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ARTICLE TYPE

Evaluation of Electrostatic Binding of PAMAM Dendrimers and Charged Phthalocyanines by Fluorescence Correlation Spectroscopy

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We have assessed host-guest interactions between PAMAM dendrimers and charged phthalocyanine probes by Fluorescence Correlation Spectroscopy (FCS). Our results show strong binding in water at low ionic strength with an affinity that decreases from $K_B \sim 10^9$ to 10^8 M^{-1} upon decreasing the phthalocyanine charge of $z = -4, -2$ and -1 . The binding affinity also decreases significantly by salt addition leading to K_B values of ca. $10^5 - 10^6$ M^{-1} . The changes of binding affinity probed by varying the phthalocyanine charge, and by changing the ionic strength or pH conditions, allowed us to evaluate the electrostatic contribution (K_{el}) in dendrimer-phthalocyanine interactions. In particular, this approach afforded values of electrostatic potential for PAMAM dendrimers in water at low ionic strength and at dendrimer concentrations in the nanomolar range. The electrostatic potential of PAMAM generations 4 and 7 are around 50 mV in close agreement with theoretical estimates using Poisson-Boltzmann cell model. Interestingly, the nonelectrostatic binding is significant and contributes even more than electrostatic binding to dendrimer-phthalocyanine interactions. The nonelectrostatic binding contributes to an affinity of K_B above 10^5 M^{-1} , as measured in conditions of low dendrimer charge and high ionic strength, which makes these dendrimers promising hosts as drug carriers.

Introduction

PAMAM dendrimers are spherically symmetric macromolecules built by iteratively branching out of an amidamine moiety around an ethylenediamine core.¹ Each complete layer of amidamine moieties forms a full generation ($n = 0, 1, 2, \dots$) and dendrimers are referred to by their generation number (D_nG). PAMAM dendrimers with flexible branches have a globular shape and a size of a few nanometers, e.g., D4G has a hydrodynamic radius of ca. 2.3 nm.² The molecular structure of PAMAM dendrimers comprises several ionizable amine groups that confer them polyelectrolyte character. The terminal primary amines of these dendrimers are all protonated at neutral pH, while the interior tertiary amines are protonated in the pH range between 6 and 3.^{1,3,4}

Dendrimers find applications in many leading-edge fields, e.g., catalysis, drug delivery, photodynamic therapy, biosensing and multi-purpose scaffolds, among others.⁵ Pioneering studies have compared larger dendrimers with micelles because both systems can act as supramolecular host molecules capable of binding small guest molecules (guest@host complexes).⁶ PAMAM dendrimers have received most attention as potential transfection agents for gene and drug delivery, as these macromolecules can bind DNA and drugs at physiological pH. Initial studies of dendrimers as potential delivery agents focused on their use for noncovalent encapsulation of drug molecules.⁷⁻¹⁰

Phthalocyanines are adaptable macrocycles which are used as

dyes, sensors, in non-linear optics and photodynamic therapy of cancer (PDT).^{11,12} The physico-chemical properties of metal phthalocyanines may be altered by simply varying the central metal ion and substituents on the periphery.^{13,14} The inherent insolubility of phthalocyanines in aqueous media can be improved by introducing a variety of charged substituents, thereby enhancing their solubility and avoiding or decreasing self-aggregation. For instance, in this work we have used sulfonated aluminum phthalocyanines as charged fluorescent probes to study electrostatic interactions with PAMAM dendrimers in aqueous solution.

The nanoenvironment of the inner dendrimer structure is well suited for host-guest complexes, based on entrapment, hydrophobic and ionic interactions. The binding constant reveals the strength of host-guest interactions, and thus it is a good approach to probe these interactions upon changing medium conditions. Routinely, the binding constant of fluorescent probes with a macromolecule is obtained by conventional spectroscopic techniques such as steady state UV-Vis absorption and fluorescence emission. For instance, the interaction of Pc4 with Cytochrome c ($z = +8$) in water (3.3×10^5 M^{-1}) has been assessed by ensemble fluorescence measurements.¹⁵ A large binding constant (5×10^7 M^{-1}) was also found for the interaction of D4G with a bivalent sulfonate dye in buffered aqueous solutions,¹⁶ as well as for the interaction of HSA with D4G in PBS at pH 7.4 (1.67×10^6 M^{-1}).¹⁷

However, the measurement of binding affinities by conventional spectroscopic techniques may be impaired by low

probe solubility or self-aggregation,¹⁴ or even by the absence of significant changes in their spectroscopic properties, i.e., similar spectra of free and bound probe, the lack of quenching or wavelength shift, low molar absorption coefficient, etc.¹⁸ In this case, the technique of Fluorescence Correlation Spectroscopy (FCS) may offer an interesting alternative. The FCS technique measures fluctuations of fluorescence intensity during the Brownian diffusion of a fluorescent probe through the detection volume. When the hydrodynamic radii of free and bound probe are different enough, the technique of FCS provides a way to follow the binding of a fluorescent probe to a host macromolecule or colloid. Furthermore, it has the advantage that it can be performed with very small sample volumes and at very low concentrations of fluorophore probe, eventually at single-molecule level. Thus, it allows to obtain information at concentration ranges that are not easily accessible to conventional techniques. Several examples of reaction rates, formation of aggregates, conformational changes and dynamics of proteins have been determined by FCS, which is by now a well-established technique.¹⁸⁻²⁷

Recently, we have studied the electrostatic swelling of PAMAM dendrimers by FCS.²⁸ This study revealed changes in dendrimer diffusivity upon changing pH and/or ionic strength, which reflect both dendrimer swelling and electrolyte dissipation effects. The dendrimer swelling upon its protonation is not very pronounced, but it has been suggested to induce a change from a “dense-core” to a “dense-shell” type of structure.²⁹ This structural change may also affect the dendrimer’s ability to interact with guest molecules.

