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

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Electronic Supplementary Information

Contents:

1. Determination of binding affinity K_B by FCS technique

In this section, we give further details on the approach used to obtain the binding affinity K_B of dendrimer-phthalocyanine complexes in aqueous solution from FCS technique. The FCS measurements were performed either: *i*) with a drop on top of a glass coverslip, or *ii*) by titration in a home-made liquid cell. In this case, a home-made liquid cell was built by gluing a pipette tip to a glass coverslip, thoroughly cleaned prior to use (Scheme S1). The advantage of performing titrations is that it allows us to keep always the same measurement position on the coverslip glass for a series of dendrimer concentrations, or salt addition. This procedure avoids experimental uncertainties in the diffusion coefficient measured by FCS due to variations in coverslip thickness, as theoretically evaluated in [Enderlein *et al. ChemPhysChem,* 2005, **6**, 2324].
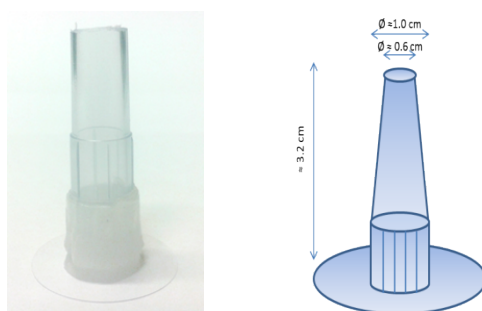
Scheme S1 – Home-made liquid cell used for the titration procedure in FCS measurements.

Each titration series begins by inserting an initial volume of phthalocyanine solution in the liquid cell, and performing an FCS measurement to calibrate the detection volume. The diffusion coefficient of each phthalocyanine, Pc4, Pc2 and Pc1, were previously calibrated with the reference dye Atto655-COOH (D= 426 µm^2/s, water at 25ºC). Subsequently, well-defined volumes of a stock solution of dendrimer are added and carefully mixed before each FCS measurement. In this way, it was possible to increase the dendrimer concentration from sub-nanomolar up to micromolar range. Some selected examples of the FCS curves measured by a titration procedure are shown in Fig. S1.
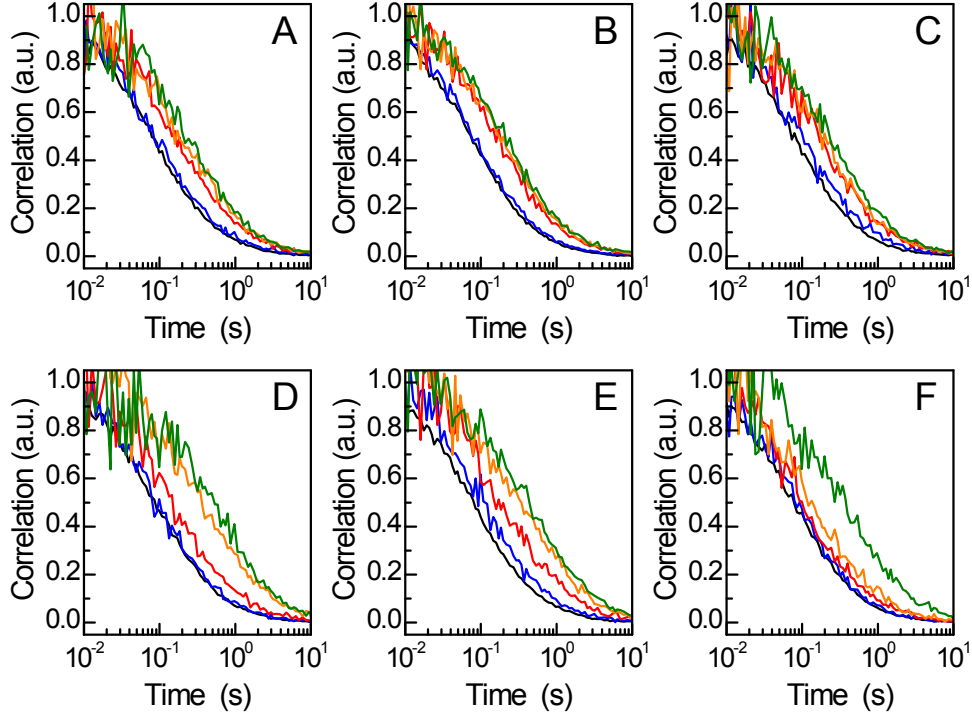
Fig. S1 Experimental FCS autocorrelation curves from selected titration series performed to determine the binding affinity of dendrimer-phthalocyanine complexes in aqueous solution free with KCl salt (60 mM). The top row was measured for dendrimer D4G and phthalocyanines (A) Pc4, (B) Pc2 and (C) Pc1, and bottom row was measured for dendrimer D7G and phthalocyanines (D) Pc4, (E) Pc2 and (F) Pc1. The values of bound molar fraction retrieved from these titration experiments are plotted in Fig. 1B of the main text and in Fig. S2, B and E.

