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Interaction between PAMAM-NH_2 G4 dendrimer and 5-fluorouracil in aqueous solution

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ABSTRACT

The formation equilibrium of poly(amidoamine) dendrimer (PAMAM-NH_2 G4) complex with an oncologic drug such as 5-fluorouracil (5-FU) in water at room temperature was examined. Using the results of the drug solubility in dendrimer solutions and the method of equilibrium dialysis, the maximal number of drug molecules in the dendrimer–drug complex and its equilibrium constant were evaluated. Solubility results show that PAMAM-NH_2 G4 dendrimer can transfer tens 5-fluorouracil molecules in aqueous solution. The number of active sites in a dendrimer macromolecule being capable of combining the drug, determined by the separation method, amounts to $n = 30 \pm 4$. The calculated equilibrium constant of the 5-FU-active site bonding is equal to $K = (400 \pm 120)$.

1. Introduction

Poly(amidoamine) dendrimers (PAMAM) are polymeric macromolecules that can find their use as carriers of biologically and medically important molecules such as fragments of genetic material (Pavan et al., 2010a,b; Peng et al., 2010; Shakhbazau et al., 2010; Wang et al., in press), drugs (Cheng and Xu, 2005a; Cheng et al., 2008c; D'Emanuele and Attwood, 2005; Gupta et al., 2006b; Medina and El-Sayed, 2009; Najlah and D'Emanuele, 2006) or vitamins (Boisselier et al., 2010). Special expectations are associated with the use of these polymers as carriers of oncologic drugs (Cheng and Xu, 2008; Thomas et al., 2010), including among others 5-fluorouracil (Bhadra et al., 2003; Mei et al., 2009; Singh et al., 2008; Venuganti and Perumal, 2008, 2009; Zhuo et al., 1999). The most frequently tested polymers of this kind include dendrimers of the PAMAM class, especially those belonging to the fourth (G4) and fifth (G5) generation. The surface groups in PAMAM dendrimers of these generations allow ligand molecules to penetrate the dendrimer interior and to react with the groups localized in it.

Drug molecules can be transferred either as covalently bonded to the functional groups on the dendrimer surface or by the formation of non-covalent complexes with dendrimers (Cheng and Xu, 2008; Cheng et al., 2008c; Patri et al., 2005). In the second case, the complex bonding forces can include: hydrogen bonds (Beezer et al., 2003; D'Emanuele and Attwood, 2005; Gupta et al., 2006a; Svenson and Tomalia, 2005; Zeng and Zimmerman, 1997), electrostatic interactions between oppositely charged fragments of drug molecule and dendrimer macromolecule (Beezer et al., 2003; Cheng et al., 2008b; D'Emanuele and Attwood, 2005; Gupta et al., 2006a; Zeng and Zimmerman, 1997) as well as hydrophobic interactions (D'Emanuele and Attwood, 2005; Esfand and Tomalia, 2001; Gupta et al., 2006a; Svenson and Tomalia, 2005; Zeng and Zimmerman, 1997). It was also observed that selected compounds including drugs in aqueous solution in the presence of dendrimers show increased solubility (Cheng et al., 2005, 2008a,b,c; Cheng and Xu, 2005a,b; Gupta et al., 2006a; Hu et al., 2009; Milhem et al., 2000).

A single molecule of PAMAM-NH_2 G4 with ethylendiamine core contains about 250 potential bonding sites, which comprise 64 surface primary amine groups and 62 internal tertiary amine groups and 124 amide groups. The complexity of a dendrimer–ligand system is first of all connected with the presence of many functional groups that can play the role of active sites capable of bonding a ligand. These groups can participate in the acid–base equilibria, which imparts the character of weak polyelectrolyte to the dendrimer molecule. The above mentioned properties of the complex cause that with regard to the processes of bonding a ligand with dendrimer macromolecules it is difficult to talk about a precisely defined stoichiometry. Such a system shows to a large extent a non-stoichiometric character.

