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Thermochemical and spectroscopic studies on the supramolecular complex of PAMAM-NH_2 G4 dendrimer and 5-fluorouracil in aqueous solution

Adam Buczkowski[a,*], Pawel Urbaniak[b], Bartlomiej Palecz[a,*]

[a] Department of Physical Chemistry, University of Lodz, Pomorska 165, Lodz 90-236, Poland
[b] Department of Inorganic and Analytical Chemistry, University of Lodz, Tamka 12, 91-403 Lodz, Poland

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ABSTRACT

The equilibrium of the formation of polyamidoamine dendrimer (PAMAM-NH_2 G4) and an oncological drug, 5-fluorouracil (FU) in water at room temperature has been examined. Using calorimetric titration, the number of active sites in the dendrimer combining the drug molecules and the equilibrium constant of the dendrimer–drug complex were estimated. The addition of the drug to the dendrimer active sites is an exothermic process. This process is accompanied by a beneficial change in entropy. The number of drug molecules combined by the polymer was confirmed by means of ^{1}H NMR spectroscopy. ^{1}HNMR measurements show that the dendrimer macromolecule binds the drug molecules with superficial protonated or unprotonated amine groups.

1. Introduction

Polyamidoamine dendrimers (PAMAM) are polymeric macromolecules that can be used as carries of molecules of biological and medical importance, such as fragments of genetic material (Pavan et al., 2010a, 2010b; Peng et al., 2010; Shakhbazau et al., 2010; Wang et al., 2010) or drugs (Cheng and Xu, 2005; Cheng et al., 2008b; D'Emanuele and Attwood, 2005; Gupta et al., 2006b; Medina and El-Sayed, 2009; Najlah and D'Emanuele, 2006), including 5-fluorouracil (Bhadra et al., 2003; Jin et al., 2011; Mei et al., 2009; Singh et al., 2008; Venuganti and Perumal, 2008, 2009; Zhuo et al., 1999). One of the most frequently studied polymeric carriers is the PAMAM dendrimer of the fourth generation (G4), in which a ligand molecule can interact with both superficial and internal groups of the dendrimer. The supramolecular dendrimer-drug complex can be maintained by 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 the oppositely charged fragments of the drug molecules and dendrimer macromolecules (Beezer et al., 2003; Cheng et al., 2008a; D'Emanuele and Attwood, 2005; Gupta et al., 2006a; Zeng and Zimmerman, 1997) and hydrophobic interactions (D'Emanuele and Attwood, 2005; Esfand and Tomalia, 2001; Gupta et al., 2006a; Svenson and Tomalia, 2005; Zeng and Zimmerman, 1997).

In one molecule of PAMAM-NH_2 G4 with an ethylenodiamine core, the potential binding sites include: 64 superficial primary amine groups, 62 internal tertiary amine groups and 124 amide groups. Due to this complexity it is difficult to speak about a precisely defined stoichiometry with reference to the processes of binding a ligand with the dendrimer macromolecule.

Several research centers have determined the number of ligand molecules transferred by a dendrimer macromolecule using spectroscopic measurements (Kolhe et al., 2003; Yang et al., 2009) and the method of equilibrium dialysis (Sekowski et al., 2009; Shcharbin et al., 2007). The results obtained were interpreted with the use of a model of the same active sites (Buczkowski et al., 2011).

The aim of our study was to estimate the parameters of binding 5-fluorouracil molecules by the active sites of PAMAM-NH_2 G4 in aqueous solution by means of an isothermal titration calorimeter and ^{1}H NMR spectroscopy. A particular attention was focused on the thermodynamic characteristics of binding the drug investigated by the active macromolecules of PAMAM-NH_2 G4.

2. Material 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.

* Corresponding author. Fax: +48 42 635 58 14.
E-mail addresses: buczkowski_adam@tlen.pl (A. Buczkowski), paleczb@uni.lodz.pl (B. Palecz).

