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Determination of equilibrium constant of C₆₀ fullerene binding with drug molecules

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Abstract

We report a new analytical method that allows the determination of the magnitude of the equilibrium constant of complexation, K_h , of small molecules to C₆₀ fullerene in aqueous solution. The developed method is based on the up-scaled model of C₆₀ fullerene-ligand complexation and contains the full set of equations needed to fit titration datasets arising from different experimental methods (UV-Vis spectroscopy, ¹H NMR spectroscopy, diffusion ordered NMR spectroscopy, DLS). The up-scaled model takes into consideration the specificity of C₆₀ fullerene aggregation in aqueous solution and allows the highly dispersed nature of C₆₀ fullerene cluster distribution to be accounted for. It also takes into consideration the complexity of fullerene-ligand dynamic equilibrium in solution, formed by various types of self- and hetero-complexes. These features make the suggested method superior to standard Langmuir-type analysis, the approach used to date for obtaining quantitative information on ligand binding with different nanoparticles.

Introduction

C₆₀ fullerene is now one of the most interesting and well-known allotropic forms of carbon particularly through its association with a large range of applications at the interface of biology, chemistry and physics [1-4]. A particular issue associated with C₆₀ fullerene is its ability to non-covalently bind various biologically active compounds (ligands) associated with the extended π -electron system of the C₆₀ fullerene surface. This arises as a consequence of strong π -stacking interactions with other small molecules in aqueous solution [5]. The effect of complexation had mainly been investigated in terms of the formation of supramolecular C₆₀ fullerene-ligand ordered structures [6,7], host-guest phenomena [8,9] or C₆₀ fullerene binding with micelles [10,11]. However, during the past five years the complexation of pristine C₆₀ fullerene with small drug molecules in aqueous solution has become a major focus of scientific investigation. In part, this is due to recent successes in combining co-administered C₆₀ fullerene with antitumor drugs, resulting in improvement of medico-biological effects both *in vivo* and *in vitro* [12-15].

The dynamic equilibrium in solution, containing the mixture of C₆₀ fullerenes and ligand molecules able to non-covalently bind with fullerene, in its simplest case may be quantified using two principal physico-chemical parameters, viz. the equilibrium microscopic binding constant, K_h , characterizing the reaction of ligand-fullerene complexation, and the equilibrium microscopic aggregation constant, K_F , characterizing the fullerene aggregation. Although the

dynamic equilibrium properties of C₆₀ fullerene aqueous solutions appear to be complex and involve two stages of C₆₀ fullerene cluster formation, viz. fast (diffusion-limited) and slow (reaction-limited) modes [16,17], it is considered that within short experiment times (i.e. the fast mode), the C₆₀ fullerene aqueous solution may be considered to exist in an equilibrium state. A few attempts to measure K_h in non-aqueous systems are available in the literature (see [5] for a recent review). However, to the best of our knowledge the first experimental determination of K_h for various ligands in water was reported in [18,19] using light absorption spectrophotometry. Two major difficulties were noted, namely:

- (i) the existence of ‘ligand-induced C₆₀ fullerene aggregation’ resulting in dramatic changes to the titration curve. This effect cannot currently be accounted for resulting in the undesired consequence of being forced to operate with significantly reduced working concentration ranges;
- (ii) the very approximate evaluation of the C₆₀ fullerene aggregation, considering just a limited number of small C₆₀ fullerene clusters.

It is also worth adding that the two difficulties mentioned do not exhaust the problems by which the investigator may be challenged when addressing the quantification of fullerene-ligand interactions. The ligand adsorption into the fullerene clusters apparently results in very complex molecular equilibria comprising various cluster-ligand complexes, which is difficult to evaluate strictly even for relatively simple ligand-ligand hetero-associations (see [20] for a review). It should thus be recognized that the existing approaches do not allow for the evaluation of fullerene-ligand interaction at wholly satisfactory level, and the method suggested in [18,19], although yielding the first measurement of K_h in aqueous solution, gives very approximate values of the equilibrium complexation constants.

In follow-up investigations, the complexation of C₆₀ fullerene with doxorubicin [21, 22] and cisplatin [23] showed that the problems encountered when working with C₆₀ fullerene-ligand systems run much deeper than first realized. It was recognized that the range of classical physico-chemical methods typically utilized for determining binding constants for different molecules, such as ¹H NMR spectroscopy, UV-Vis spectroscopy and isothermal titration calorimetry, appears to be generally useless for quantifying the C₆₀ fullerene-ligand equilibrium. On the other hand, diffusion properties of the interacting molecules had been shown to act as a good measure of the interaction, which has provided a clue for the present study.

In summary, it may be concluded that the existing elaboration of the methodology for determining C₆₀ fullerene-ligand binding constants enables very approximate magnitudes of K_h to be obtained, originating from the lack of accurate complexation models or any clear understanding of what experimental method is best suited for studying such a system. In the

present work we report the development of analytical approaches which enable K_h to be measured for any C₆₀ fullerene-ligand complexation in aqueous solution, which accounts for the specificity of C₆₀ fullerene cluster formation.

Materials and Methods

The highly stable purified C₆₀ fullerene aqueous colloid solution (C₆₀FAS, concentration 0.15 mg/ml) was prepared according to the method reviewed in [24] and based on the technology of transferring C₆₀ molecules from toluene to an aqueous phase (heavy water) with the help of ultrasonic treatment. Full characterization of the morphology of the resultant C₆₀FAS is given in [24, 25].

Doxorubicin, ethidium bromide and Hoechst33256 (Sigma, Germany, Fig.1) were dissolved in C₆₀FAS at concentrations of 2 mM. Titration of the prepared solution was accomplished from a stock solution of drug prepared at the same concentration of 2 mM in heavy water. This procedure was used in order to maintain the concentration of drug and vary the C₆₀ fullerene concentration.

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 ¹H resonance frequency of 600.13 MHz and working under TopSpin version 2.1 (Bruker Biospin, Rheinstetten, Germany).