In the present work, we have assessed from FCS measurements the binding interactions between PAMAM dendrimers and oppositely charged phthalocyanine probes in aqueous solution. The number of sulfonate substituents in the phthalocyanine was varied from tetra-, bis- and mono-sulfonate substitution, in order to have a series of probe charges of $z = -4$, -2 and -1 . The changes in binding affinity of dendrimer-phthalocyanine upon varying the phthalocyanine charge, and the ionic strength or pH conditions, were used to probe the contribution of electrostatic interactions to the pair binding. This approach allowed us to retrieve the electrostatic potential of the PAMAM dendrimers in water at low ionic strength and at dendrimer concentrations in the nanomolar range. The electrostatic potential of macromolecules or colloids is an important parameter to assess their stability in solution or predict supramolecular interactions. In this work, by applying FCS technique to probe electrostatic interactions, it was possible to retrieve the electrostatic potential of PAMAM dendrimers in very dilute conditions, which are not easily accessible to other techniques. It was also interesting to find that nonelectrostatic interactions contribute as much as, or even more than, electrostatic interactions to dendrimer-phthalocyanine binding, which renders these dendrimers promising vehicles for drug carrying in physiological conditions, in which the ionic strength may strongly screen electrostatic interactions.

Experimental

Materials

Aluminium(III) phthalocyanine tetrasulfonate chloride (Pc4) was purchased from Porphyrin Products, Inc. and was used as received. Bisulfonate and monosulfonate phthalocyanines, Pc2 and Pc1 were synthesized according to Ambroz *et al.*³⁰ PAMAM dendrimers (*DnG*) with ethylenediamine core of generations 4 and 7 were supplied from Sigma-Aldrich as methanolic solutions with concentrations of 10% and 5% wt, respectively. Sodium phosphate dibasic heptahydrate $\geq 98\%$, ACS reagent; Citric acid anhydrous, puriss. p.a., ACS reagent; and Hydrochloric acid (HCl), puriss. p. a. 37%, were supplied from Sigma-Aldrich. Disodium hydrogen phosphate anhydrous, puriss. p.a. $>99.0\%$ was purchased from Fluka. Sodium chloride Reagent Plus $\geq 99.5\%$ was purchased from Riedel-de Haën (Sigma Aldrich). Potassium chloride $\geq 99.5\%$ was supplied from Fluka. Ultrapure water was obtained from a Milli-Q Gradient equipment (Merck Millipore). Resistivity after filtration was $\geq 18\text{M}\Omega\cdot\text{cm}$ (conductivity $\leq 5\mu\text{S}\cdot\text{cm}^{-1}$). Microscope cover glasses of $\text{Ø}22\text{mm}$ were produced by Menzel-Gläser (Gerhard Menzel GmbH). Atto655-COOH was supplied by Atto-Tec GmbH.

Sample preparation

All samples were freshly prepared at room temperature after solubilizing the required amount of product, or from dilution of stock solutions in ultrapure water. FCS samples of phthalocyanine solutions were diluted to the nanomolar concentration range typical in FCS. HCl/KCl stock solutions at pH 2 were prepared by mixing of 10.6 mL of HCl (0.2M), 50 mL of KCl (0.2M) and subsequently diluted to 200mL of water. Buffer Citrate/Phosphate (BCP) aqueous solutions were prepared from the mixture of appropriate amounts of stock solutions of 0.1M citric acid and 0.2M dibasic sodium phosphate in 100mL of water, and Buffer Phosphate (BP) was prepared from aqueous solutions of 0.2M monobasic sodium phosphate and 0.2M dibasic sodium phosphate diluted in 200mL of water.³¹ The pH of solutions was measured after and before dendrimer addition to test the buffer capacity at desired concentrations. The pH measurements were performed on a Crison microPH 2001 pH-meter, with electrodes Orion 910SBNWP from Thermo Electron Corporation. To prepare stock aqueous solutions of *DnG*, commercial methanolic solutions were evaporated first by a jet of N_2 and subsequently left under vacuum overnight. Finally, the viscous dendrimer oil was reconstituted with water to the desired concentration. FCS measurements were prepared by deposition of a drop ($\sim 10\mu\text{L}$) of each sample over glass coverslips. FCS titrations of phthalocyanines with dendrimer or salt were carried out in a home-made plastic cuvette immobilized to the coverslips with heat, as referred to previously.²⁸ A certain volume of initial sample was deposited in this “cuvette” and, subsequently, volumes of reactants were added and gently mixed before each measurement. Coverslips were thoroughly cleaned prior to use.

Methods and instruments

Fluorescence Correlation Spectroscopy: Time-traces of fluorescence intensity from two independent detectors were cross-correlated to give correlation curves, $G(\tau)$, which are free from artifacts of detector after-pulsing. The correlation curves