The autocorrelation curves were fitted with a model equation that considers two populations, one of "free" and another of "bound" fluorescent probe,

$$G(\tau) = \frac{N_{free}Q_{free}^2\left(1+\dfrac{\tau}{\tau_{free}}\right)^{-1}\left(1+\dfrac{\tau}{\kappa\cdot\tau_{free}}\right)^{-1/2} + N_{bound}Q_{bound}^2\left(1+\dfrac{\tau}{\tau_{bound}}\right)^{-1}\left(1+\dfrac{\tau}{\kappa\cdot\tau_{bound}}\right)^{-1/2}}{\left[N_{free}Q_{free}+N_{bound}Q_{bound}\right]^2}$$

(S1)

here, N_{free} and N_{bound} are the average number of free and bound species in the detection volume, respectively. Q_{free} and Q_{bound}, are the brightness of each species, and τ_{free} and τ_{bound} are the respective diffusion times.

The brightness Q depends on the excitation cross-section and emission quantum-yield of the fluorophore, and also on optical and instrumental parameters. In fitting Eq. S1, we have taken into consideration eventual changes in the emission quantum-yield of

phthalocyanine bound to PAMAM dendrimers, as previously observed by some of us [Paulo *et al. J. Phys. Chem. C,* 2010, **114**, 19035]. This was done by adjusting the relative brightness Q_{free}/Q_{bound} that accounts for differences in the emission quantum-yield assuming that other parameters are not changed.

We have also determined the following values of diffusion coefficient of 325±7, 339±4 and 345±6 µm²/s for phthalocyanines Pc4, Pc2, and Pc1, respectively, in aqueous solution. These values were used to calibrate the detection volume at the beginning of each titration. The diffusion times of the "bound" species, τ_{bound}, was used to calculate the respective diffusion coefficient, which afforded values of 89±6 µm²/s for D4G and 49±1 µm²/s for D7G dendrimers, as previously determined by some of us [Garcia-Fernandez *et al. J. Phys. Chem. Lett.,* 2014, **5**, 1472].

Within each titration series, the several FCS curves were fitted using a global analysis procedures by defining Q_{free}, Q_{bound}, τ_{free} and τ_{bound} as common parameters, and allowing N_{free} and N_{bound} to be individually fitted. The average numbers of free and bound species were used to calculate a molar fraction of bound phthalocyanine probe,

$$\chi_B = \frac{N_{bound}}{N_{free} + N_{bound}} \tag{S2}$$

Assuming a simple 1:1 equilibrium model for the binding of phthalocyanine (guest) to dendrimer (host) in aqueous solution,

$$Guest + Host \overset{K_B}{\leftrightarrow} Complex \tag{S3}$$

Then, the bound fraction χ_B can be related to the binding affinity K_B through,

$$K_B = \frac{\chi_B}{(1 - \chi_B)([Host] - \chi_B[Guest])} \tag{S4}$$

here [Guest] and [Host] are the analytical concentrations of guest and host, respectively (that differ from equilibrium concentrations by the amount of complex formed).