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Several research centers have determined the number of ligand molecules transferred by a dendrimer macromolecule using spectroscopic measurements of the content of bonded ligands (Kolhe et al., 2003; Yang et al., 2009) and the number of active sites capable of bonding a ligand in dendrimer macromolecule using the method of equilibrium dialysis (Sekowski et al., 2009; Shcharbin et al., 2007). The release of 5-fluorouracil from its combination with PAMAM G4 dendrimer modified on its surface with polyethylene glycol chains (PEG) has been examined in order to reduce toxicity (Bhadra et al., 2003). It was found that the drug was slowly released under both *in vitro* and *in vivo* conditions. PAMAM G4 dendrimer substituted with PEG chains combined higher amounts of the drug and showed a lower homoliticity than its unsubstituted equivalent. It was shown (Venuganti and Perumal, 2008) that the addition of PAMAM G4 dendrimer facilitated the diffusion of 5-fluorouracil from lipophile vesicles through skin. The use of PAMAM dendrimers modified with PEG chains and folic acid radicals as carriers of 5-fluorouracil (Singh et al., 2008) and the combinations of PAMAM dendrimer with 5-fluorouracil and anti-rational microRNA in order to reduce the cancer cell development were examined (Mei et al., 2009).

The aim of our study was to evaluate the number of 5-fluorouracil molecules, an oncologic drug, combined by PAMAM-NH_2 G4 macromolecule and the equilibrium constant of the 5-FU combination with the active sites of this dendrimer in aqueous solution.

2. Materials and methods

2.1. Materials

PAMAM-NH_2 G4 dendrimer (m.w. ~14 kDa, Sigma–Aldrich) with ethylenediamine core, 5-fluorouracil (m.w. = 0.13 kDa, Sigma–Aldrich, ≥99%), water distilled three times and degased, benzoylated dialysis tubing (MWCO 2 kDa, Sigma–Aldrich)

2.2. Methods

2.2.1. Measurements of drug solubility

The increase in 5-FU solubility in dendrimer solutions was determined by spectrophotometry (Specord 50 Analytic Jena). The concentration range of dendrimer was of 2.5–50 μM. The μM dendrimer solution prepared from 5 mM methanol solution of dendrimer was evaporated to 1/3 volume and made up with water to remove methanol. Aqueous solutions of dendrimer with specified concentrations were prepared from a 50 μM aqueous dendrimer solution. Prior to measurement, dendrimer solutions were saturated with the drug for one week at room temperature. The concentration of 5-fluorouracil in the solutions tested was determined by the spectrophotometric method using a calibration curve determined at wavelength λ_{max} = 266 nm, within the drug concentration range of 10–450 μM, described by the equation $y = (6920 \pm 40)x (R^2 = 0.9988)$. The directional coefficient of the equation is equal to the absorption coefficient of 5-fluorouracil, $\varepsilon_{max} = 6920\ M^{-1}\ cm^{-1}$. The value of the molar absorption coefficient of 5-fluorouracil reported in literature amounts to $7000\ M^{-1}\ cm^{-1}$ (Mallano et al., 2008). The drug concentration in the solutions tested was determined by the spectrophotometric method from the difference in two measurements: drug absorbance in the water–dendrimer mixture and residual absorbance of the aqueous solution of dendrimer. The samples of initial saturated solutions of 5-fluorouracil to be spectrophotometrically measured were diluted 500 times.

Table 1
Solubility of 5-fluorouracil in PAMAM-NH_2 G4 dendrimer solutions with various concentration.

c G4 [μM]	S 5-FU [μM]
0	79,300
2.5	78,840
5	80,250
10	80,040
20	79,750
30	82,530
40	81,360
50	83,290

2.2.2. Equilibrium dialysis

Equilibrium dialysis was performed in two-chamber microdialyzers (Harvard Apparatus – USA) with a membrane of molecular weight cut off 2 kDa (Sigma–Aldrich) at room temperature. In one chamber of the dialyzer, we placed the aqueous mixture of PAMAM G4 dendrimer with a concentration of 10 μM with 5-fluorouracil with concentrations from 100 μM to 4000 μM. The second chamber contained the solution of 5-fluorouracil with the same concentration as in the first chamber. The dialysis was performed for 3 days. For particular concentrations, dialyses were carried out 6 times. Following the dialysis, the drug concentration in both chambers was spectrophotometrically determined in the same way as described in Section 2.2.1.