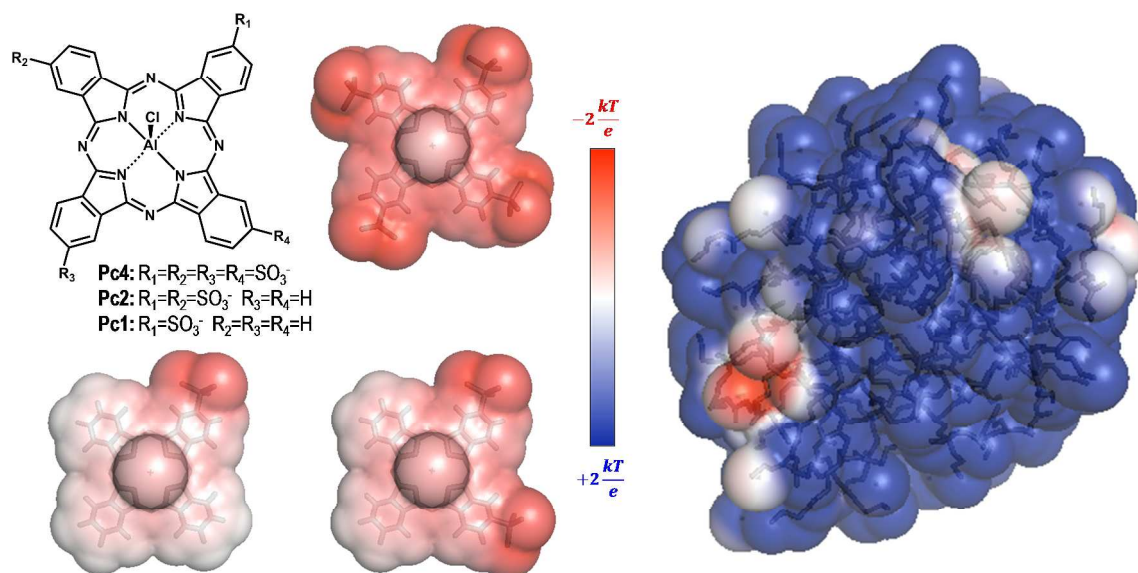
were analyzed in the time range longer than 10 μ s, where intensity fluctuations are exclusively attributed to translational motion of the probe across the detection volume. The correlation decays due to triplet-state transitions or eventual inclusion-exclusion reactions that occur at shorter times were disregarded. A pure diffusion model was used to fit the experimental correlation curves (see Electronic Supplementary Information, ESI).³² We have fitted the correlation curves of samples DnG-Pc either using a two-population model with distinct diffusion times for free and bound fluorescent probe,³³ or alternatively using a single average diffusion time.¹⁸⁻²⁰ In the latter case, when two interchanging fluorescent molecules coexist with diffusion coefficients of the same order of magnitude, we have considered the binding model proposed by Al-Soufi *et al.* (see ESI). We have assumed a 1:1 stoichiometry because phthalocyanine concentration is in the nanomolar range and dendrimer concentration is at least 10-fold greater, besides, no evidence of higher aggregates was found.^{20,21} Acquisitions times of 300 seconds were used in each measurement. Data analysis of individual correlation curves was performed using the software SymPhoTime (PicoQuant), or in a programmed spreadsheet. The focal area and detection volume were calibrated using as reference Atto655-COOH with known diffusion coefficient in water at 25°C ($D=426\ \mu\text{m}^2/\text{s}$).³⁴ FCS Measurements were carried out at room temperature of $(25 \pm 1)^\circ\text{C}$ in a Microtime 200 setup from PicoQuant GmbH. Equipment technical details were previously described.²⁸

Zeta-potential: The experimental zeta potential values of D4G

and D7G in water were determined in a Doppler electrophoretic light scattering analyser, Zetasizer Nano ZS from Malvern Instruments Ltd. The zeta-potential (ζ) was calculated from electrophoretic mobility according to the Henry's relation and Helmholtz-von Smoluchowski approximation. PAMAM Dendrimers are non-ideal surfaces and ζ -potential obtained herein should be considered as "effective" ζ -potential values.³⁵ Electrophoretic mobilities were automatically processed using Zetasizer software from Malvern Instruments Ltd. At least 3 different measurements were carried out for each dendrimer solution.

Results and Discussion

The interaction between positive PAMAM dendrimers and negative aluminium phthalocyanines originates a complex that has strong electrostatic character due to their opposite charge. The binding affinity of the dendrimer-phthalocyanine complex depends on experimental variables, such as pH and ionic strength, which strongly affect electrostatic interactions. The number of sulfonate substituents in the phthalocyanine also affects its binding affinity toward the dendrimer. In Chart 1, the electrostatic potential of phthalocyanines with one, two and four sulfonates – Pc1, Pc2 and Pc4, respectively – is mapped on the solvent accessible surface showing the gradual increase of negative charge.



Scheme 1 (Left, clockwise) Chemical structure of phthalocyanines dyes, and electrostatic potential on the solvent accessible surface of Pc4, Pc2 and Pc1 – red indicates negatively charged regions. (Right) Electrostatic potential surface of D4G dendrimer with all terminal amines protonated and condensed chlorine counterions – blue indicates positively charged regions.[†]

[†] Electrostatic potential on the solvent accessible surface were generated with the software "The PyMOL Molecular Graphics System", Version 1.3, Schrödinger, LLC. Molecular geometries for phthalocyanines were previously optimized using ZINDO method and atomic charges were determined from a single point DFT calculation using CHelpG scheme as implemented in Gaussian 98.³⁶ Molecular structure of D4G dendrimer was sampled from molecular dynamics simulations, and atomic charges were taken from the force field used in these simulations.³⁷

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The dendrimers' surface is strongly positively charged due to the terminal protonated amines, although, the condensation of chlorine counterions on the dendrimer partially compensates its positive charge. This shows up as regions of neutral or negative charge on the electrostatic potential surface of the dendrimer (see Scheme 1). The dendrimer's structural heterogeneity and its hydrophobic domains also play an important role in host-guest complexation and other type of interactions, of nonelectrostatic character, should not be disregarded, as it will later be shown.