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2.2. Methods

2.2.1. Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry (ITC) was conducted using a VP-ITC instrument (MicroCal, USA). Aliquots of 3 μl of 20 mM 5-fluorouracil in water were injected via a 287.37 μl syringe at intervals of 600 s into 1.4275 ml of 10 μM PAMAM-NH_2 G4, stirring at 416 rpm. Titrations were done at 25 °C. All solutions used in the experiments were degassed. For background correction, water (in the cell) was titrated with 5-fluorouracil in water (in the syringe) at the same concentrations, and the background was subtracted from the final curves. The thermal effect of diluting the dendrimer aqueous solution under the titration conditions was neglected.

2.2.2. ^{1}H NMR spectroscopy

The samples of mixtures were prepared from mother solutions: 2.8 mM PAMAM-NH_2 G4 in D_2O and 20 mM 5-fluororacil in D_2O. Before the preparation of the mother solution of dendrimer, methanol was removed by drying the sample for 3 days at room temperature.

^{1}H NMR spectra of 5-fluorouracil and PAMAM-NH_2 G4 dendrimer mixtures with different molar ratios in D_2O were obtained on a Bruker Avance III 600 MHz NMR spectrometer at room temperature. Each spectrum is an average of 16 scans of the given samples. The spectra recorded were analyzed within the range of 3.5–2 ppm.

3. Results and discussion

3.1. Isothermal titration calorimetry (ITC)

The isothermal titration calorimetry (ITC) technique was used to determine the thermal effects of the titration of a 10 μM solution of PAMAM-NH_2 G4 (in a cell) with 20 mM solution of 5-fluorouracil (in a syringe) and the corresponding thermal effects of diluting the drug in water. Their difference was used to calculate the thermal effect of dendrimer-drug interaction corrected by the dilution effect (Fig. 1).

In order to estimate the binding parameters: number of active sites, n, equilibrium constant of ligands–active site K and corresponding molar combining enthalpy ΔH, Saboury's dependences were used (Divsalar et al., 2006; Saboury et al., 2006a, 2006b):

$$\frac{q_{max}-q}{q_{max}}r = \left(\frac{q_{max}-q}{q}\right)l\frac{1}{n} - \frac{1}{Kn} \quad (1)$$

$$\Delta H = \frac{q_{max}}{nrV} \quad (2)$$

where q – cumulative heat value at a certain total ligand concentration, q_{max} – cumulative heat value upon saturation of all active sites, r – total concentration of macromolecule, l – total concentration of ligand, n – receptor valence or the number of active sites of the macromolecule, V – volume of solution after the addition of titrant.

The values of the parameters of binding the drug with the active sites of dendrimer estimated from Saboury's dependence: $n = 26 \pm 1$, $K = 4080 \pm 310$, $\Delta H = -1.2 \pm 0.1$ kcal mol^{-1} were used as initial values describing the enthalpogram with the Origin Lab software (USA) for the VP-ITC calorimeter. The binding and thermodynamic parameters–binding constant (K), number of binding centers per one molecule (n), enthalpy (ΔH) and entropy (ΔS) – were computed from actual calorimetric data by non-linear fitting using Origin Lab software (USA) for the VP-ITC calorimeter (Fig. 1):

$$n = 25 \pm 3 \quad K = 32,800 \pm 300 \quad \Delta H = -1.5 \pm 0.2 \text{ kcal mol}^{-1}$$
$$\Delta S = 10.7 \pm 0.4 \text{ cal mol}^{-1}\text{ K}^{-1}$$

The binding of 5-fluorouracil by PAMAM-NH_2 G4 is a process controlled by both enthalpy and entropy.