The values of bound molar fraction χ_B obtained from fitting Eq. S1 to several titration series performed are shown in Fig. S2. The experimental values of χ_B obtained from each titration series were adjusted using Eq. 4 to find the respective binding affinity. The values of K_B obtained using this approach are shown in Fig. 2 of the main text, and are given below in Table S1.
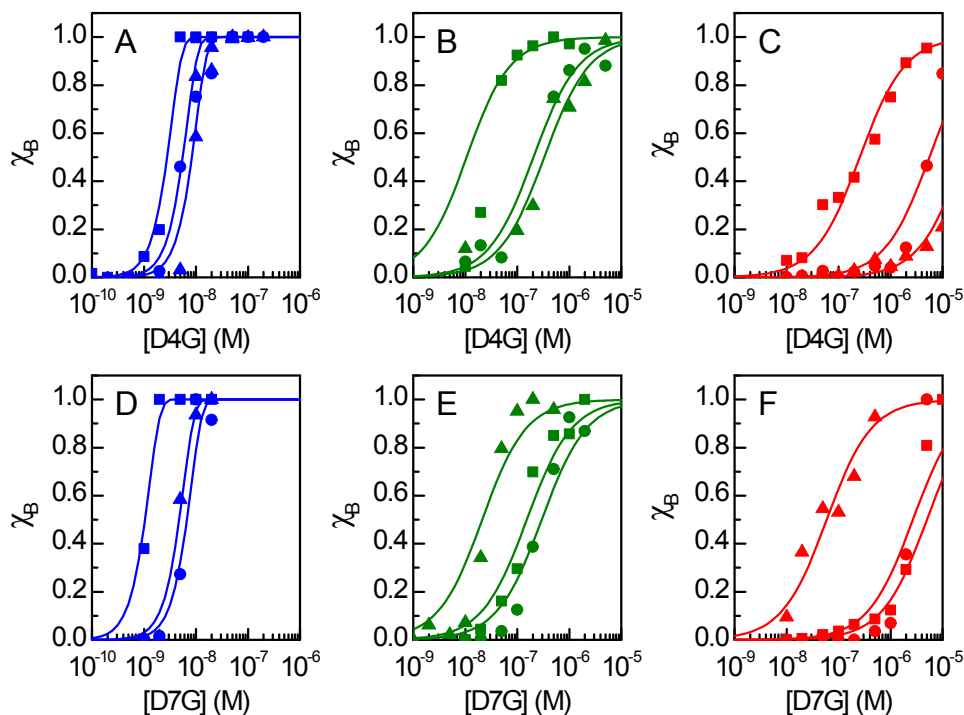
Fig. S2 Bound molar fraction (χ_B) of Pc4 (squares), Pc2 (circles) and Pc1 (triangles) obtained at several dendrimer concentrations in aqueous solution from selected titration series. The first row shows the titration series with dendrimer D4G in (A) non-buffered water, (B) aqueous solution with KCl salt (60 mM), and (C) in buffer HCl/KCl at pH2. The second row shows the same results for dendrimer D7G (D-F).

Table S1 Values of binding affinity K_B of dendrimer-phthalocyanine complexes obtained from data of Fig. S2 for conditions of non-buffered water, with KCl (60 mM) and at pH 2 buffer KCl/HCl.

	K_B / M^{-1}		
	Pc1	Pc2	Pc4
	D4G		
Water (non-buffered)	1.6×10^8	3.3×10^8	2.0×10^9
with KCl (60 mM)	3.0×10^6	5.0×10^6	1.0×10^8
at pH 2, HCl/KCl	7.6×10^4	1.8×10^5	3.9×10^6
	D7G		
Water (non-buffered)	2.9×10^8	3.1×10^8	2.0×10^{10}
with KCl (60 mM)	6.6×10^6	3.4×10^6	5.0×10^7
at pH 2, HCl/KCl	3.7×10^5	3.7×10^5	1.7×10^7

2. Analysis of FCS curves using average diffusion times

Al-Soufi and co-workers have proposed an alternative analysis that is more suitable for the single curve fitting procedure [Al-Soufi *et al. J. Am. Chem. Soc.*, 2005, **127**, 8775]. In eq. S3 it is shown the host-guest binding reaction, in which K_B is the ratio of association (k_a) and dissociation (k_d) rate constants, respectively. They proposed that a mean diffusion time ($\bar{\tau}_D$) is obtained when relaxation time of binding ($1/k_a$) and unbinding reaction ($1/k_d$) of eq. S3 is much faster than the typical transit time of the probe, (τ_R)

$$\tau_R = (k_a[Host] + k_d)^{-1} \tag{S5}$$

The diffusion time obtained in this way will be an averaged diffusion time, depending on the molar fractions of free (guest) and bound (complex) fluorescent probes.