PAMAM dendrimers show a strong binding affinity toward sulfonated phthalocyanines even in the nanomolar concentration range. This is experimentally observed through a sudden change of correlation curves and thus of the diffusion coefficient of phthalocyanine upon adding dendrimer in aqueous solution, as measured by FCS (Fig. 1A, see caption and ESI). The diffusion coefficient decreases from values of 325 – 345 $\mu\text{m}^2/\text{s}$ for free phthalocyanines to about 89 or 49 $\mu\text{m}^2/\text{s}$ for bound complexes with D4G or D7G dendrimers, respectively. This reflects a change in the hydrodynamic radius from free phthalocyanine, which is around 0.7 nm, to that of the dendrimer-phthalocyanine pair, which practically coincides with the dendrimer radius of 2.3 and 4.4 nm for the same generations.

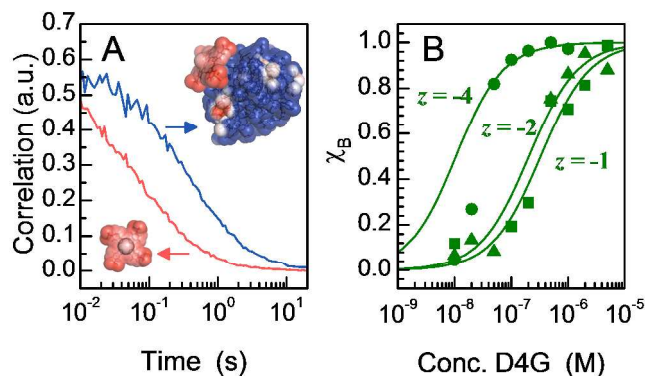


Fig. 1 (A) Experimental FCS autocorrelation curves for Pc4 in aqueous solution free (red) and bound to D4G (blue). (B) Bound molar fraction (χ_B) of phthalocyanines Pc4, Pc2 and Pc1 obtained at several dendrimer D4G concentrations in aqueous solution with KCl salt (60 mM). The phthalocyanines Pc4, Pc2 and Pc1 are identified by their charges, $z = -4$, -2 and -1 , respectively. Symbols are experimental data and lines are the best fit to a 1:1 binding model (see ESI).

By assuming a static two-population model of free phthalocyanine and bound dendrimer complex, and fitting it to experimental correlation curves, it is possible to obtain the fraction of bound phthalocyanine for each dendrimer concentration. The fraction of bound phthalocyanine (χ_B) increases with the concentration of dendrimer until full complexation of phthalocyanine in solution is achieved, as shown in Fig. 1B. The binding affinity can be obtained from these

curves of phthalocyanine bound fraction (χ_B) as a function of dendrimer-host concentration. In Fig. 1B, it is shown that the binding affinity of dendrimer-phthalocyanine pair decreases as the phthalocyanine charge decreases, which is perceived by a shift of bound fraction curves toward higher dendrimer concentrations.

The binding affinity of dendrimer-phthalocyanine was first measured in water at low ionic strength, i.e. in non-buffered media. In these conditions, the electrostatic interactions between dendrimer and phthalocyanine have their strongest contribution, as the dendrimer displays its maximum effective charge and ion screening of electrostatic interactions is minimum. For instance, the binding affinity of the pair D4G-Pc4 in water (non-buffered) is higher than $2 \times 10^9 \text{ M}^{-1}$ and, although it decreases along the series $\text{Pc4} > \text{Pc2} > \text{Pc1}$, it still has a value of $1.6 \times 10^8 \text{ M}^{-1}$ for the monosulfonated derivative (see Fig. 2A and Table S1). The dendrimers' effective charge depends strongly on pH and ionic strength due to their ionisable groups.²⁸ In distilled water, D4G is expected to have the primary amines almost completely protonated, and tertiary amines partially protonated. Therefore, the structural charge of D4G should be at least +64, although the effective charge is much lower due to the charge screening by counterion condensation. For instance, Chen *et al.* report an effective charge of ca. +30 at pH 5.³⁸ Indeed, the dendrimer charge reaches a maximum effective value as the ionization degree increases and further protonation of the dendrimer amines at low pH does not lead to an effective increase of dendrimer charge, as previously reported.²⁸ On the other hand, the sulfonic groups of phthalocyanine should be totally deprotonated in the whole range of pH, assuming the pKa of -2.7 for p-toluenesulfonic acid moiety.³⁹ In these conditions, a strong electrostatic interaction was expected between dendrimer and phthalocyanine due to the high charge density of PAMAM dendrimers (see Scheme 1).

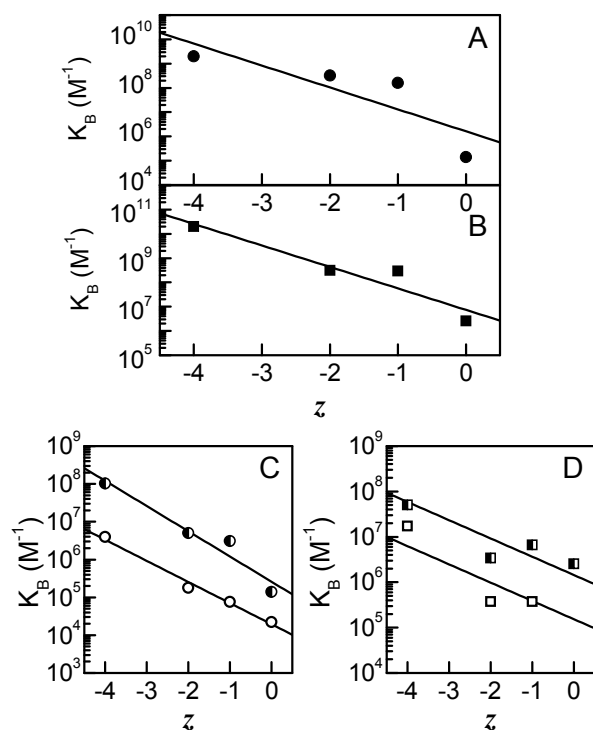


Fig. 2 Binding affinity (K_B) of dendrimer-phthalocyanine complexes in water at low ionic strength (non-buffered) for dendrimers (A) D4G and (B) D7G. The series are plotted as a function of phthalocyanine charge (z). For the point at $z = 0$, it was taken the value of the nonelectrostatic binding constant, K_{nel} , as obtained from salt addition experiments (inset Fig. 3), according to our data analysis (see text for further details). (C)

Binding affinity (K_B) of dendrimer-phthalocyanine complexes for dendrimer D4G in water with KCl salt, 60 mM (half-filled circles) and at pH 2 with HCl/KCl (empty circles). (D) The same for dendrimer D7G in water with KCl salt, 60 mM (half-filled squares) and at pH 2 with HCl/KCl (empty squares). Lines are linear regressions according to our data analysis with eq. 3 (see text for further details).