1D ¹H 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 chemical shift timescale at $T=298$ K. Chemical shifts were measured relative to an internal reference standard 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, g , was varied linearly in the range $2 \leq g \leq 95\%$ 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 and Discussion

As discussed in the introductory section the principal difficulty of determining the magnitude of the equilibrium constant of ligand complexation with C₆₀ is the need to take into consideration the specific non-linear effects in C₆₀ fullerene-ligand complexes and strong C₆₀ fullerene aggregation. The latter results in highly dispersed solutions with formally indefinite stoichiometry $m:n$ ($m,n \rightarrow \infty$) of the C₆₀ fullerene-ligand complexes. Such systems are typically analyzed in terms of the ligand's adsorption into C₆₀ fullerene clusters using Langmuir and related analyses (see [5] for review) or using one-dimensional hetero-association models (see [20] for review). However, the standard adsorption models do not seem to be appropriate for such highly dispersed systems as C₆₀ fullerene in aqueous solution. For this reason the development of a complexation model that takes into account the specificity of C₆₀ fullerene aggregation is required. The most detailed analysis of complexation accounting for fractal and continuous C₆₀ fullerene aggregations was accomplished in [18]. However, the severe limitation to the number of C₆₀ molecules immobilized in a cluster was introduced. It does not allow large C₆₀ fullerene clusters with dimensions ~100 nm and greater seen in probe microscopy images and dynamic light scattering spectra (DLS) to be taken into consideration [16-18,24,25]. We faced a virtually identical problem in [26] when trying to quantify the C₆₀ fullerene aggregation in aqueous solution in terms of equilibrium aggregation constant K_F . The so-called 'up-scaled aggregation model' was suggested, considering C₆₀FAS as a solution resembling that of micelles whose dimensions are discretely increased layer on layer with increasing solute concentration. The fundamental assumptions behind this model are the spherical shape of the clusters being formed as a result of aggregation and the non-specific nature of monomer binding. As a consequence, based on DLS titration data the value $K_F = 56000 \text{ M}^{-1}$ was obtained which is in a good agreement with the existing theoretical estimations and the results of energetic analyses. In the present work the basic idea behind the 'up-scaled aggregation model' was used for the development of the 'up-scaled C₆₀ fullerene-ligand' complexation model.

The general formulation of any complexation model must contain the set of equations enabling experimental titration data to be fitted by means of adjusting the set of model parameters, of which K_h is the principal search parameter (see [20,27] for review). These equations are (i) the set of mass balance equations, and (ii) the set of equations linking the experimentally observable parameter with the parameters of dynamic equilibrium in aqueous solution (concentrations, equilibrium complexation constants). As set out below, these equations will be derived for the C₆₀ fullerene-ligand complexation, and attested to by experimental data.

Derivation of the mass balance equations of the up-scaled model.

According to the up-scaled model of C₆₀ fullerene aggregation [26] the formation of C₆₀ fullerene clusters is considered as simultaneous binding of R monomeric C₆₀ molecules (termed ‘conglomerate’) to the C₆₀ fullerene core micelles containing M C₆₀ fullerenes, with macroscopic binding constant, K_R . Such binding results in formation of a C₆₀ fullerene layer on the micelle which causes growth of C₆₀ fullerene clusters and eventually leads to the setup of their equilibrium distribution. The titration of the C₆₀ fullerene aqueous solution with the ligand is considered in terms of dilution of the initial C₆₀ fullerene concentration, C_0 ($C_0=0.14$ mM), by a dilution factor, r . Consequently, the initial concentrations of the micelles, C_{M0} , and conglomerates, C_{R0} , are diluted by r .

Let us consider the complexation of the ligand with C₆₀ fullerene clusters as the binding of conglomerate containing D ligands with macroscopic complexation constant, K_H . Hence, the growth of C₆₀ fullerene clusters is determined by the attachment of C₆₀ molecule and ligand layers (Fig.2). In such an approach, the searchable microscopic complexation constant may be defined as $K_h = K_H/D$. It follows that the full system of mass balance equations in the three-component system ‘C₆₀ fullerene clusters, C_M ’ – ‘ligand conglomerates, C_D ’ – ‘C₆₀ fullerene conglomerates, C_R ’ can be written as:

$$\begin{cases} C_0 = MC_{M0} + RC_{R0} \\ \frac{C_{M0}}{r} = \sum_{i=0}^{\infty} \sum_{j=0}^{\infty} C_{M1} (K_R C_{R1})^i (K_H C_{D1})^j \\ \frac{C_{R0}}{r} = C_{R1} + \sum_{i=0}^{\infty} \sum_{j=0}^{\infty} i C_{M1} (K_R C_{R1})^i (K_H C_{D1})^j \\ C_{D0} = C_{D1} D + \sum_{i=0}^{\infty} \sum_{j=0}^{\infty} j \cdot D \cdot C_{M1} (K_R C_{R1})^i (K_H C_{D1})^j \end{cases} \quad (1)$$

where C_{D1} is the concentration of ligand conglomerates non-complexed with C₆₀ fullerene clusters; C_{D0} is the total ligand concentration; C_{R1} and C_{M1} are the concentrations of non-complexed C₆₀ fullerene conglomerates and C₆₀ fullerene core micelles, respectively; indexes i and j denote the number of C₆₀ fullerene and ligand conglomerates, respectively, bound with the micelles containing M C₆₀ molecules.

Let us further execute the procedure of up-scaling by analogy with that performed in [26] by multiplying each term in the first three expressions of eqs.(1) by M . The fourth equation is multiplied by D . The parameters inside the brackets, $(K_R C_{R1})^i$, are multiplied and divided by M . The parameters inside the brackets, $(K_H C_{D1})^j$, are multiplied and divided by D . The

corresponding transformation of eqs.(1) yields

$$\begin{cases} C_0 = C'_{M0} + B \cdot C'_{R0} \\ \frac{C'_{M0}}{r} = \sum_{i=0}^{\infty} \sum_{j=0}^{\infty} C'_{M1} (K'_R C'_{R1})^i (K_h C'_{D1})^j \\ \frac{C'_{R0}}{r} = C'_{R1} + \sum_{i=0}^{\infty} \sum_{j=0}^{\infty} i C'_{M1} (K'_R C'_{R1})^i (K_h C'_{D1})^j \\ C_{D0} = C'_{D1} + \sum_{i=0}^{\infty} \sum_{j=0}^{\infty} j \cdot H \cdot C'_{M1} (K'_R C'_{R1})^i (K_h C'_{D1})^j \end{cases} \quad (2)$$

where

$$B = \frac{R}{M}, \quad K'_R = \frac{K_R}{M}, \quad H = \frac{D}{M}, \quad C'_{M0} = C_{M0} \cdot M, \quad C'_{R0} = C_{R0} \cdot M, \quad C'_{M1} = C_{M1} \cdot M, \quad C'_{R1} = C_{R1} \cdot M, \\ C'_{D1} = C_{D1} \cdot D, \quad K'_h = \frac{K_H}{D}.$$