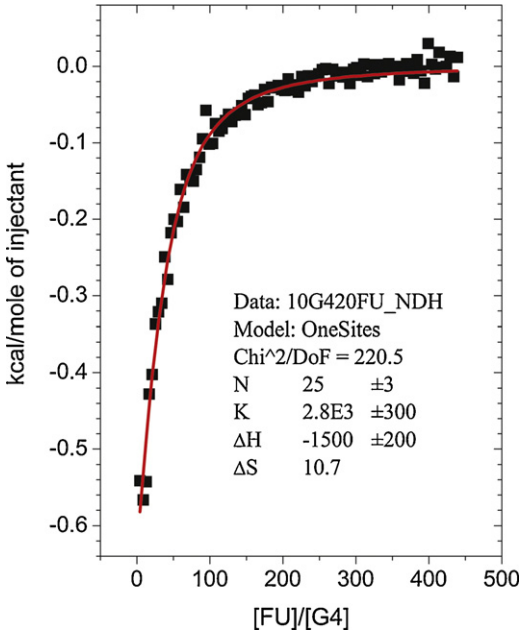

Fig. 1. Thermal effect of the interaction between PAMAM-NH_2 G4 and 5-fluorouracil corrected with the dilution effect and calculated per one mole of the drug.

3.2. ^{1}H NMR spectroscopy

The ^{1}H NMR spectroscopic method (a Bruker Avance III 600 MHz apparatus) was used to record a series of spectra, in heavy water as solvent, of PAMAM-NH_2 G4 solution with a concentration of 140 μM, 5-fluorouracil solution with a concentration of 12,6 mM and mixtures of PAMAM-NH_2 G4 (140 μM) and 5-fluorouracil within the range of the molar drug to dendrimer ratio from 10/1 to 130/1 (Fig. 2).

Groups of protons were assigned to particular peaks within the spectrum range analyzed for the PAMAM-NH_2 G4 as in (Hu et al., 2010). Band III (2.8 ppm) corresponds to protons of methylene groups at the terminal amine groups of dendrimer and to protons of methylene groups at the internal tertiary amine groups. Band IV (3.2 ppm) corresponds to protons located at the amide bond on the amine group side.

The spectrum of 5-fluorouracil within the range of 3.5–2 ppm is poor in signals and constitutes no significant obstruction in the interpretation of the signals of the dendrimer-drug mixture spectra. With the increase in the concentration of 5-fluorouracil band III shows the greatest shift and it was selected for further analysis (Fig. 2).

For bands III of successive dendrimer-drug mixtures, we calculated the difference between the shifts ($\Delta\delta$) of the band position in the dendrimer-drug mixture in relation to the position of corresponding band in the solution of dendrimer.

The dependence of the difference between shifts $\Delta\delta$ of the signal in the spectrum of the ligand-macromolecule mixture on the ligand concentration according to the model of the same active sites, assumes the following form (Fielding, 2007; Hu et al., 2010):

$$\Delta\delta = \frac{\Delta\delta_{max}}{2}\left[\left(1+\frac{l}{nr}+\frac{1}{Knr}\right) - \sqrt{\left(1+\frac{l}{nr}+\frac{1}{Knr}\right)^2 - \frac{4l}{nr}}\right] \quad (3)$$