The values of binding affinity for D7G dendrimer are within one order of magnitude of those obtained for D4G dendrimer (Fig. 2B and Table S1). In most cases, the binding constants for these dendrimer generations have comparable values despite the large difference in size and charge numbers between these two generations, e.g. dendrimer D4G has a size of ca. 2.3 nm and 126 ionizable amines, while D7G has ca. 4.4 nm and 1022 amines.²⁸ The similarity between binding affinities could be explained if both generations have comparable surface charge densities, which would be a consequence of charge renormalization by counterion condensation, as it will be developed further ahead. About the nonelectrostatic contribution to dendrimer-phthalocyanine binding, this type of interactions are intrinsically akin between generations due to the self-similar structure of dendrimer molecules, although the larger surface area of D7G compared to D4G would, in principle, favour the higher generation.

With the aim of gaining further insight into electrostatic and nonelectrostatic contributions to dendrimer-phthalocyanine binding, we have measured the binding constants of the same phthalocyanine series: Pc4 – Pc2 – Pc1, with both dendrimer

generations, D4G or D7G, in other conditions besides non-buffered water, e.g. in the presence of salt or at low pH (Fig. 2 C,D). The selected conditions were in aqueous solution of KCl salt (60 mM) and acidic medium (HCl/KCl, pH2). The binding constants decrease more than one order of magnitude with added salt (KCl, 60 mM) due to screening of electrostatic interactions, although large values of K_B are still observed, such as $3.0 \times 10^6 \text{ M}^{-1}$ for D4G-Pc1 complex. In acidic medium (HCl/KCl, pH2), the values of binding constant decrease another order of magnitude relatively to conditions of added salt (KCl, 60 mM). This result was surprising because it was expected that full protonation of dendrimers at pH 2 would at most increase slightly their charge number and favour electrostatic interaction with phthalocyanines. As previously mentioned, the dendrimer's effective charge reaches a limit due to charge renormalization by counterion condensation, and it does not change appreciably from half- up to full protonation states.²⁸ This means that the dendrimer's effective charge should be approximately the same for the conditions of added salt (KCl, 60 mM) and in acidic medium (HCl/KCl, pH2). Also, the ionic strength, and thus charge screening, is similar for these two conditions. The contribution of electrostatic interactions is not enough to explain the discrepancy of one order of magnitude between the binding constants in added salt (KCl, 60 mM) and in acidic medium (HCl/KCl, pH2). We hypothesize that this discrepancy is due to a change in dendrimer structure from counterion uptake that occurs with protonation of the dendrimer's amine groups. This has been previously reported as a transition from a "dense-core" and a "dense-shell" type of structures.²⁹

In another set of experiments, the ionic strength of the medium was gradually increased by addition of salt to a solution of dendrimer-phthalocyanine completely bound ($\chi_B = 1$), and the gradual decrease of the bound fraction was followed by FCS (see Fig. 3). The charge screening of electrostatic interactions by the added ions significantly decreases the binding affinity of the dendrimer-phthalocyanine pair. However, even at large concentrations of salt of $\sim 0.5 \text{ M}$, the binding affinity is not completely screened off (see Table 2 and related discussion below). This means that, besides electrostatic interactions, there is a significant contribution of non-electrostatic interactions to dendrimer-phthalocyanine binding.

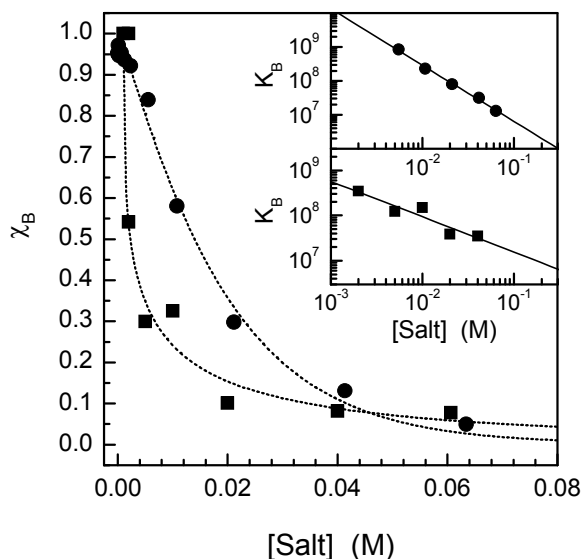


Fig. 3 Bound molar fraction (χ_B) of Pc4 with dendrimers D4G (circles) and D7G (squares) in water at several concentrations of added KCl salt – the dotted curves are guidelines. The inset shows the Record-Lohman plots and the best fits obtained with Eq. 1 to the binding affinity of Pc4 with D4G (top, circles) and D7G (bottom, squares).