2.2.3. pH measurements

To assess the extent of protonation of terminal amine groups in the dendrimer macromolecule, a series of pH-metric measurements (pH-METER N5172) was carried out within the dendrimer concentration range of 5–50 μM at room temperature.

3. Results and discussion

The measurements of 5-fluorouracil solubility in water in the presence of PAMAM-NH_2 G4 dendrimer point approximately to a linear increase in the drug solubility with dendrimer concentration within the polymer concentration range of 2.5–50 μM (Table 1, Fig. 1). The dependence of 5-fluorouracil solubility in dendrimer solution (Fig. 1) on the dendrimer concentration was described with the linear equation $y = (75 \pm 15)x + (79190 \pm 420)$ ($R^2 = 0.7891$). This dependency points approximately to a linear character. The lack of linearity is due to the several hundred-dilution of the saturated 5-fluorouracil solutions under testing taken for the determination of the drug absorbance.

One can ascribe a physical sense to the coefficients of this straight line. The free term (79.2 mM) is close to the solubility of 5-fluorouracil in pure water (96.9 mM) (Singh, 2005). The direc-

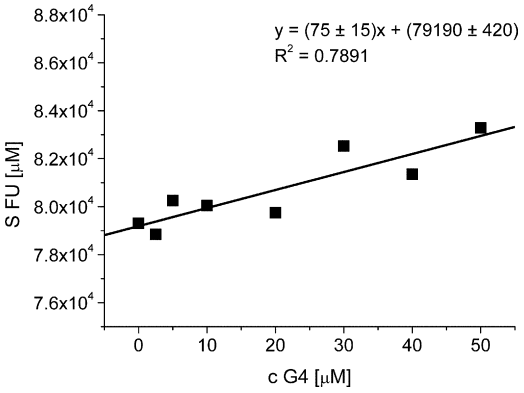

Fig. 1. Dependence of 5-fluorouracil solubility on the concentration of PAMAM-NH_2 G4 dendrimer solution.

Table 2
Equilibrium distribution 5-fluorouracil concentration in solution with 10 μM PAMAM-NH_2 G4 dendrimer and in solution of the drug on the opposite side.

[FU]/[G4]	c FU [μM]	c FU [μM]	c [μM]	f [$\times 10^{-6}$ M]	b
Initial molar ratio	On PAMAM side	On opposite side	Bound FU concentration	Free FU concentration	c/[G4]
10	79	69	10	69	1.00
15	178	148	31	148	3.06
20	152	134	18	134	1.84
30	331	292	40	292	3.97
40	425	387	38	387	3.79
50	367	339	28	339	2.83
60	639	573	66	573	6.62
100	921	855	66	855	6.63
150	1401	1306	94	1306	9.44
200	2017	1911	106	1911	10.58
250	2505	2339	166	2339	16.62
300	2986	2809	177	2809	17.70
350	3486	3318	168	3318	16.84
400	3971	3805	166	3805	16.62

tional coefficient ($n = 75 \pm 15$) can be interpreted as a number of drug molecule combined by one molecule of dendrimer (Yang et al., 2009). Based on spectrophotometric measurements determining the increase in 5-fluorouracil solubility in dendrimer solutions within the concentration range of 2.5–50 μM, the number of the drug molecules combined by a single macromolecule of dendrimer was determine to be equal to 75 ± 15. This means that PAMAM-NH_2 G4 dendrimer possessing 250 possible active sites (64 primary amine groups, 62 tertiary amine groups and 124 amide groups) can combined maximum 90 molecules of the drug. The values measured comprise the range of drug to dendrimer molar ratio from about 1500:1 to 30000:1, which indicates that the drug molecules are combined not only with the surface protonated and unprotonated amine groups but also with those being inside.

