Supplementary information

Fluorescent supramolecular nanoparticles signal the loading of electrostatically charged cargo

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EXPERIMENTAL PART

Materials and equipment

Chemicals and solvents were obtained from Sigma-Aldrich and used as received. PEI was purchased from Polysciences. *N,N*-Diisopropylethylamine (DIPEA) was obtained from Biosolve. PiBMA-CD and PiBMA-TBP were prepared as described before.[1] Deuterated NMR solvents were purchased from Cambridge Isotope Laboratories Inc. 6-Monodeoxy-6-monotosyl-β-cyclodextrin, 6-monodeoxy-6-azido-β-cyclodextrin, and 6-monodeoxy-6-monoamino-β-cyclodextrin were synthesized according to literature procedures.[2-4] MilliQ water with a resistivity of 18.2 MΩ cm at 25 °C was used in all experiments. ^{1}H-NMR spectroscopy was performed on a Bruker 400 MHz NMR spectrometer. Dynamic light scattering (DLS) measurements were performed on a Zetasizer NanoZS (Malvern Instrument Ltd, Malvern, United Kingdom) with a laser wavelength of 633 nm and a scattering angle of 173˚. UV/VIS absorption spectra were recorded using a Perkin Elmer Lambda 850 UV-VIS spectrometer. Fluorescence spectra were recorded using a Perkin Elmer LS 55 fluorescence spectrophotometer equipped with a high energy pulsed Xenon source for excitation. High resolution scanning electron microscopy (hrSEM) pictures were taken on a Carl-Zeiss 1500 hrSEM.

Synthesis of the components

Synthesis of PiBMA-CD-RhB

To a solution of PiBMA (20 mg, 3.33 µmol) in dry DMSO (2 mL) was added a solution of Lissamine RhB ethylene diamine (2.5 mg, 0.005 mmol) in dry DMSO (2.5 mL). The mixture was allowed to react at 45 °C for 1 day, after which a solution of 6-monodeoxy-6-monoamino-β-cyclodextrin (60.5 mg, 0.053 mmol) and DIPEA (81 µL, 0.107 mmol) in DMSO (1.5 mL) was added. The mixture was reacted for 2 days at 60 ˚C. To the crude reaction mixture, water (10 mL) was added and the unreacted anhydride

rings were opened with 0.1 M $NaOH_{aq}$ (1.3 mL). Subsequently, the mixture was purified by dialysis (SpectraPor membrane, MWCO 6-8 kD) for 1 week. Pure product grafted with 5 cyclodextrins and 1.5 RhB (PiBMA-CD-RhB) units was obtained by freeze-drying (10 mg, 42% yield).The degree of grafting of RhB was calculated with a calibration curve (Fig. S2).The degree of grafting of CD was calculated from the ratio between the CH_3 protons of the polymer and the C1-H of β-CD. ^{1}H-NMR (400 MHz, D_2O, pH 11): δ 0.25-1.4 (br, 239 H, CH_3), 3.01-4.0 (m, 207 H, C2-6H of CD), 4.88-5.1 (br, 39.6 H, C1-H of CD), 7.25 (br, RhB), 8.5 (br, RhB).

Synthesis of PEI-FL

To a solution of PEI10000 (138 mg, 0.01 mmol) in dry DMSO (10 mL) was added a solution of fluorescein isothiocyanate (55.9 mg, 1.104 mmol) in dry DMSO (10 mL). The mixture was allowed to react at room temperature for 2 days. To the crude reaction mixture, water (10 mL) was added. Afterwards, the mixture was purified by dialysis (SpectraPor membrane, MWCO 1000 kD) for 1 week. Pure product grafted with FL (PEI-FL) was recovered by freeze-drying (50 mg, 36% yield).The degree of grafting of FL was calculated with a calibration curve (Fig. S2).

Preparation of the supramolecular nanoparticles

The SNPs were prepared in phosphate buffer (5 mM, pH 7.4), maintaining the host : guest ratio at 1 : 1 at 15 μM CD, from stock solutions of PiBMA-CD-RhB (6.44 μM polymer), PiBMA-CD (6.44 μM polymer), PiBMA-TBP (4.28 μM polymer) by mixing the components with a vortex. The final composition of these SNPs was: PiBMA-CD-RhB (3.3 μM CD, 1 μM RhB), PiBMA-CD (11.7 μM CD), and PiBMA-TBP (15 μM TBP). To the prepared SNPs, PEI/FL or PEI was added from stock solutions of 10 or 100 μM PEI. At each addition of PEI or PEI/FL, the samples were allowed to equilibrate for 10 min prior to DLS, UV-VIS or fluorescence measurements.

