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Does fluorescence of ANS reflect its binding to PAMAM dendrimer?

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Abstract

The analysis of binding between cationic PAMAM G5 dendrimer and anionic fluorescent probe using fluorescence and equilibrium dialysis has been made. It was found that at low concentrations of ANS the double fluorimetric titration technique can be successfully used for quantitative analysis of binding of ANS to dendrimer. Based on fluorescence and dialysis data the constants of binding and the number of binding centers were calculated for binding of ANS to PAMAM G5 dendrimer: K_b is approx. $(0.5–1) \times 10^5$ M^{-1} and n is (0.5–0.7).

Keywords: ANS; PAMAM dendrimer; Equilibrium dialysis; Fluorescence; Binding

1. Introduction

Dendrimers are a class of three-dimension polymers suitable for a wide range of biomedical applications: drug delivery, RNA, DNA and oligonucleotide targeting, detoxication, microarray systems, catalysis [1,2]. Dendrimers possess empty internal cavities and many functional end groups which are responsible for high solubility and reactivity. Drugs (guests) can either be attached to dendrimers' end groups or encapsulated in the macromolecule interior (host) (Meijer 'dendritic box') [3,4]. One of useful techniques for studying dendrimer-guest complexes is based on studying dendrimers interaction with

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fluorescent probes [3,4]. It was shown that the size of both the guest and the dendrimer cavity determines the complex stoichiometry and that the liberation of guests can be achieved by selective removal of the protecting terminal groups [3–5]. Earlier we studied the interaction between polyamidoamine (PAMAM) dendrimers and anionic fluorescent probe ANS. It had been shown that double fluorimetric titration technique can be successfully used for determination of parameters of binding [6,7]. Other researchers proposed a single titration fluorimetric assay for detection and quantitation of polyamidoamine dendrimers [8]. However, many reviewers asked a question: does fluorescence of ANS reflect its binding to PAMAM dendrimers? To answer this question we studied the interaction between anionic fluorescent probe ANS and cationic PAMAM G5 dendrimer (MW 28825 Da, diameter of 5 nm and 128 surface amino groups) by equilibrium dialysis and fluorescence.

2. Materials and methods

Polyamidoamine (PAMAM) G5 dendrimer (ethylenediamine core, amine surface) was purchased from Dendritic NanoTechnologies Inc. (USA), 1-anilinonaphthalene-8-sulfonic acid was obtained from Sigma (USA). All other chemicals were of analytical grade. Double distilled water was used to prepare the solutions. Phosphate-buffered saline (PBS 150 mM NaCl, 1.9 mM NaH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4) was used for all measurements. Before experiments PAMAM G5 dendrimer was dialyzed overnight at +4 °C against 0.15 M Na-phosphate buffer, pH 7.4. For equilibrium dialysis Micro-Equlibrium Dialyzer (Harvard Apparatus, USA) was used with 5 kDa ultra-thin membranes (Harvard Apparatus, USA). Dialysis was performed for 24 h at 25 °C in PBS in two chambers divided by ultra-thin membrane. In the first chamber the solution of pure ANS was added, in the second one the solution of "ANS+dendrimer" was added. Initially, ANS was added in both chambers in equal concentrations. Concentration of ANS after dialysis was determined spectrophotometrically using its molar extinction coefficient at 350 nm of $5 \times 10^3\ M^{-1}\ cm^{-1}$. For samples containing dendrimer the correction for possible absorbance of dendrimer at this wavelength was made. The concentrations of ANS bound to dendrimer, free and total ANS were calculated according to the equation:

$$C_{\text{bound}} = \frac{A - A_{\text{pure\_G5}}}{\varepsilon \cdot l} - \frac{A_{\text{pure\_ANS}}}{\varepsilon \cdot l} = C_{\text{total}} - C_{\text{free}} \quad (1)$$

where A—the absorbance of the "ANS + dendrimer" solution (from the second chamber), $A_{\text{pure\_ANS}}$—the absorbance of pure ANS (from the first chamber), $A_{\text{pure\_G5}}$—absorbance of pure PAMAM G5 dendrimer in the same conditions, ε—molar extinction coefficient of ANS, l—cuvette optical path in centimeter.

Fluorescence emission intensity and synchronous spectra were taken with a Perkin-Elmer LS-50B spectrofluorometer at 25 °C. The excitation wavelength was set at 360 nm and the emission spectra were registered between 400 and 700 nm. It was checked that dendrimers were not excited by 360 nm wavelength and did not emit fluorescence. The excitation and emission slit widths were 7 and 4 nm, respectively. Samples were contained in 1 cm path length quartz cuvettes and were continuously stirred. The binding constant (K_b) and the number of binding centers per one dendrimer molecule (n) were determined by a double fluorimetric titration technique and calculated by Scat-

chard–Klotz analysis [9]. The corrections of fluorescence intensity for double fluorimetric titration were made as described previously [10]. All data are expressed as a mean value ± SD of 6–8 independent experiments. Statistical significance was assessed using Student–Fisher test.

3. Results and discussion

Equilibrium dialysis is a direct technique for determination of binding parameters between ligands and hosts [11]. We performed the equilibrium dialysis for different concentrations of ANS in presence and absence of 50 μM PAMAM G5 dendrimer. After dialysis the difference in absorbance between "dendrimer + ANS" system and "only ANS" system (after taking into account the absorbance of pure dendrimer in the same conditions) was measured. It allowed for calculating the concentration of ANS that was bound to PAMAM dendrimer. The data are presented in Fig. 1A. Increasing of the total concentration of ANS led to the increase of concentration of ANS bound to dendrimer. The extrapolation of experimental points by Hill function (Fig. 1A) allowed to obtain the maximal concentration of ANS bound to dendrimer. For 50 μM PAMAM G5 dendrimer it was equal to 34 μM, i.e., *n*—the number of molecules of ANS per one molecule of dendrimer—was approximately 0.68. According to Scatchard–Klotz analysis (Fig. 1B) the experimental points of equilibrium dialysis are well approximated by single linear fit with K_b equaled to $0.47 \pm 0.27 \times 10^5$ M^{-1} and *n*—0.65 ± 0.22.