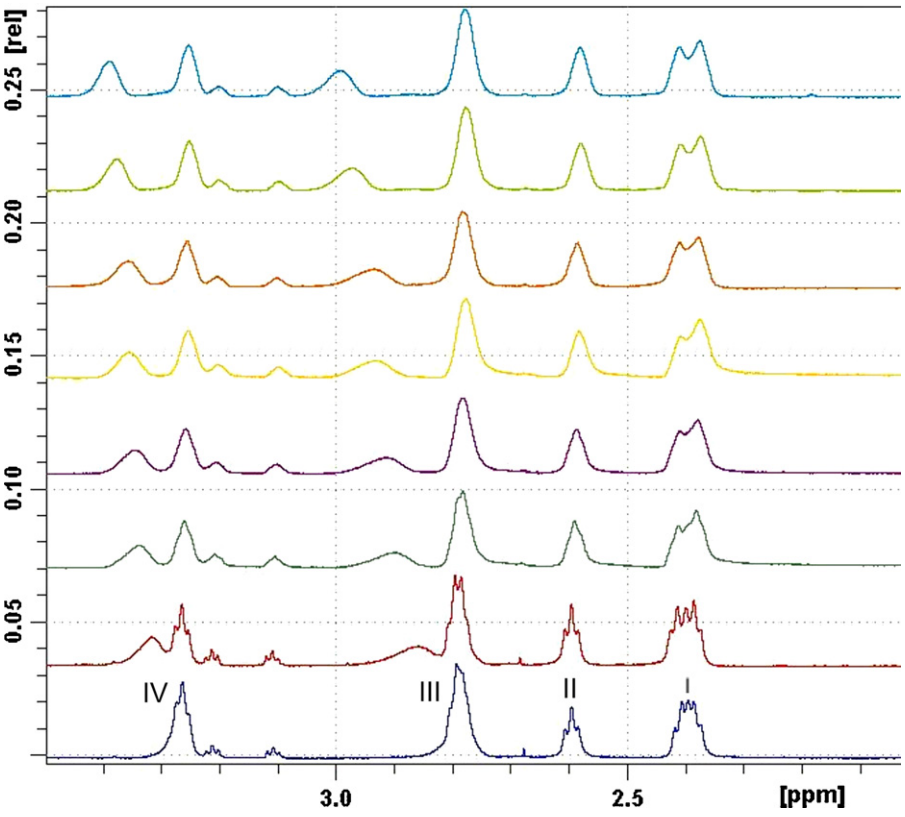

Fig. 2. The first curve is the spectrum of the PAMAM-NH_2 G4 solution with a concentration of 140 μM in heavy water. Next curves are selected ^{1}H NMR spectra of the mixtures of 5-fluorouracil and PAMAM-NH_2 G4 (140 μM). The molar drug to dendrimer ratio in successive solutions: 15/1, 25/1, 30/1, 35/1, 40/1, 70/1 and 90/1. The positions of bands III and IV are shifted with increasing drug concentration.

where $\Delta\delta$ – change in the chemical shift observed after the addition of ligand, $\Delta\delta_{max}$ – maximum change in the chemical shift after the saturation of macromolecule with ligand.

The dependence of the difference between shifts $\Delta\delta$ for band III on the concentration of 5-fluorouracil (Table 1) was described with the model of the same active sites using the method of non-linear three-parameter regression (Fig. 3, Eq. (3)), and the Origin 7.0 program.

As initial parameters of Eq. (3) the following data were used: the $\Delta\delta$ value of the point with the highest 5-fluorouracil concentration examined (as the maximum difference between the band positions $\Delta\delta_{max}$) and the parameters of binding determined according to the assumptions of the model of the same active sites (Buczkowski et al., 2011). The following values of selectable parameters were obtained (Fig. 3):

$$n = 30 \pm 10 \quad K = 400 \pm 200 \quad (R^2 = 0.9915)$$

The relatively great changes in the chemical shifts of the protons in methylene groups at the terminal primary and internal tertiary amine groups of the dendrimer (band III) and the weaker ones at the amide group (band IV) suggest that these groups can be directly involved in binding 5-fluorouracil (Fig. 2). The changes in the shifts of band IV are probably connected with the addition of 5-fluorouracil to amide groups, while those of band III can be

Table 1
Chemical shifts of selected proton groups of the solutions of PAMAM-NH_2 G4 and 5-fluorouracil with various compositions.

[FU]/[G4]	c G4 [μM]	c FU [μM]	Band III δ $CONHCH_2CH_2NH_2 + NCH_2CH_2CO$
–	140	0	2793
10	140	1400	2833
15	140	2100	2860
20	140	2800	2876
25	140	3500	2900
30	140	4200	2914
35	140	4900	2932
40	140	5600	2935
45	140	6300	2945
70	140	9800	2970
90	140	12,600	2991
130	140	18,200	3016