The examination of equilibrium dialysis was aimed at the evaluation of the number of active groups and the equilibrium constant of the addition of drug molecules. The determined concentrations of 5-fluorouracil in both chambers: the aqueous mixture of PAMAM-NH_2 G4 and 5-fluorouracil as well as the aqueous solution of 5-fluorouracil are given in Table 2. The concentration of combined 5-fluorouracil was calculated from the difference between the concentrations of both solutions. From the measurement results it follows that with the increase in 5-fluorouracil concentration from 100 μM to 2500 μM in solution, the number of drug molecules associated with dendrimer macromolecule is gradually increased (Table 2). When the concentration of fluorouracil exceeded 2500 μM the active sites of PAMAM-NH_2 G4 were saturated with the drug (Fig. 2) and successive changes in the concentration of the drug combined were not observed.

The dependence of the number of moles of combined ligand per 1 mole of receptor on the free ligand concentration has a hyperbolic character (Bobrovnik, 2002):

$$b = \frac{Knf}{1 + Kf} \quad (1)$$

where b is the number of moles of combined ligand per 1 mole of receptor; K is the equilibrium constant of the ligand–active site complex formation; n is the receptor valence or the number of active sites of the macromolecule; f is the concentration of free ligand.

The linearization of Eq. (1) in the doubly inversely proportional system is called Scatchard–Klotz' equation (Bobrovnik, 2002; Scatchard, 1949):

$$\frac{1}{b} = \frac{1}{Kn} \times \frac{1}{f} + \frac{1}{n} \quad (2)$$

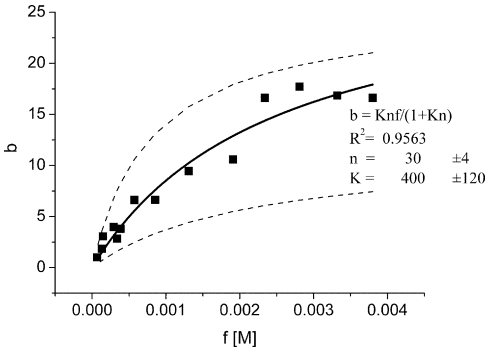

Fig. 2. Dependence of 5-fluorouracil combined by 10 μM PAMAM-NH_2 G4 dendrimer (Eq. (1)) on the concentration of free 5-fluorouracil in aqueous solution at room temperature. Experimental points were described with hyperbolic dependence (1) continuous line. The lower and upper limits of the range obtained as Scatchard–Klotz's solution (2) is denoted with broken line.

Considering its simplicity and graphic visual value of the results obtained, Scatchard–Klotz' equation is still use to evaluate the number of active sites of macromolecules and the bonding constant, especially in biochemical and biophysical studies, despite the fact that it considerably amplifies the measurement errors (Munson and Rodbard, 1983).

The experimental results of equilibrium dialyses obtained (Table 2) were described with Scatchard–Klotz's Eq. (2) to evaluate the range of variation, in which one should seek for the number of active sites and the equilibrium constant of the 5-FU-PAMAM-NH_2 G4 combination in aqueous solution. The dependence of the ratio of the concentration of dendrimer and combined 5-FU ($1/b$) on the inverse of free 5-FU concentration ($1/f$) (Fig. 3) is described with the equation of straight line: $y = (6.30 \pm 0.41) \times 10^{-5}x + (5.4 \pm 2.1) \times 10^{-2}$ ($R^2 = 0.9512$). The number of active sites n in dendrimer and equilibrium constant K of the bond between 5-fluorouracil and a single active site of the polymer evaluated from Scatchard–Klotz's Eq. (2) amount to:

$$n = 18.5 \pm 7.0 \quad K = 860 \pm 380$$

The results of equilibrium dialysis indicate that in aqueous solution at room temperature, one macromolecule of PAMAM-NH_2 G4 contains up 25 active sites that can combine 5-fluorouracil with a bonding constant of about 900. The values obtained are encumbered with considerable uncertainty, which seems to result from the fact that in a doubly inversely proportional system, the points obtained within the range of higher concentration condense near the origin of coordinates. Thus it is the points obtained for the lowest concentrations and encumbered with the greatest error that decide to the largest extent about the dependence. The description