In the case of the controls, the SNPs were prepared maintaining the same concentration of host, guest, RhB, PEI, and FL.

Calculation of the concentrations of charged groups induced by the SNP components and the cargo

The amount of charges in the SNPs was calculated based on the concentration of the different species in the SNPs, which are composed of PiBMA-CD-RhB (3.3 μM CD, 1 μM RhB), PiBMA-CD (11.7 μM CD), and PiBMA-TBP (15 μM TBP).

1) The concentration of carboxylate groups (COO^-), which remains always constant, was calculated by addition of the carboxylic groups of the 3 components:

PiBMA-CD-RhB: The total concentration of COO^- in the polymer PiBMA-CD-RhB is 71.5 μM. Taking into account that there are 5 CDs per polymer backbone, the concentration of PiBMA-CD-RhB is:

$$\frac{3.3}{5} = 0.67\ \mu M$$

Therefore, the concentration of COO^- in the particles coming from this component is :

$$0.67 \times 71.5 = 48\ \mu M$$

PiBMA-CD: The total concentration of COO^- in the polymer PiBMA-CD is 69 μM. Taking into account that there are 9 CDs per polymer backbone, the concentration of PiBMA-CD is:

$$\frac{11.7}{9} = 1.3\ \mu M$$

Therefore, the concentration of COO^- in the particles originating from this component is :

$$1.30 \times 69 = 90\ \mu M$$

PiBMA-TBP: The total concentration of COO^- in the polymer PiBMA-TBP is 69 μM. Since there are 9 TBPs per polymer backbone, the concentration of PiBMA-TBP is:

$$\frac{15}{9} = 1.67\ \mu M$$

Therefore, the concentration of COO- in the particles coming from this component is :

$$1.67 \times 69 = 115\ \mu M$$

Therefore, the concentration of COO^- in the SNPs is:

$$48 + 90 + 115 = 253\ \mu M$$

2) The concentration of PEI after its addition to the SNPs was calculated from the concentration of FL taking into consideration that there are on average 3 fluoresceins per PEI polymer backbone. The concentration of FL at each data point was calculated from the calibration curve of FL by UV-VIS (Fig. S2).

3) The concentration of amines at each data point was calculated from the concentration of PEI taking into account the stoichiometry of the polymer and by addition of the concentrations of the primary, secondary, and tertiary amines. The PEI of MW 10 kDa has a concentration of 55 μM of primary amines, 116 μM of secondary amines, and 58 μM of tertiary amines. Therefore, the total amount of amines in PEI is : 229 μM. Taking into account the proton sponge effect,[5,6] it was assumed that half of the total amount of amines are protonated at physiological pH. For example, after addition of 0.2 μM of PEI into the SNPs, the concentration of amines is (Table S1):

$$0.2 \times 229 \div 2 = 23\ \mu\text{M}$$

DLS and ζ potential measurements

To perform the DLS and ζ potential measurements, the refractive index of the material was fixed to 1.465 and the absorbance to 0.01. The parameters for the dispersant (water) were taken from the database of the machine. The temperature was set to 25 ˚C. The measurements were automated. PEI or PEIFL was added to the pre-prepared SNPs from concentrated stock solutions (10 or 100 μM PEI) and allowed to equilibrate for 10 min before measuring.

UV-Vis measurements

PEI or PEIFL was added to the SNPs from concentrated stock solutions (10 or 100 μM PEI). At each concentration, the samples were allowed to equilibrate for 10 min prior to the UV-Vis measurements.

Fluorescence measurements

PEI or PEIFL was added to the SNPs from concentrated stock solutions (10 or 100 μM PEI). At each concentration, the samples were allowed to equilibrate for 10 min prior to the fluorescence measurements. At each concentration, the emission of RhB (685 nm) after FL excitation (450 nm), and the direct RhB excitation (560 nm) were recorded. The emission of FL (520 nm) after FL excitation (450 nm) was also recorded.