Evaluation of the sums in eqs.(2) gives

$$\begin{cases} C_0 = C'_{M0} + B \cdot C'_{R0} \\ \frac{C'_{M0}}{r} = \frac{C'_{M1}}{(1 - K'_R C'_{R1})(1 - K_h C'_{D1})} \\ \frac{C'_{R0}}{r} = C'_{R1} + \frac{C'_{M1} K'_R C'_{R1}}{(1 - K'_R C'_{R1})^2 (1 - K_h C'_{D1})} \\ C_{D0} = C'_{D1} + \frac{C'_{M1} H K_h C'_{D1}}{(1 - K'_R C'_{R1})(1 - K_h C'_{D1})^2} \end{cases} \quad (3)$$

Re-arranging the first and second equations in eqs.(3) with respect to C'_{R0} and C'_{M1} yields

$$\begin{cases} \frac{C_0 - C'_{M0}}{rB} = C'_{R1} + \frac{K'_R C'_{R1} C'_{M0}}{r(1 - K'_R C'_{R1})} \\ C_{D0} = C'_{D1} + \frac{K_h H C'_{D1} C'_{M0}}{r(1 - K_h C'_{D1})} \end{cases} \quad (4)$$

Further solution of eqs.(4) results in the full set of monomer concentrations

$$\begin{cases} C'_{R1} = \frac{r + C'_{M0} B K'_R + (C_0 - C'_{M0}) K'_R - \sqrt{[r + C'_{M0} B K'_R + (C_0 - C'_{M0}) K'_R]^2 - 4r K'_R (C_0 - C'_{M0})}}{2r B K'_R} \\ C'_{D1} = \frac{r + C'_{M0} H K_h + C_{D0} K_h r - \sqrt{[r + C'_{M0} H K_h + C_{D0} K_h r]^2 - 4r^2 K_h C_{D0}}}{2r K_h} \\ C'_{M1} = \frac{C'_{M0}}{r} (1 - K'_R C'_{R1})(1 - K_h C'_{D1}) \end{cases} \quad (5)$$

where $K'_R=56000 \text{ M}^{-1}$, $B=0.914$, $C_0=0.14 \text{ mM}$, $C'_{M0} = 0.009 \text{ mM}$ are known from previous studies of C_{60} fullerene aggregation by means of the up-scaled model. Here, two quantities remain as

unknowns, viz. H and K_h , which may act as adjustable parameters during the fitting of experimental data. As long as H was introduced in eq.(2) as a ratio of ligand number in ligand conglomerates, D , to fullerene number in micelles, M , this parameter may thus be associated with stoichiometry of the fullerene-ligand binding reaction.

The specificity of the first expression in eqs.(5) is that it is identical to the same equation derived in [26] with respect to the C_{60} fullerene aggregation in the absence of the ligand. This is quite expected as in the complexation model developed, the binding of the C_{60} fullerene conglomerates (R) does not depend on the binding of the ligand conglomerates (D), i.e. the process of complexation does not affect the process of aggregation.

Eqs.(5) represent the central point of the up-scaled model of C_{60} fullerene-ligand complexation. Further analysis will be devoted to obtaining equations describing the experimental observable for various experimental methods which can be utilized for the measurement of the complexation constant.

Application to UV-Vis spectrophotometry.

The observed absorption of the ligand in the visible range of the electromagnetic spectrum (where the overlap of the C_{60} fullerene and the ligand spectra is minimal) in UV-Vis experiments is written as

$$A = (\varepsilon_m - \varepsilon_h) C'_{D1} + \varepsilon_h C_{D0} \quad (6)$$

where the extinction coefficient in the C_{60} fullerene-ligand complex, ε_h , goes as the third adjustable parameter in the up-scaled model, i.e. $[K_h, H, \varepsilon_h]$; ε_m is the extinction coefficient of the ligand in a monomer state, commonly known from the literature or it may be measured at infinite ligand dilution.

Application to 1H NMR experiment.

The observed 1H NMR proton chemical shift must include the possibility of ligand self-association at high concentrations typically used in NMR (\sim mM). In the most general case of indefinite self-association with equilibrium constant K_D [28] the chemical shift measured for the C_{60} fullerene-ligand complexation is given as

$$\delta = \frac{C'_{D1}}{C_{D0}(1 - K_D C'_{D1})} \left[2\delta_m - 2\delta_d + \frac{2\delta_d - \delta_m}{1 - K_D C'_{D1}} \right] + \delta_h \left[1 - \frac{C'_{D1}}{C_{D0}(1 - K_D C'_{D1})^2} \right] \quad (7)$$

where the chemical shift in the C_{60} fullerene-ligand complex, δ_h , goes as the third adjustable parameter in the up-scaled model, i.e. $[K_h, H, \delta_h]$; δ_m is the ligand proton chemical shift in monomer state, commonly known from the literature or it may be measured at infinite ligand

dilution. The first term in eq.(7) stands for the ligand self-association, and the second term represents the C₆₀ fullerene-ligand binding.

Application to DLS experiment.

The light scattering intensity recorded in typical DLS experiments is recalculated into the hydrodynamic diameter, d_z , of light scattering particles, and is determined by the square of molecular weight of the C₆₀ fullerene clusters which had adsorbed the ligand. According to the up-scaled model these clusters comprise the core fullerene micelle, i ligand conglomerates and j C₆₀ fullerene conglomerates attached to it

$$\frac{1}{d_z} = \frac{\sum_{i=0}^{\infty} \sum_{j=0}^{\infty} (M + iR + jD)^2 f_{ij} \frac{1}{d_{ij}}}{\sum_{i=0}^{\infty} \sum_{j=0}^{\infty} (M + iR + jD)^2 f_{ij}} \quad (8)$$

where $f_{ij} = C'_{M1} (K_F B C'_{R1})^i (K_h C'_{D1})^j$ is the mole fraction of the C₆₀ fullerene-ligand clusters in aqueous solution; $d_{ij} = d_1 \cdot \sqrt[3]{M + iR + jD} = d_0 \cdot \sqrt[3]{1 + i \cdot B + j \cdot H}$ is the diameter of cluster containing i and j layers. Here it is assumed that the hydrodynamic dimensions of the ligand and C₆₀ fullerene are commensurable and equal to d_1 ; d_0 is the diameter of the core micelle equal to 280 nm [26].