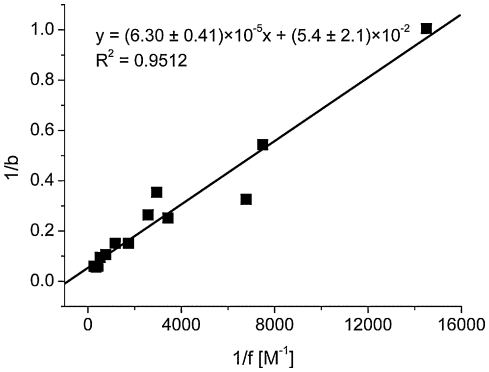

Fig. 3. Dependence of the ratio of dendrimer and combined 5-fluorouracil concentrations ($1/b$) on the reverse of free 5-fluorouracil concentration ($1/f$).

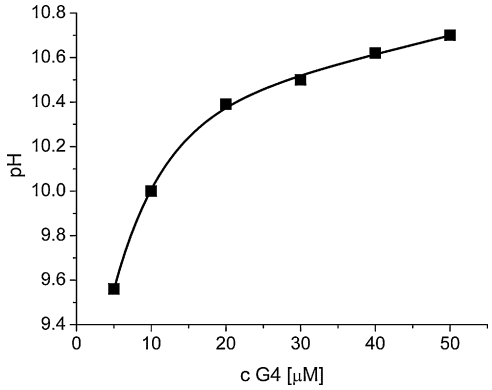

Fig. 4. Dependence of pH of PAMAM-NH_2 G4 dendrimer aqueous solution on its concentration.

of equilibrium dialysis results with Scatchard–Klotz's Eq. (2) allows one to evaluate the area where one should seek parameters n and K. The hyperbolic curves described with Eq. (1), calculated for the values of the number of active sites n and equilibrium constant K determined from Eq. (2) are presented in Fig. 2.

However, the values of K calculated on the basis of Scatchard–Klotz's Eq. (2) fail to fully describe the system under discussion. Therefore the experimental values of the equilibrium dialysis introduced to hyperbolic Eq. (1) were described by the method of non-linear two-parameter regression, using the Origin 7.0 program. It is an advantage of this method that particular experimental points make the same contribution to the parameter values under calculation. The following values of selectable parameters were obtained:

$$n = 30 \pm 4 \quad K = 400 \pm 120 \quad (R^2 = 0.9563)$$

In order to determine the extent of the protonation of terminal amine groups, a series of pH-metric measurements was performed. The aqueous solution of PAMAM-NH_2 G4 dendrimer with a concentration of 10 μM shows pH = 10 (Fig. 4). According to dependence proposed by Niu (2003), 15% of the surface amine groups of PAMAM-NH_2 G4 in its aqueous solution are protonated to ammonium groups. Probably the intramolecular tertiary amine groups with a lower basicity (Cakara and Borkovec, 2007; Cakara et al., 2003; Diallo et al., 2004; Niu et al., 2003) are practically not protonated under these conditions. One should assume that one macromolecule of PAMAM-NH_2 G4 dendrimer in aqueous solution of pH = 10 contains about 10 surface cationic centers.

