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

Adam Buczkowski[a], Szymon Sekowski[b], Aleksandra Grala[a], Danuta Palecz[b], Katarzyna Milowska[b], Pawel Urbaniak[c], Teresa Gabryelak[b], Henryk Piekarski[a], Bartlomiej Palecz[a,*]

[a] Department of Physical Chemistry, University of Lodz, Pomorska 165, Lodz 90-236, Poland
[b] Department of General Biophysics, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland
[c] Department of Inorganic and Analytical Chemistry, University of Lodz, Tamka 12, 91-403 Lodz, Poland

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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.

* Corresponding author. Fax: +48 42 635 58 14.
E-mail address: paleczb@uni.lodz.pl (B. Palecz).

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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-

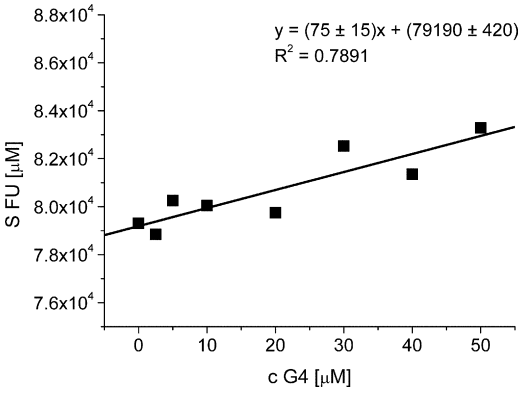

Fig. 1. Dependence of 5-fluorouracil solubility on the concentration of PAMAM-NH_2 G4 dendrimer solution.