Taking into account the ‘interaction effect’ originating from the long-range interaction of C₆₀ fullerene clusters with each other and dramatically affecting their diffusion properties (see [26] for more detail), the following equation should be written

$$d_z = \frac{d_0}{1 + A' \frac{C'_{M0}}{r}} \cdot \frac{\sum_{i=0}^{\infty} \sum_{j=0}^{\infty} (1 + i \cdot B + j \cdot H)^2 f_{ij}}{\sum_{i=0}^{\infty} \sum_{j=0}^{\infty} (1 + i \cdot B + j \cdot H)^{5/3} f_{ij}} \quad (9)$$

where A' is given as the second virial coefficient characterizing the cluster-cluster long-range interaction [26], which may depend on the charge of the ligand. This model contains three adjustable parameters, i.e. [K_h, H, A'].

Application to diffusion ordered NMR spectroscopy.

A powerful alternative to DLS is diffusion ordered NMR spectroscopy (DOSY) allowing measurement of the translational diffusion coefficient, D_z . The up-scaled model for this type of experiment is similar to DLS, although the weighing of the diffusion coefficient (or the hydrodynamic diameter as in DLS) ($D_{ij} = \frac{kT}{3\pi\eta d_{ij}}$) must be accomplished against the first power

of molecular mass

$$D_z = kT \cdot \frac{1 + A' \frac{C'_{M0}}{r}}{3\pi\eta d_0} \cdot \frac{\sum_{i=0}^{\infty} \sum_{j=0}^{\infty} (1 + i \cdot B + j \cdot H)^{2/3} f_{ij}}{\sum_{i=0}^{\infty} \sum_{j=0}^{\infty} (1 + i \cdot B + j \cdot H) f_{ij}} \quad (10)$$

where η is the solvent viscosity; k is the Boltzmann constant; T is the absolute temperature; d_0 is the diameter of the solution component used to acquire NMR magnetization decay, which in case of DOSY is the characteristic ligand dimension; A' is the second virial coefficient [26]; $f_{ij} = C'_{M1} (K_F B C'_{R1})^i (K_h C'_{D1})^j$. This model formally contains three adjustable parameters, i.e. [K_h, H, A'] although d_0 could also be taken as an adjustable quantity.

Data analysis in the up-scaled model.

The set of search binding parameters in the up-scaled model, specific to each experimental method and described above, can be determined by means of fitting the experimental titration curves given as a dependence of the experimental observable on concentration. The fitting may be accomplished by standard computer procedures, implemented in mathematical software such as Matlab, Mathematica or MathCAD, which minimize a sum of square deviations of the experimental and theoretically calculated [eqs.(6),(7),(9),(10)] observable at each concentration point (i.e. the discrepancy). Thus the discrepancy may be used as a measure of goodness of fit. In the present work the fitting procedure was carried out in Matlab.

Experimental study of C₆₀ fullerene-drug complexation.

In order to test the up-scaled model developed in the present work we used the set of aromatic drug molecules representing a good model system to investigate the mechanism of C₆₀ fullerene-drug interaction [12-15,18-22]: doxorubicin (DOX), methylene blue (MB), proflavine (PF), ethidium bromide (EB) and Hoechst33258 (HT) (Fig.1). All of these drugs absorb in the visible range of the electromagnetic spectrum suggesting that UV-Vis spectrophotometry is appropriate for studying the complexation with C₆₀ fullerene (due to minimal spectral overlap between the C₆₀ and drug molecules), whereas the standard DLS method will not be appropriate for such analysis (due to overlap of the wavelengths of the incident laser beam and absorption maximum).

The measurement of the UV-Vis experimental titration curve for DOX, PF and MB drugs was accomplished in [18] and outlined in Fig.3. The curves are differential (i.e. obtained as a difference of the absorptions in C₆₀ fullerene-drug and C₆₀FAS) and are characterized by initial

decrease of the optical density (due to direct drug binding to C₆₀ molecules), replaced by further increase (due to additional scattering from ‘ligand-induced C₆₀ fullerene aggregation’, see Fig.4b in [18]). The observed changes reflect two distinct processes, of which the latter is cooperative. Full account of both processes within a single model is difficult. Hence, the titration range was reduced in [18] and the computation of the binding parameters accomplished. In the present work the same reduced dataset was fitted by means of the up-scaled model (eqs.(5)-(6)). The resultant parameters given in Table 1 evidence a good quality of fit and correlation of the parameters derived from the simplified model [18] and from the accurate up-scaled model developed here.

Further analysis was performed with respect to ¹H NMR (Fig.4) and DOSY NMR (Fig.5) titration data for DOX, EB and HT drugs binding with C₆₀ fullerene. It is seen from Fig.4 that the changes in proton chemical shifts with concentration of C₆₀ fullerene are relatively small (~0.01...0.04 ppm) as compared with the error of reproducibility of the NMR experiment. This result was also noted in [21] and explained by increased distance between the C₆₀ fullerene and drug surfaces in the complex thereby minimizing the effective magnetic shielding of the drugs’ protons. This indicates that the use of ¹H NMR titration data for aromatic drugs binding to C₆₀ fullerene is inappropriate. In contrast, the DOSY curves (Fig.5) demonstrate quite strong concentration dependence. Moreover, the trend is different (i.e. decreasing or increasing). Previously the unexpected shape of the diffusion curves was noted for DOX binding with C₆₀ fullerene [21] and explained by the ‘interaction effect’ discussed above with respect to the DLS (eq.(9)) and DOSY (eq.(10)) models. The decrease/increase of the curves observed in Fig.5 appears to be also the consequence of the interplay between the ‘interaction effect’ (leading to increase of D_z) and the drug’s adsorption into C₆₀ fullerene clusters (leading to a decrease of D_z). Fitting of the data in Fig.5 by means of eqs.(5),(10) results in the set of binding parameters outlined in Table 2. The discrepancy between the theoretical model and experimental data (~10⁻³) is good enough to indicate the appropriateness of the up-scaled model (corresponding to the goodness of fit $R^2 \approx 0.95$). The magnitudes of K_h weakly depend on the type of ligand evidencing non-specific adsorption of the drugs into the C₆₀ fullerene clusters. This is expected because the drugs have similar dimensions and electrostatic charge states (bearing positive charges under neutral solution conditions). Similar conclusions may be drawn with respect to the magnitude of parameter H , which is weakly dependent upon the type of ligand and reflects the ratio of the number of particles in the ligand conglomerates and core micelle (eq.(2)). The ability to get reliable K_h values for the C₆₀ fullerene-drug systems, demonstrated here, without any severe restrictions to conditions of experiment or model assumptions is the principal value of the developed up-scaled model, which thereby makes it superior to standard Langmuir-type

adsorption analyses.