The pure ANS in aqueous solution had a weak fluorescence in a range 400–600 nm with a maximum at 525 nm. Adding of PAMAM G5 dendrimer to ANS solution led to a significant increase of ANS fluorescence intensity and to a blue shift of the emission maximum to about 500 nm. It indicates that ANS was placed in a more hydrophobic environment due to its interactions with dendrimer. The double fluorimetric titration of

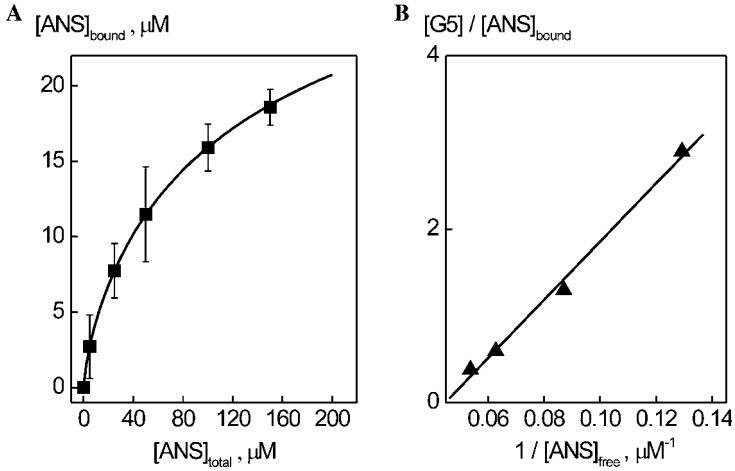

Fig. 1. (A) The dependence of concentration of bound ANS on concentration of total ANS in solution after equilibrium dialysis. Concentration of PAMAM G5 dendrimer is 50 μM. PBS, pH 7.4, 25 °C. (B) Scatchard–Klotz plots (data on equilibrium dialysis) of the dependence of concentration of bound ANS on concentration of free ANS in solution after dialysis. Concentration of PAMAM G5 dendrimer is 50 μM. PBS, pH 7.4, 25 °C.

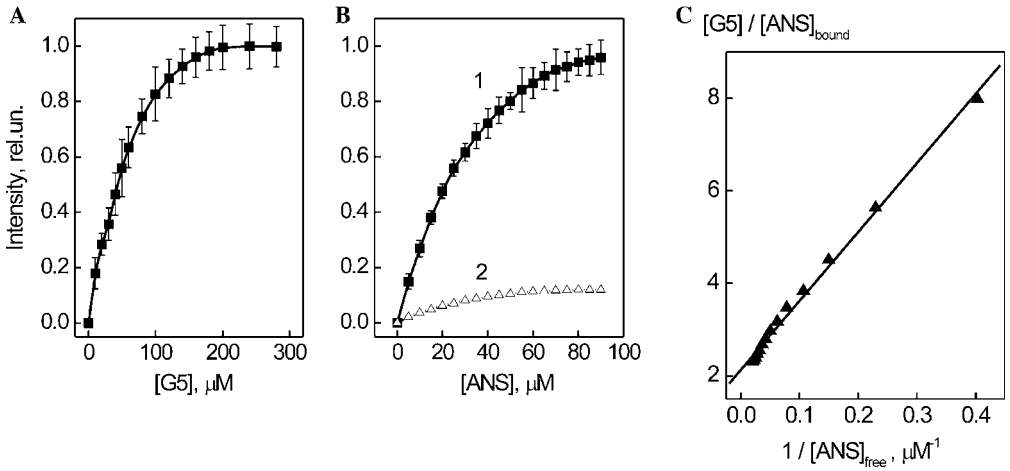

Fig. 2. (A) The dependence of fluorescence intensity of ANS upon addition of PAMAM G5 dendrimer. $\lambda_{exc} = 360$ nm, $\lambda_{em} = 500$ nm. Concentration of ANS is 10 μM. PBS, pH 7.4, 25 °C. (B) The dependence of fluorescence intensity of ANS upon its addition to 60 μM of PAMAM G5 dendrimer solution (curve 1). For comparison the dependence of fluorescence intensity of pure ANS upon its concentration in solution is presented in the same conditions (curve 2). $\lambda_{exc} = 360$ nm, $\lambda_{em} = 500$ nm. PBS, pH 7.4, 25 °C. (C) Scatchard–Klotz plots (data on fluorescence) of the dependence of concentration of bound ANS on concentration of free ANS in solution. Concentration of PAMAM G5 dendrimer is 50 μM. PBS, pH 7.4, 25 °C.

ANS by PAMAM G5 dendrimer is presented in Fig. 2. Scatchard–Klotz plots (Fig. 2C) can be well approximated by a single linear fit with $K_b = 1.43 \pm 0.27 \times 10^5\ M^{-1}$ and $n = 0.47 \pm 0.16$.

The comparison of results on equilibrium dialysis and fluorescence shows that these parameters are very close to each other. Some difference can be explained by different timescale of measurements: equilibrium dialysis was made after 24 h of equilibrium while fluorescence measurements were made in 15 min after addition of ANS (or PAMAM dendrimer).

Thus, double fluorescence titration technique can successfully be used for the fast and cheap preliminary quantitative analysis of binding parameters of newly synthesized dendrimers.

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