In order to assess the non-electrostatic binding component, K_{nel} , it was employed the Record-Lohman model to analyse the data in Fig. 3.^{40,41} This model predicts a log-log relation between the observed binding constant and the concentration of bulk salt,

$$\ln K_B = \ln K_{nel} + a \ln[\text{Salt}] \quad (1)$$

here K_{nel} is the binding constant extrapolated to a large salt concentration (1M), and a is related with the number of released counterions, which is treated here as an empirical fitting parameter. Briefly, the values of bound fraction χ_B in Fig. 3 were used to calculate binding constants K_B shown in the insets assuming a 1:1 equilibrium for the dendrimer-phthalocyanine binding (see ESI for further details). The log-log plots in the insets of Fig. 3 show a linear relation over a couple of orders of magnitude in the binding constant. The fits performed with Eq. 1 are also shown here for generations D4G (top) and D7G (bottom). These have afforded K_{nel} values of $1.4 \times 10^5 \text{ M}^{-1}$ and $2.6 \times 10^6 \text{ M}^{-1}$ for generations D4G and D7G, respectively. As a test of internal consistency, the values of binding constant obtained from the last point of each series shown in Fig. 3, corresponding to a ionic strength of $\sim 60 \text{ mM}$, were compared to those of Fig. 2 C,D obtained for the same phthalocyanine (Pc4) at similar ionic strength (half-filled symbols). The larger value of K_{nel} found for D7G probably reflects the larger size of this generation compared to D4G, which provides more points of hydrophobic contact or hydrogen bonding at its surface, thus leading to a larger nonelectrostatic contribution to the binding constant.

According to our interpretation, the value of K_{nel} should be the same for all phthalocyanines. Thus, we have obtained an independent estimate of K_{nel} from the salt dependence of the binding constant between Pc2 and D7G. This afforded a K_{nel} of

$5 \times 10^5 \text{ M}^{-1}$, which agrees within experimental uncertainty with the value of $\sim 10^6 \text{ M}^{-1}$ given above for the salt dependence of the binding constant of Pc4 and D7G.

Data in Fig. 2 were also analysed assuming that binding energy (ΔG_B) has the contribution from two separate components: an electrostatic and a nonelectrostatic one,^{42,43}

$$\Delta G_B = \Delta G_{nel} + \Delta G_{el} = -RT \ln K_{nel} - RT \ln K_{el} \quad (2)$$

Furthermore, if we assume that the electrostatic component, K_{el} , may be described by a Coulombic expression,⁴⁴ then we obtain a linear correlation in log-scale of the total binding constant, K_B , with the charge, z , of guest species,

$$\ln K_B = \ln K_{nel} - z \cdot \frac{F \Delta \psi}{RT} \quad (3)$$

where F stands for the Faraday constant, R is the ideal gas constant, T is the temperature, and $\Delta \psi$ is the electrostatic potential of the host. According to this model, the component K_{el} will account for variations of the charge of host and guest, as well as screening of electrostatic interactions by added salt. On the other hand, K_{nel} will account for binding due to hydrogen bonding and hydrophobic interactions, among others. Fig. 2 shows a log plot of the binding constant obtained from FCS, as a function of the charge z of the phthalocyanine probe. According to Eq. 3, the intercept at $z = 0$ is given by $\ln K_{nel}$, which has been independently determined from Eq. 1 using the data of Fig. 3. Therefore, this value of $\ln K_{nel}$ was introduced at $z = 0$ in the plots of Fig. 2. This approach is fully consistent with our analysis using Eq. 3. These fits performed are shown in Fig. 2, and the values found for parameters K_{nel} and electrostatic potential $\Delta \psi$ of dendrimer host are given in Table 1.

Table 1 Parameters obtained from fitting Eq. 3 to results shown in Fig. 2: non-electrostatic binding constant, K_{nel} , and the dendrimer electrostatic potential, $\Delta \psi$. The values are given for dendrimer D4G and D7G in water at low ionic strength (non-buffered) and with added salt (KCl, 60 mM).

		$K_{nel} (\text{M}^{-1})$	$\Delta \psi (\text{mV})$
D4G	water (non-buffered)	$\sim 10^5$	53
	added salt (KCl, 60mM)		39
D7G	water (non-buffered)	$\sim 10^6$	52
	added salt (KCl, 60mM)		24

It is worth noticing that K_{nel} gives a significant contribution to the binding affinity of dendrimer-phthalocyanine complexes, being larger than K_{el} in the most favourable conditions of low ionic strength and a phthalocyanine charge of $z = -4$. The PAMAM dendrimers have relatively nonpolar pockets and highly reactive surface functionalities such as amines, carboxyl and hydroxyl groups. Different types of nonelectrostatic interactions have been reported concerning the interaction of non-steroidal anti-inflammatory drugs with PAMAM dendrimers, such as: *i*) hydrophobic or π - π interactions between the interior cavities of the dendrimer and the hydrophobic region of the drug; *ii*)

hydrogen-bond, van der Waals, etc.⁴⁵ It is well-known the capacity of PAMAM dendrimers to dissolve small hydrophobic and non-charged molecules.^{46,47} The flexible structure of these dendrimers with its many functional groups spread throughout is likely to create environments prone for hydrophobic or hydrogen bond interactions. The phthalocyanines are macrocyclic molecules and it is not likely that these molecules are completely internalized in D4G or D7G dendrimers, but the entrapment in pockets or grooves formed on the dendrimer's surface is likely to occur. Therefore, the component K_{nel} may be related to interactions with inner parts or the folded branches of the dendrimer.

Interestingly, K_{nel} decreases by one order of magnitude when the pH is reduced to 2 with HCl/KCl. This result agrees with the hypothesis suggested above that it is a change in the dendrimer's structure from a "dense core" to a "dense shell" conformation that is responsible for a decrease of binding constant at low pH, because it would affect mainly K_{nel} , but not necessarily the K_{el} contribution. The counterion uptake by the dendrimer structure at low pH seems to impair nonelectrostatic interactions of the phthalocyanine guest molecules with either hydrophobic contacts or hydrogen bonds of the dendrimer host.