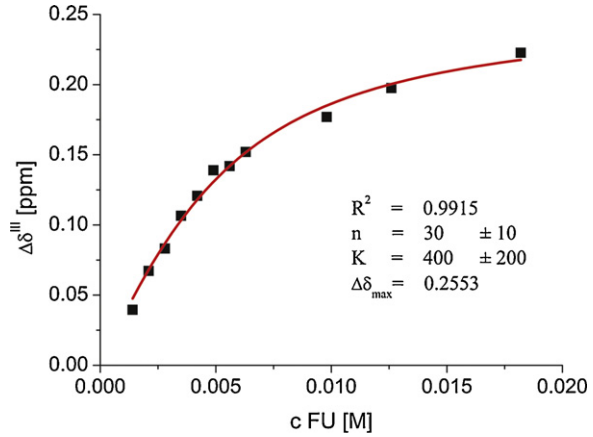

Fig. 3. Dependence of the change in chemical shift on the 5-fluorouracil concentration for methylene protons of band III.

Table 2
Parameters of binding 5-fluorouracil to the active sites of PAMAM-NH_2 G4 determined by various techniques.

	n	K	ΔH [kcal mol^{-1}]
Isothermal titration calorimetry, $t = 25\,^{\circ}C$			
Single set of identical sites	25 ± 3	2800 ± 300	-1.5 ± 0.2
^{1}H NMR spectroscopy, $t = 20\,^{\circ}C$			
Single set of identical sites	30 ± 10	400 ± 200	
Micro-equilibrium dialysis,[a] $t = 20\,^{\circ}C$			
Single set of identical sites	30 ± 4	400 ± 120	

[a] Ref. Buczkowski et al. (2011).

connected with the drug binding by the superficial primary amine groups or internal tertiary amine groups.

Not all the peak maxima in ^{1}H NMR spectra describing the protons of methylene groups directly adjoining the internal tertiary amine group are shifted during increasing the concentration of 5-fluorouracil in the mixture. The change in the shift occurs in the case of protons of two dendrimer methylene groups on the carbonyl side of amide bond. The methylene group (2.6 ppm) situated on the amine side of the amide bond practically is not shifted. This suggests that the internal tertiary amine groups do not constitute active sites for bonding 5-fluorouracil. Then the changes in band III shift would be connected with the binding of the drug by the superficial protonated or unprotonated amine groups.

The values of the PAMAM-NH_2 G4 – drug complex formation constant and the numbers of drug molecules bound by the dendrimer are similar to those calculated from the model of the same active sites obtained from the results of equilibrium dialyses (Buczkowski et al., 2011) (Table 2).

4. Conclusions

From the calorimetric and ^{1}H NMR spectroscopic tests of 5-fluorouracil and PAMAM-NH_2 G4 dendrimer in aqueous solution it follows that this dendrimer forms supramolecular complexes with the oncologic drug: 5-fluorouracil. The parameters of bonding 5-fluorouracil with PAMAM-NH_2 G4 obtained by various techniques are listed in Table 2.

^{1}H NMR spectroscopic measurements indicate that the molecule of PAMAM-NH_2 G4 has about 30 active sites binding 5-fluorouracil with an equilibrium constant of $K = 400 \pm 200$. Similar binding parameters were obtained from the calculations based on the model of the same active sites that was used to describe the results of equilibrium dialyses (Buczkowski et al., 2011) (see Table 2).

The calorimetric examinations show that the molecule of the dendriner under investigation has about 25 ± 3 sites bonding 5-fluorouracil with an equilibrium constant of $K = 2800 \pm 300$. The binding of the drug by the dendrimer active sites is an exothermal process accompanied by a beneficial change in entropy.

The discrepancy between the values of equilibrium constant K obtained by the above discussed methods probably results from the different characters of the test techniques used, i.e. firstly a dynamic character of the calorimetric titration (an experimenter never knows if the system under examination has fully reached the equilibrium state) and a static character of the ^{1}H NMR spectroscopic measurements and micro-equilibrium dialysis (a thermostating period of tens hours was needed to reach the equilibrium state) (see Table 2), secondly the ITC parameters show non-equilibrium system of two processes: classical static binding (like NMR and equilibrium dialysis) and heat changes of process of dynamic binding-dissociation of 5-fluorouracil molecules (Markova et al., 2010; Pavel et al., 2005) with end groups of PAMAM G4 dendrimer.

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