological solution (volume ratio as 1:2). The *solid lines* illustrate the calculated scattering

systems. A power law decreasing of the X-ray scattering intensity with a power exponent −4 was revealed. It suggests the spherical shape of the nanoparticles (aggregates). From the SAXS curves one can estimate the gyration radius of the particles in C_{60} fullerene with Dox in physiological solution and C_{60} fullerene in physiological solution, which appeared to be similar in both systems, viz. $R_g \approx 14$ nm. The mean diameter of the spherical nanoparticles can be calculated from the gyration radius and its value was found to be about 36 nm. This value is lower than that obtained from DLS which is quite expected because of different sensitivity of these methods to different sizes (i.e., SAXS is more sensitive to smaller sizes whereas DLS better 'sees' large aggregates).

Taken together, the results of probe microscopy and light scattering presented above evidence the complexation of C_{60} fullerene with Dox in physiological solution. It supports the previously published hypothesis (Evstigneev et al. 2013, 2014) based on the experiment carried out in non-salted aqueous media which states that Dox molecule may induce additional C_{60} fullerene aggregation in physiological stock solution prepared for administration in biosystem. When such mixture is injected into biosystem the C_{60} fullerene clusters incorporate antibiotic molecules and thereby act as nano-containers protecting the antibiotic molecule from binding to plasma proteins and scavenging molecules in biological fluid, and deliver Dox to target cells. Such mechanism resembles the well-described binding of C_{60} fullerene to micelles (Dallavalle et al. 2014) and may explain the observed in vivo and in vitro biological synergism (see "Introduction" section) when the Dox and C_{60} molecules are administered together. On the other hand, it is worth noting that C_{60} fullerene itself exerts cytotoxicity (Aschberger et al. 2010), membranotropic action (Prylutska et al. 2012), and possesses antioxidant properties (Gharbi et al. 2005; Prylutska et al. 2008) which may also be responsible, in part, for the biological interaction of fullerene and Dox. However, at least two indirect evidences support the hypothesis of complexation as the predominant mechanism:

(i) The biological synergism was shown to be the most pronounced specifically when Dox and C_{60}FAS enter the biosystem as a mixture, and is less pronounced when they are administered sequentially (Panchuk et al. 2015),

(ii) The biological synergism of various aromatic molecules (including Dox), originating from their direct non-covalent interaction in biological fluid, has long been known and termed 'the interceptor action' (Traganos et al. 1991; Piosik et al. 2002; Woziwodzka et al. 2013; Evstigneev 2014). The interceptor action suggests that the observed features of drugs' biological interaction should be more dependent on physico-chemical parameters of their interaction (i.e., affinity constants and concentrations) rather than on the chemical properties of each of the interacting molecules (e.g., the ability to generate/absorb reactive oxygen species). In fact this statement has recently received preliminary confirmation due to the existence of correlation of the observed change in biological effect in vitro with the parameters of intermolecular interaction for various aromatic drug molecules (including Dox) in the presence of three potential interceptor molecules, viz. caffeine, chlorophylline, and C_{60} fullerene, (Skamrova et al. 2014; Buchelnikov and Evstigneev 2014).

Although understanding of the mechanism of biological interaction of Dox and C_{60} fullerene requires further investigation, results of the present work, complemented by literature data, point out that this mechanism may be due to their complexation in biological fluid. If so, the complexation of fullerene with other important aromatic antitumor drugs may be expected, e.g., with mitoxantrone, topotecan, actinomycin D etc., and, hence, similar biological interaction may be anticipated. Investigation of this issue is currently under way in our laboratory.

Conclusions

Based on literature data, the complexation of C_{60} fullerene with antitumor antibiotic Dox is currently considered to be the key step in the mechanism of antitumor effect being developed during simultaneous administration of C_{60} and Dox in vivo. Previous physico-chemical investigations of C_{60}–Dox complexation have been performed in non-physiological environment, thus making it difficult to project the obtained results onto biological system.

In the present work the structural self-organization of C_{60} fullerenes, Dox molecules, and their complex has been investigated using probe microscopy and light scattering techniques in physiological solution (0.9 % NaCl). It was found that C_{60} molecules form densely packed monolayer clusters. Such structure was found to differ considerably from the structure of C_{60} fullerene layers, precipitated from water solution. The observed difference originates from shielding of the C_{60} fullerene negative charge by the salt ions. It was also established that within the 'salt' spot (the range of high concentration) the structure of the layer of the C_{60} fullerene–Dox-containing system is similar to the structure of the Dox layer, which consists of ordered long-chain branched nano-objects with inclusions from salt nanocrystals. However, in the region away from the 'salt' spot (the range of low concentration) the structure of the layer of the C_{60} fullerene–Dox-containing system demonstrates an island-like type, which is qualitatively different from the Dox layer structure and is quantitatively different from the structure of C_{60} fullerene precipitated from physiological solution. These results point out on complex formation between C_{60} fullerene and Dox molecules occurring when these compounds present together in physiological solution. SAXS data clearly demonstrate the existence of particles with a characteristic size <100 nm in the studied systems. Along with that the main fraction of nanoparticles has diameters in the range of 36 nm that well agrees with DLS data for C_{60} fullerene in physiological solution.

In general, the obtained data have confirmed the existence of well-pronounced complexation between the fullerene and Dox in solution close by its properties to physiological one, which allows further discussion of the effect of complexation in projection onto biological system. In this context the most important feature of the obtained data is the increase of the average C_{60} fullerene cluster size on addition of Dox in physiological solution, suggesting that these clusters incorporate antibiotic molecules and, presumably, act as nano-containers protecting the antibiotic molecule from binding to plasma proteins and scavenging molecules in biological fluid. This result is in agreement with literature data on C_{60} fullerene complexation with Dox performed in non-salted media, and supports the previously suggested mechanism of C_{60}–Dox complexation as the key step in biological synergistic interaction of fullerene and Dox

on their simultaneous administration in biosystem. It may therefore act as a starting hypothesis in any evaluation of the in vivo and in vitro biological synergism when aromatic drugs and C_{60} molecules are administered together.

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