The electrostatic potential $\Delta\psi$ shown in Table 1 for dendrimers D4G and D7G in non-buffered water are about 50 mV for both generations. These values agree in order of magnitude with those reported from a theoretical study, which predict electrostatic potentials of 30 – 40mV for D4G and of 60 – 80 mV for D7G.⁴⁸ Actually, this study reported on calculated values of ζ -potential, which does not necessarily have to coincide with the electrostatic potential $\Delta\psi$ from Eq. 3. Another study reported an experimental value of 77 mV for the ζ -potential of PAMAM dendrimers of generation 6 at neutral pH and ionic strength of 0.15 M.⁴⁹ This value is also of the same order of magnitude, but again larger than our $\Delta\psi$ values. However, both theoretical and experimental studies report on conditions that are quite different from our measurements done at very dilute dendrimer and salt concentrations. Moreover, we have measured the ζ -potential for D4G and D7G in water at low ionic strength (non-buffered) and for dendrimer concentrations of 10^{-5} and 10^{-6} M. These measurements afforded values around 30 mV for both generations (see Fig. 4). The values of ζ -potential are similar for both generations and relatively lower than those from the literature mentioned above.

We have also used Poisson-Boltzmann cell model in order to theoretically estimate the dendrimer electrostatic potential for the same conditions as our measurements. The details of these calculations are given elsewhere.²⁸ From the calculated electrostatic potential, it is possible to obtain the radial distribution of counterions, $N_{\text{ci}}(r)$, and assuming that counterions strongly attracted become condensed on the dendrimer, then the effective charge may be estimated. The criterium of Bjerrum has been used to define a distance below which counterions are assumed to be condensed by using the inflection point of the radial distribution of counterions, $(d^2N_{\text{ci}}/dr^2)_{r=r^*} = 0$. In Fig. 4, the values of electrostatic potential calculated from Poisson-Boltzmann cell model at the inflection point, $\phi(r^*)$, are shown for several dendrimer concentrations (grey symbols connected by lines). Curiously, we find a close agreement between this

estimate of the electrostatic potential $\phi(r^*)$ and the measured ζ -potentials for both generations. This result is not obvious because the slipping plane defined for the ζ -potential does not have to necessarily coincide with the inflection point at which the electrostatic potential from Poisson-Boltzmann cell model was calculated.

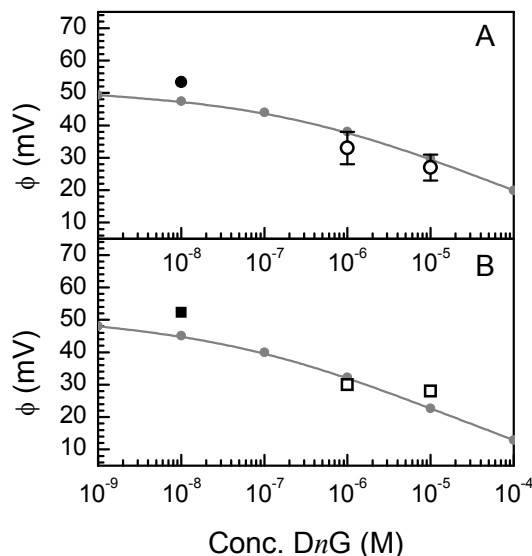


Fig. 4 Estimated values of the electrostatic potential calculated from Poisson-Boltzmann cell model for dendrimers: (A) D4G and (B) D7G (see text for further details). The empty circles and squares show the experimental ζ -potential values measured for D4G and D7G, respectively. The black-filled circle and square show the electrostatic potential $\Delta\psi$ obtained from Eq. 3 for generations D4G and D7G in water at low ionic strength (non-buffered).

Another interesting agreement is found between the electrostatic potentials calculated from Poisson-Boltzmann, $\phi(r^*)$, and those obtained from Eq. 3 of $\Delta\psi \sim 50$ mV for generations D4G and D7G in non-buffered water (black-filled symbols in Fig. 4). In this case, the values are compared in the nanomolar concentration range, because it is the typical concentration range of titration curves measured by FCS for dendrimer-phthalocyanine binding in water (non-buffered). The definition of electrostatic potential from Eq. 3 is related to the binding of a charged guest to the dendrimer host. On the other hand, the potential from Poisson-Boltzmann, $\phi(r^*)$, is a long-ranged potential, and we hypothesize that the coincidence between these values may be explained by $\phi(r^*)$ being close to an effective potential that is probed by the charged guest upon binding.

Some critical remarks are due about separating the binding constant in two independent contributions of electrostatic and nonelectrostatic interactions, as assumed in Eq. 2. The component K_{el} depends on the host and guest charges, as well as on the bulk ionic strength, whereas K_{nel} is assumed to be constant between the different guest molecules. In the case of the phthalocyanines used as guest molecules, the charged sulfonic groups are spread on the periphery of the macrocycle. Thus, the molecule's hydrophobic

surface increases when a charged group is replaced by a non-charged group along the series Pc4 – Pc2 – Pc1. This may lead to an increase of the nonelectrostatic contribution to the binding constant along the same series $Pc4 < Pc2 < Pc1$, which otherwise is assumed to be invariable in Eq. 2. This increase may affect the values of K_{el} retrieved from the linear fits of Figs. 2, then leading to artificially lower values of the surface potential or to higher values of K_{nel} component.