In the aqueous solution of dendrimer, the surface positively charged ammonium groups and the non-dissociated amine groups combine 5-FU molecules containing a strongly negative fluorine atom by electrostatic interaction and hydrogen bonds. The number of sites combining 5-FU molecules, determined by the method of non-linear two-parameter regression, describing the results obtained from equilibrium dialysis amounts to 30 ± 4. The calculated equilibrium constant of bonding the drug with the active site amounts to $K = 400 \pm 120$ for each of those sites. The value of the equilibrium constant calculated indicates a reversible character of the bonding between 5-FU and the active sites of dendrimer. This value is of an intermediate character between strong interactions ($K > 10^3$) (Jones and Atkins, 2000), when the products of association predominate and weak interactions ($K < 10^{-3}$) (Jones and Atkins, 2000), when in the equilibrium state uncombined substrates predominate. The intermediate value of the equilibrium constant of the 5-FU-dendrimer combination shows that in aqueous solution there exist uncombined active sites of dendrimer, uncombined drug as well as the dendrimer–drug complex in equilibrium.

4. Conclusions

From the solubility tests of 5-fluorouracil in water in the presence of PAMAM-NH_2 G4 dendrimer it follows that this dendrimer forms supramolecular complexes with the oncologic drug: 5-fluorouracil. A complete saturation of PAMAM-NH_2 G4 dendrimer in aqueous solution at room temperature is connected with the combination of maximum 90 molecules of 5-fluorouracil within the range of the drug to dendrimer molar ratio of 15000:1–30000:1. The results obtained by the separation method show that about 30 molecules of 5-fluorouracil are combined by 10 μM PAMAM-NH_2 G4 dendrimer with the equilibrium constant $K = 400 \pm 120$ within the drug to dendrimer molar ratio range of 10:1–400:1.

Comparing the number of drug molecules transferred by dendrimer (75 ± 15) obtained from the measurements of 5-fluorouracil solubility with the number of active sites of the dendrimer macromolecule (30 ± 4) determined by the separation method, one can conclude that this dendrimer contains more than one type of active sites with different affinities in relation to the ligand. Thus the model of the same active sites used to describe these non-stoichiometric systems has an approximate character.

Cationic PAMAM dendrimers (of complete generation) are excellent transporters of the drugs possessing anionic groups whereas anionic PAMAM dendrimers (of half generation) can transfer the cationic drugs. Both types of PAMAM dendrimers reveal toxicity which could be reduced by dendrimers modifications using sugar or amino acids functional groups. It is also possible to substitute dendrimers surface by functional groups recognizing by the cancer cells. The investigations of interaction between drugs and dendrimer nanocontainers are mostly limited to define the equilibrium constants for only one type of active sites occurring in those molecules. The improvement of the quantitative description methods of the equilibrium existing between the dendrimer and the drug permit in future to characterize more accurately these modified and less toxic transporters it means the dendrimers molecules. Several laboratories are conducting the research concerning the utilization of modified and intact dendrimers molecules as carriers for fragments of genetic material (Pavan et al., 2010a,b; Peng et al., 2010; Shakhbazau et al., 2010; Wang et al., in press) and drugs (Cheng and Xu, 2005a; Cheng et al., 2008c; D'Emanuele and Attwood, 2005; Gupta et al., 2006b; Medina and El-Sayed, 2009; Najlah and D'Emanuele, 2006) or as nanocontainers of 5-fluorouracil carrying the drug through skin (Venuganti and Perumal, 2008).

Therefore we decided to study the 5-fluorouracil interaction with PAMAM-NH_2 G4 dendrimer which could be subject to further modifications. Not very high value of the equilibrium constant of PAMAM-NH_2 G4 and 5-fluorouracil complex, determined on the basis of the equilibrium dialysis study, can evidence about the existence of electrostatic and hydrogen bonding of macromolecule and ligand. Covalent bonding of the drug would be certainly better alternative but it would be more difficult to release the bonded ligand.

Further stage of our studies on the interaction between 5-fluorouracil and the model supramolecular system – PAMAM-NH_2 G4 – in aqueous solution concerns the thermodynamic characteristics of the process of drug combination with the active sites of the dendrimer macromolecule.

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