A final note should be given with respect to the magnitude of the virial coefficient, A' , characterizing the cluster-cluster long-range interaction. In [26] the value of A' for the C_{60} fullerene aggregation in aqueous solution was obtained as $A' \approx 2 \cdot 10^6 \text{ M}^{-1}$, whereas in the present work this value for the C_{60} fullerene-drug complexations became negative (see Table 2). This result may be explained by intrinsic repulsion of negatively charged C_{60} fullerene clusters in aqueous solution (giving a positive A' value in the case of aggregation without the ligand) and slight attraction of the clusters containing positively charged drug molecules (giving a negative A' value in the case of drugs complexing with C_{60} fullerene clusters). These conclusions are supported by the available ξ -potential measurements for C_{60} FAS without the drug and for the mixture of DOX with C_{60} FAS [21], i.e. the C_{60} fullerene clusters are characterized by deep negative values of ξ -potential, whereas the addition of positively-charged DOX to C_{60} FAS causes a significant shift of ξ -potential to small positive values. However, the A' parameter should not totally be related to the electrostatic charge of the C_{60} fullerene-drug clusters. Large particles in aqueous solution, even being electrically neutral, also interact via long-range van der Waals interactions, formed by summation of the standard $1/r^6$ attractive London potential [29]. It follows that the up-scaled model developed in the present work, in addition to measurement of K_h , may also provide unique information on the long-range cluster-cluster interaction.

Conclusions

In the present work we report a new analytical method enabling the determination of the magnitude of the equilibrium constant of complexation, K_h , of small molecules binding to C_{60} fullerene in aqueous solution. The developed method is based on the up-scaled model of C_{60} fullerene-ligand complexation and contains the full set of equations needed to fit titration dataset data for different experimental methods (UV-Vis spectroscopy, ^1H NMR spectroscopy, diffusion ordered NMR spectroscopy, DLS). The up-scaled model takes into consideration the specificity of C_{60} fullerene aggregation in aqueous solution allowing the highly dispersed nature of C_{60} fullerene cluster distribution to be accounted for. This feature makes the suggested method superior to standard Langmuir-type analysis, a method used to date for obtaining quantitative information on ligand binding with different nanoparticles.

It is suggested that measurement of the translational diffusion coefficient by means of diffusion-ordered NMR spectroscopy is probably the most suited method for measuring K_h for C_{60} fullerene-ligand systems. Many common methods such as ^1H NMR, isothermal titration calorimetry, UV-Vis and DLS do not sense interaction or involve additional severe restrictions to

the analytical procedure, thus lowering the reliability of analysis. In contrast, the DOSY method senses the translational diffusion coefficient of the drug, which is determined by interplay of two major processes, viz. the ligand adsorption into C₆₀ fullerene clusters and the cluster-cluster long-range interaction. Both of these features have been incorporated into the up-scaled model. In addition, from the value of the second virial coefficient, A' , the method provides uniquely quantitative information on the long-range cluster-cluster interaction, exerting correlation with the standard ξ -potential of nanoparticles measured in aqueous solution.

In conclusion it should be noted that one of the principal difficulties in quantifying the fullerene-ligand binding process is accounting for the non-linear cooperative processes (such as the ligand-induced fullerene aggregation), which were not considered in the present work, but is thought to be possible within the up-scaled model, and will be a matter for further studies.

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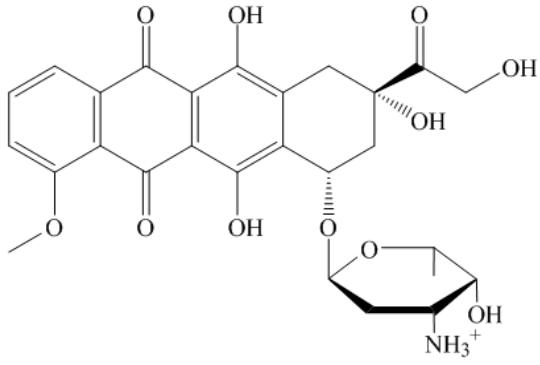
Table 1. The calculated parameters of the C₆₀ fullerene-drug complexation derived from fitting of the reduced UV-Vis titration data

Adjustable parameter	C₆₀-DOX	C₆₀-MB	C₆₀-PF
<i>H</i>	3	3	2
<i>K_h</i> , M ⁻¹	21000 (46000)	8300 (7300)	9500 (12800)
discrepancy	8.2·10 ⁻³	6.8·10 ⁻³	9.0·10 ⁻³

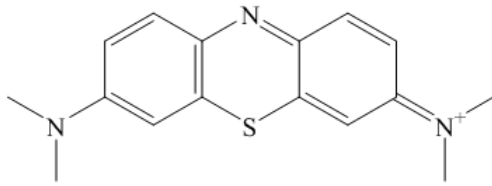
Note: the numbers in brackets represent the binding parameters computed by simplified complexation model in [18]

Table 2. The calculated parameters of the C₆₀ fullerene-drug complexation derived from fitting of the DOSY NMR titration data by means of the up-scaled model

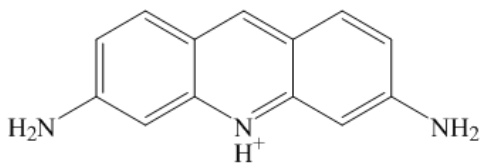
Adjustable parameter	C₆₀-DOX	C₆₀-EB	C₆₀-HT
<i>H</i>	2.75	2.90	1.65
<i>K_h</i> , M ⁻¹	500	516	490
<i>A'</i> , M ⁻¹	-16000	-16000	-14500
discrepancy	6.7·10 ⁻³	2.8·10 ⁻³	1.1·10 ⁻³