Further experiments were carried out using buffers citrate/phosphate (BCP) and phosphate (BP) to cover a wide range of pH (Fig. 5). For instance, the binding constant at pH 5 in BCP medium ($1.0 \times 10^6 \text{ M}^{-1}$) is three orders of magnitude lower than in water (non-buffered), because of the higher ionic strength of the buffer. The dendrimer-phthalocyanine binding constants were also measured at pH 3 and 8 using BCP and BP media. Furthermore, the variation of ionic strength between buffers was compensated by addition of NaCl salt, and the binding constants were again measured at comparable ionic strengths (Table 2). The values obtained at pH 3 (first column, Table 2) show a decrease by two orders of magnitude in binding affinity, as the ionic strength is increased from 0.034 to 0.540 M. The value of K_B at pH 3 in BCP and high ionic strength is comparable to K_{nel} previously obtained at pH 2 with HCl/KCl, as it would be expected. Following the values of K_B at high ionic strength (last line, Table 2), it is observed a gradual increase, up to one order of magnitude, as the pH is increased from 3 to 5 and 8. This result is also in agreement with the difference of one order of magnitude in K_{nel} between neutral and low pH conditions previously discussed, and explained with an effect of counterion uptake at low pH on the dendrimer's ability to establish nonelectrostatic interactions with guest molecules.

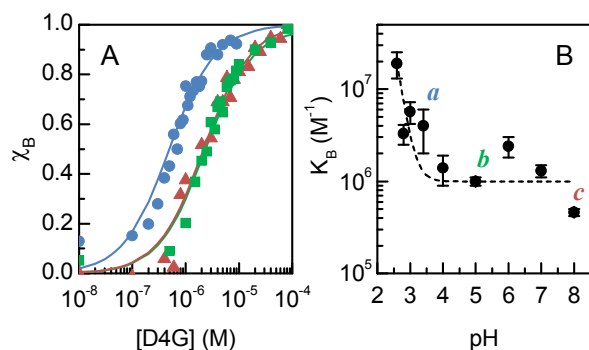


Fig. 5 Bound molar fraction (χ_B) obtained for D4G-Pc4 in buffers BCP or BP at pH 3 (blue circles), pH 5 (red triangles) and pH 8 (green squares). Symbols are experimental data and lines are best fits to a simple equilibrium model of 1:1 association. (B) Binding affinities of D4G-Pc4 in BCP solutions at different pHs calculated from several measurements identical to those shown in part A. At each pH, concentration of Pc4 was fixed at ca. 1 nM, while the concentration of D4G was varied. The letters a, b and c identify the binding affinities retrieved from the curves shown in part A using the same colour code (a, blue; b, green and c, red).

Table 2 Binding affinity (K_B) of D4G-Pc4 in BCP and BP at different pH and ionic strengths (I_s). The ionic strength was adjusted with NaCl salt.

	K_B (10^5 M^{-1})			
		pH 3	pH 5	pH 8
I_s (M)	0.034	60±15	—	—
	0.110	10±2	10±0.1	—
	0.540	0.29±0.04	1.5±0.2	4.6±0.4

In Fig. 5B, it is represented a set of measured binding constants in the range of pH 2 – 8. Below pH 4, there is a slight increase of the binding constant of D4G-Pc4 reaching a value of $2 \times 10^7 \text{ M}^{-1}$ at pH 2.6. In this pH range, the ionic strength of the buffer decreases from 76 to 18 mM (see Fig. S5), which suggests that the increase in binding constant is due to a larger contribution from electrostatic interactions. Between pH 4 and 8, the binding affinity is around 10^6 M^{-1} and does not change appreciably. In this pH range, there is an increase of the ionic strength of the buffer, and also dendrimer protonation decreases, thereby lowering the dendrimer charge. Although both factors contribute to a decrease of electrostatic interactions, the binding affinity keeps constant at a value around 10^6 M^{-1} , which suggests that nonelectrostatic binding is prevailing in these conditions. For the purpose of drug carrying in physiological media, this is a very promising result that the host-guest association between dendrimer and phthalocyanine is still very strong even at pH 7 and high ionic strength conditions.

Conclusions

We have determined apparent binding constants of dendrimer-phthalocyanine complexes by measuring their diffusion times using FCS technique. These experiments were carried out at very diluted concentrations of guest, close to single-molecule level, avoiding conditions of self-aggregation and complex stoichiometries higher than 1:1. Our results show a strong association of dendrimer-phthalocyanine pair in several conditions of pH and ionic strength, which could be useful for the application of these dendrimers as drug carriers. The changes of binding affinity observed for the several conditions probed were used to evaluate the electrostatic and nonelectrostatic components of dendrimer-phthalocyanine binding. The nonelectrostatic binding (K_{nel}) is actually the major contribution to the dendrimer-phthalocyanine binding, in spite of strong electrostatic attraction between the highly charged dendrimer-host and oppositely charged phthalocyanine-guest. The approach used here allowed to obtain estimates of the electrostatic potential of dendrimers at very dilute conditions. Although colloidal stability is not critical for dilute conditions, it is potentially interesting from a fundamental standpoint to assess electrostatic potentials in conditions that are not easily accessible to other techniques. The electrostatic potential values obtained are around 50 mV and are comparable for both generations D4G and D7G, which was attributed to an effect of charge renormalization due to counterion condensation.

In conclusion, we were able to extract significant information about the binding ability of PAMAM dendrimers as a host for

charged phthalocyanine guest molecules. The approach followed here may be applied to other studies seeking to achieve control of drug uptake and release, aiming at a future development of effective drug-delivery dendrimeric systems.

5

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† Electronic Supplementary Information (ESI) available: 1. Determination of binding affinity K_B by FCS technique; 2. Analysis of FCS curves using average diffusion times; 3. Additional results from titrations by adding salt. See DOI: 10.1039/b000000x/

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