$$\left(\frac{1}{\bar{\tau}_D}\right) = \chi_{free}\left(\frac{1}{\tau_{free}}\right) + \chi_{bound}\left(\frac{1}{\tau_{bound}}\right) \tag{S6}$$

After some straightforward algebra starting from eq. S6, we can obtain the bound molar fraction (χ_B) as a function of mean diffusion time ($\bar{\tau}_D$).

$$\chi_B = \frac{\tau_{bound}\left(\tau_{free} - \bar{\tau}_D\right)}{\bar{\tau}_D\left(\tau_{free} - \tau_{bound}\right)} \tag{S7}$$

It is also possible to write the binding constant, K_B, as a function of the mean diffusion time ($\bar{\tau}_D$), substituting Eq. S7 in S4, which can be simplified for [Host]»[Guest] conditions, giving the Eq. S8. Alternatively, Eq. S8 can be reformulated in the way of ($\bar{\tau}_D$) being a function of host concentration, as written by Al-Soufi and co-workers.

$$K_B = \frac{\tau_{bound}\left(\tau_{free} - \bar{\tau}_D\right)}{[Host]\,\tau_{free}\left(\bar{\tau}_D - \tau_{bound}\right)} \tag{S8}$$

From the fits of the autocorrelation curves, as the examples shown in Fig. 1A and Fig. S1, we obtain a mean diffusion time for each dendrimer concentration. Binding constants are then obtained by fitting ($\bar{\tau}_D$) to Eq. S8 in its alternative way (Fig. 5, main text). We have also performed some titrations in which phthalocyanine and dendrimer concentrations were fixed and bulk conditions were changed to evaluate indirectly the binding of this pair by means of Eq. S8.
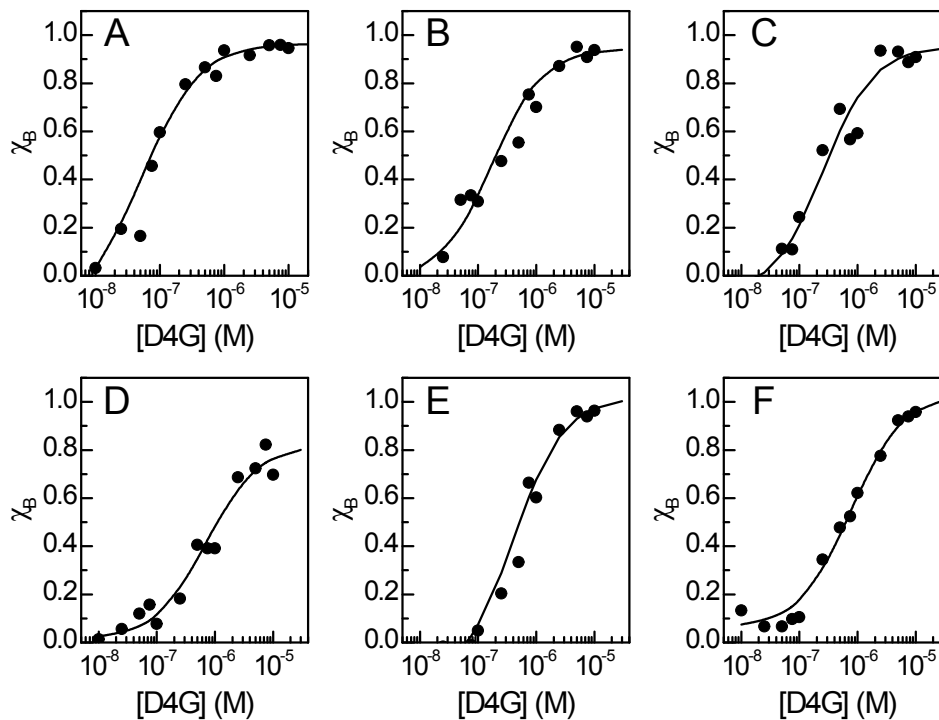
Fig. S3 Bound molar fraction (χ_B) of Pc4-D4G obtained in buffer BCP at several pHs. Circles and lines are experimental and fitted points obtained from Eqs. S7 and S8 in the same way as those of Fig. 5A. A-F panels correspond to pHs 2.6, 2.8, 3.4, 4.0, 6.0 and 7.0, respectively (see also Fig. 5). pHs 3.0, 5.0 and 8.0 can be found in Fig. 5A.