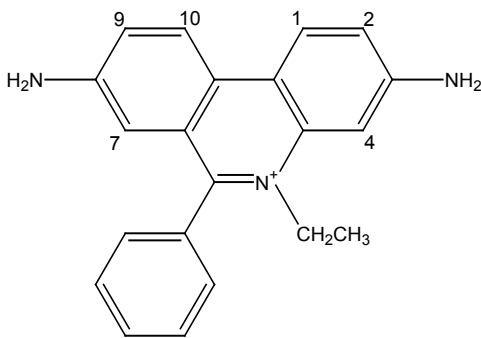
Doxorubicin



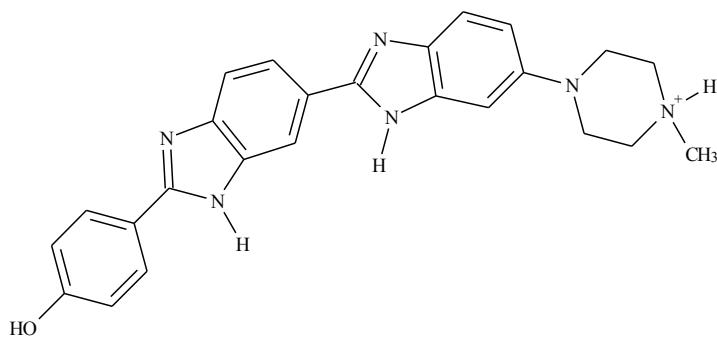
Methylene Blue



Proflavine



Ethidium bromide



Hoechst33258

Figure 1 Structures of the studied drug molecules

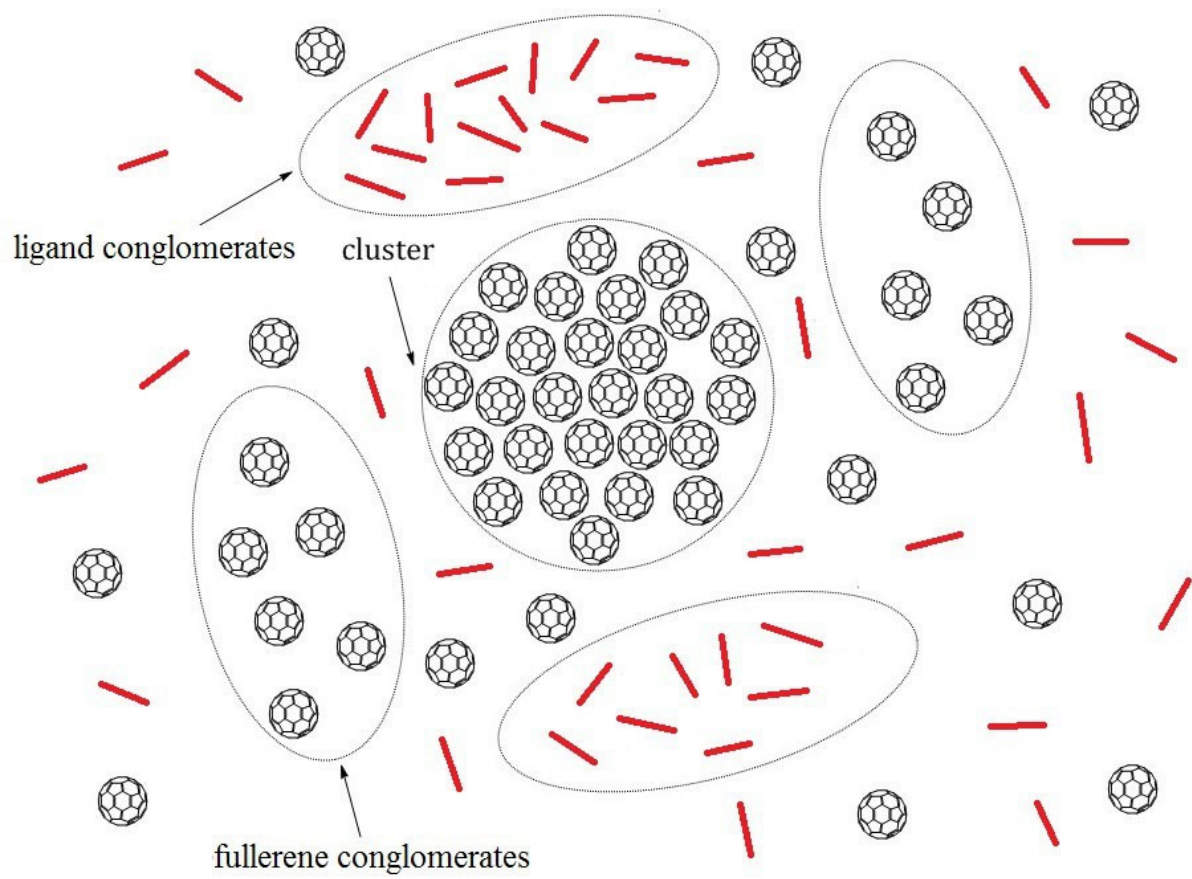


Figure 2 Schematic representation of the complexation process in the up-scaled model

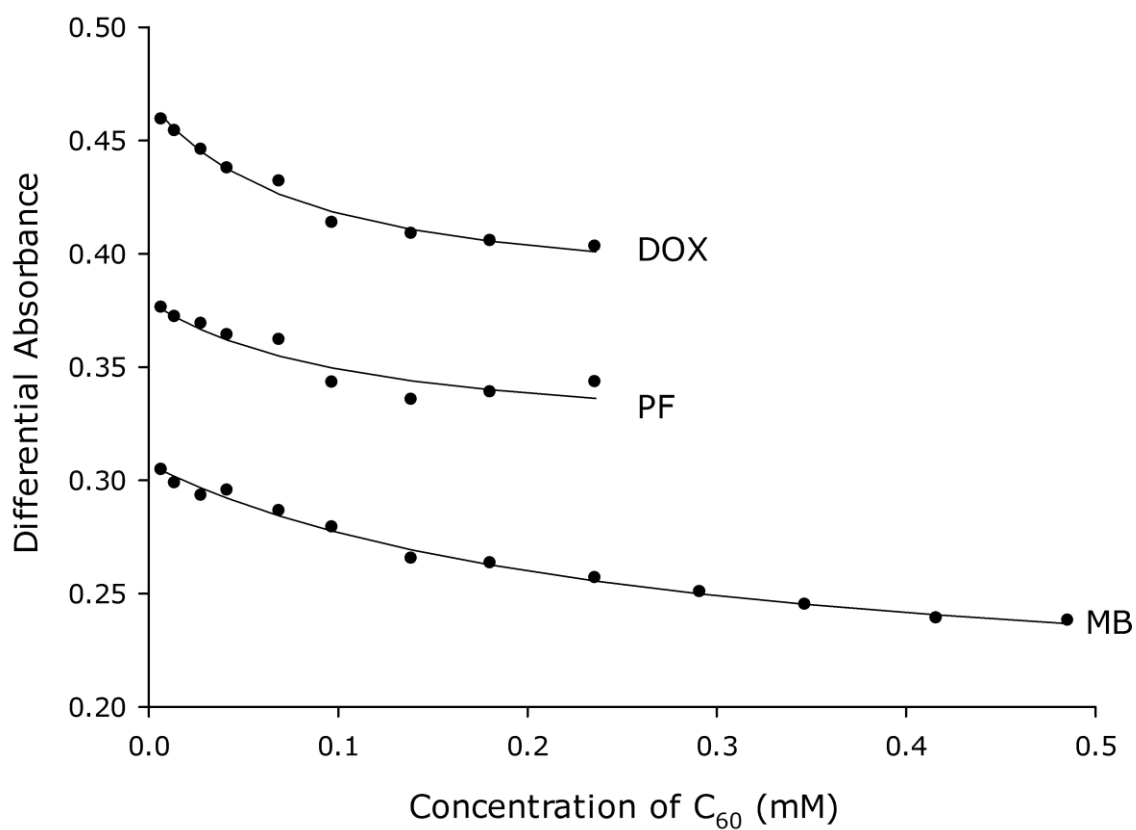
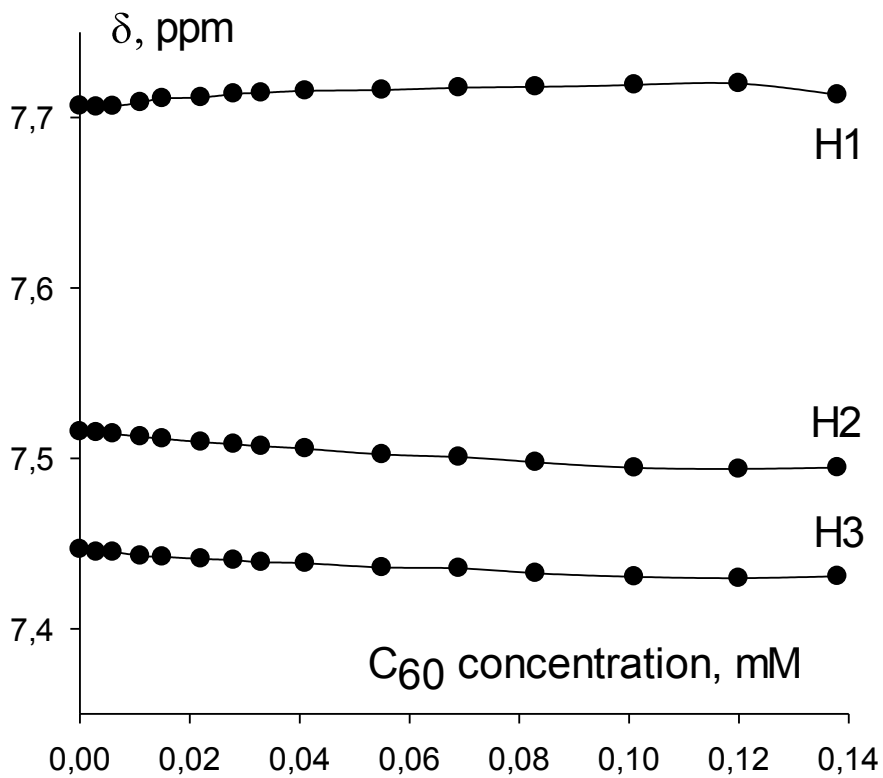
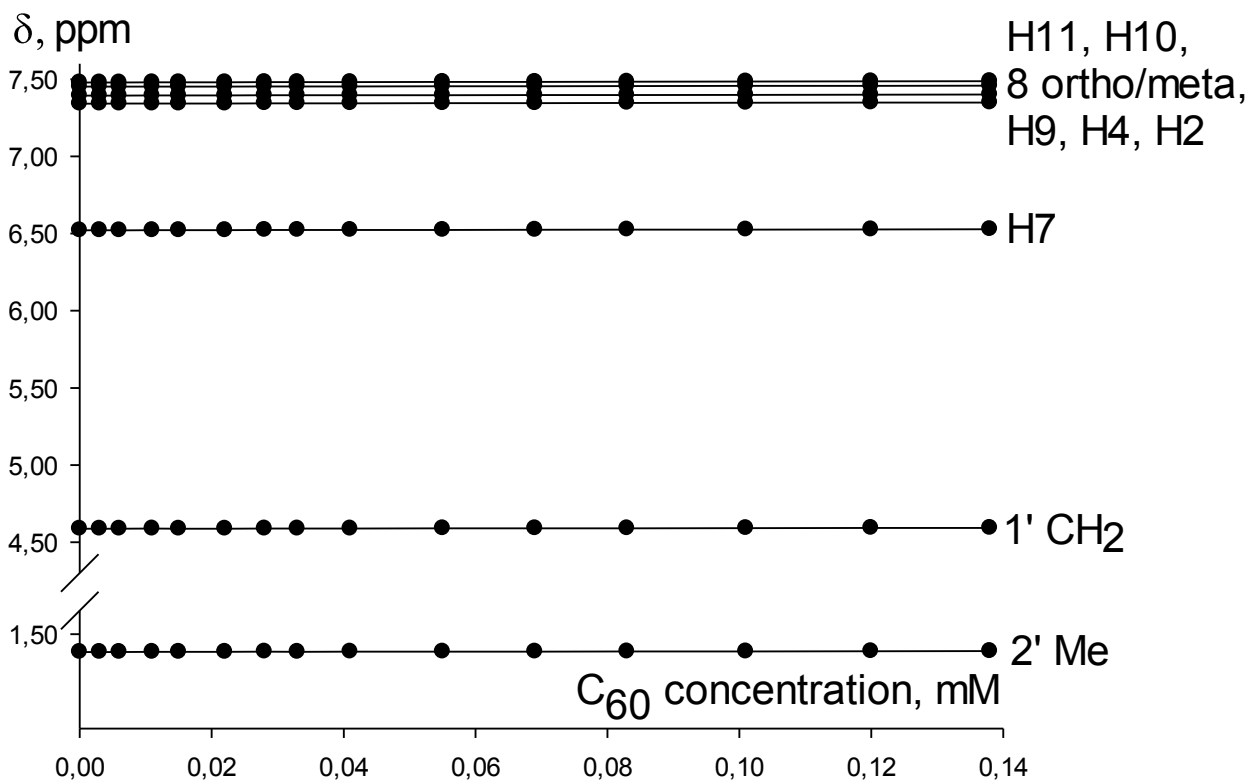


Figure 3 Experimental titration curves (reduced dataset, see discussion in the text; reprinted from [18] with permission)



(a)



(b)