3. Additional results from titration series by addition of salt

The set of experiments done by gradually adding salt to a solution of dendrimer-phthalocyanine completely bound ($\chi_B = 1$) were repeated at a concentration of D4G of 1 μM (Fig. S4). This dendrimer concentration is significantly larger than that previously used in Fig. 3 of the main text, which was in the nanomolar range. For this purpose, Pc4-D4G solutions were titrated with NaCl in non-buffered water and buffer HCl/KCl, pH2, to obtain independent values of K_{nel} and $\Delta\psi$. The approach used for data analysis was described in section 2 of this ESI. The K_{nel} obtained for Pc4-D4G in non-buffered water was ∼10^5 M^{-1}, thus, in close agreement to that obtained previously from the data in Fig. 3. On the other hand, the value of K_{nel} ∼10^4 M^{-1} for Pc4-D4G in buffer HCl/KCl, pH2, was used as point z =0 in Fig. 2C.
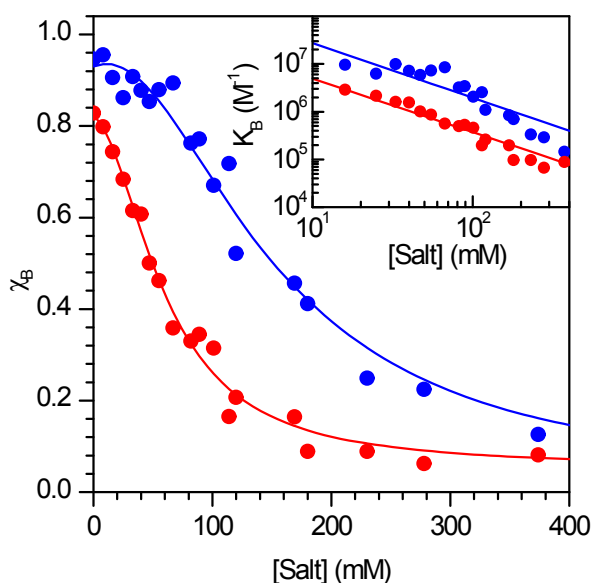

Fig. S4 Bound molar fraction (χ_B) of Pc4-D4G solutions obtained at different NaCl concentrations in non-buffered water (blue) and buffer HCl/KCl (red). Circles are experimental results obtained from Eq. S7 and solid lines are just guidelines. The inset shows the values of binding affinity estimated from Eq. S8 and fitted with Eq. 1. Pc4 and D4G concentration were kept at ca. 1nM and 1uM, respectively.
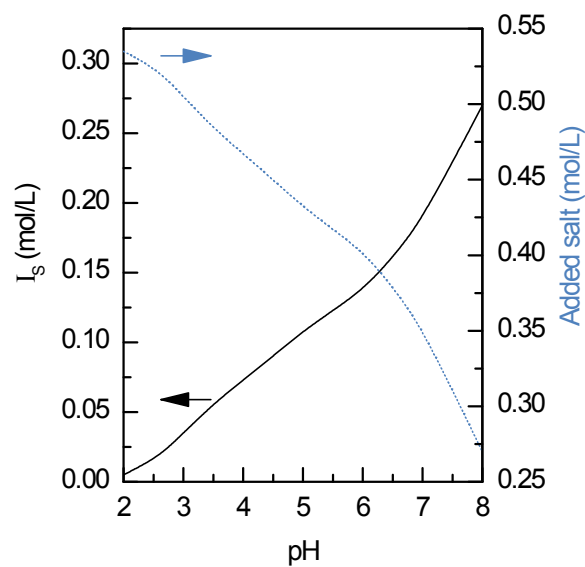
Fig. S5 Ionic Strength of the buffer (left axis) and added NaCl concentration (right axis) for BCP and BP solutions at fixed ionic strength of 0.54M (see Table 3).