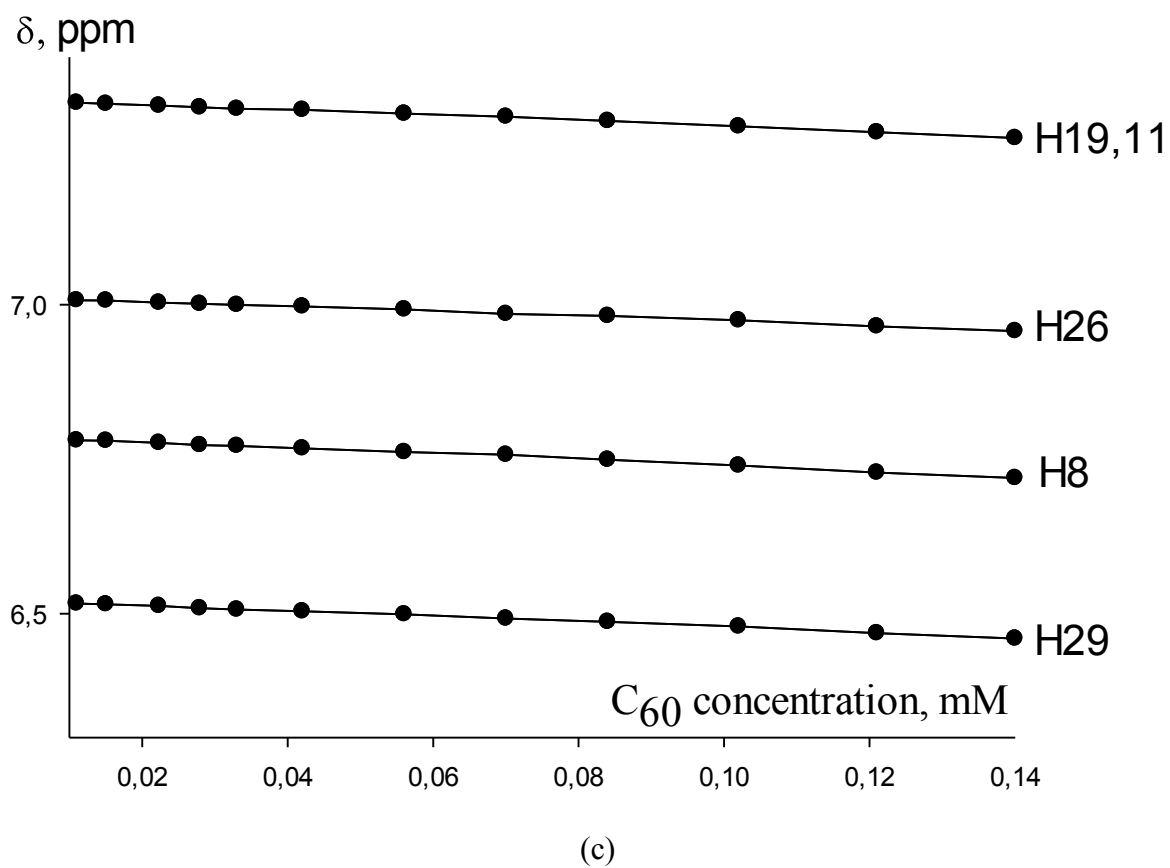


Fig.4 Experimental dependencies of ^1H NMR chemical shifts of the drug molecules on fullerene C_{60} concentration: (a) Doxorubicin, (b) Ethidium bromide, (c) Hoechst 33258

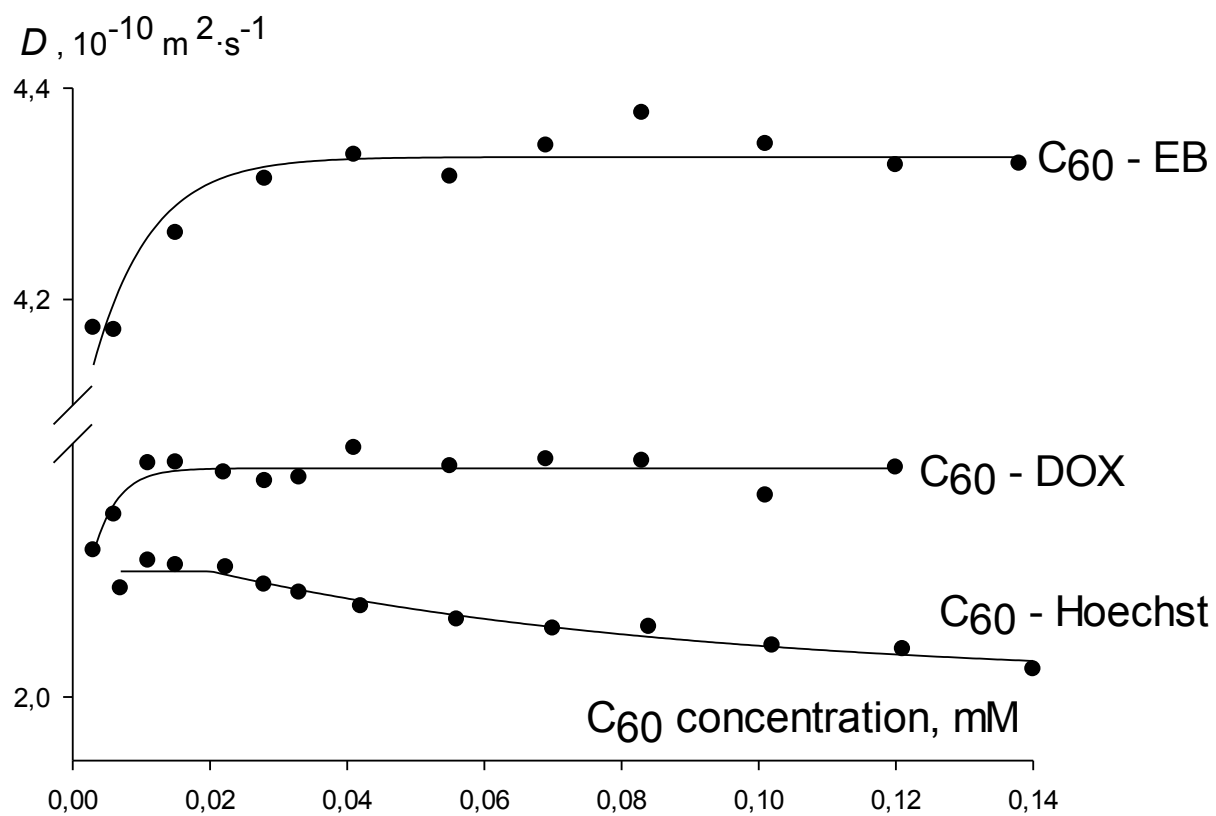


Fig.5 Experimental dependencies of translational diffusion coefficient of the drug molecules on fullerene C_{60} concentration