HR-SEM measurements

The samples were prepared by drop-casting 5 μL of a SNP solution onto a silicon wafer. After 30 s, excess of water was removed by filter paper. The samples were allowed to dry overnight in a desiccator containing silica gel beads. Original samples and 20x diluted ones were used.

Table S1. Changes in the amount of charges of the SNPs upon addition of PEI

[PEI], µM	[AMINE], µM	[FL], µM
0.0	0	0.0
0.2	23	0.5
0.3	34	1.0
0.7	80	2.0
1.3	149	4.0
1.7	195	5.0
2	229	6.0
2.3	263	7.0
2.7	309	8.0
5.3	607	16.0
10.7	1225	32.0

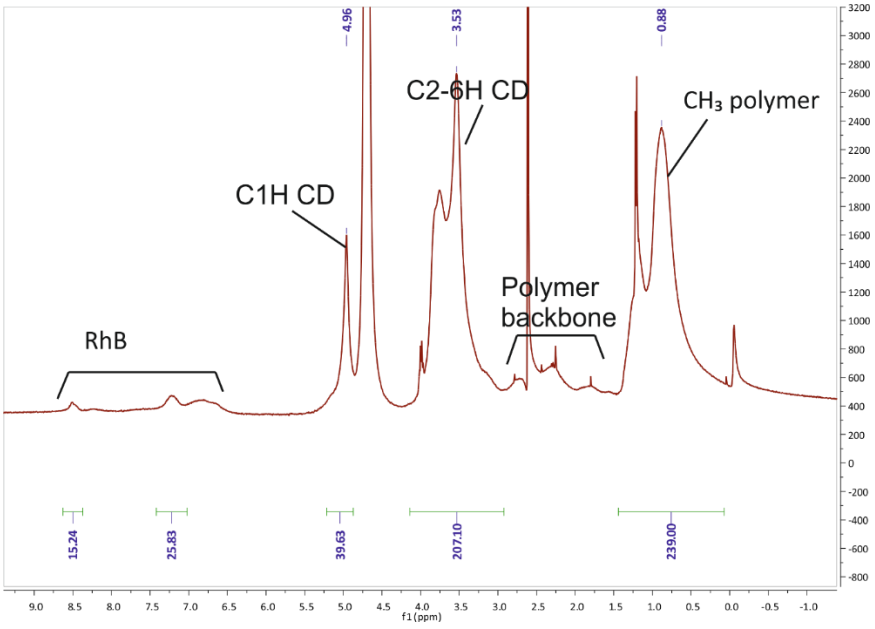

Figure S1. ^{1}H-NMR spectrum of PiBMA-CD-RhB.

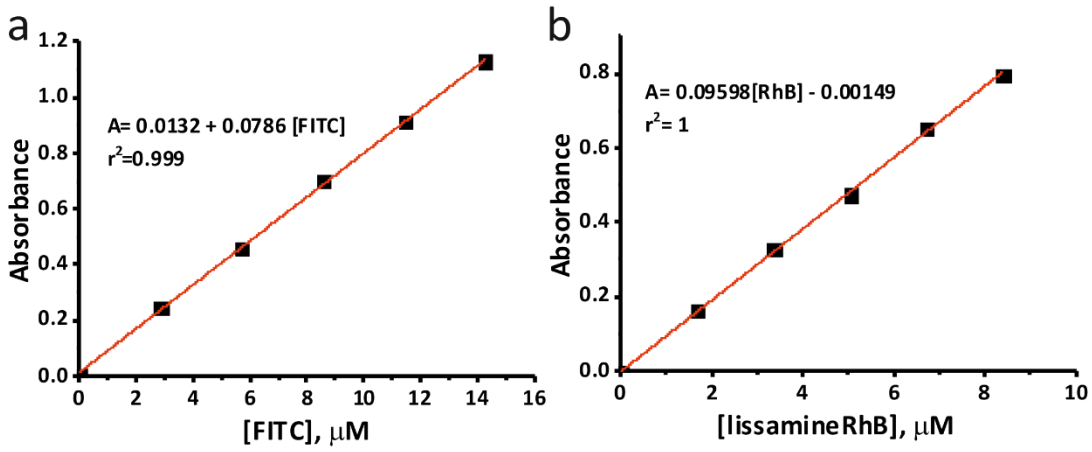

Figure S2. UV-Vis calibration curves of (a) FL and (b) RhB.

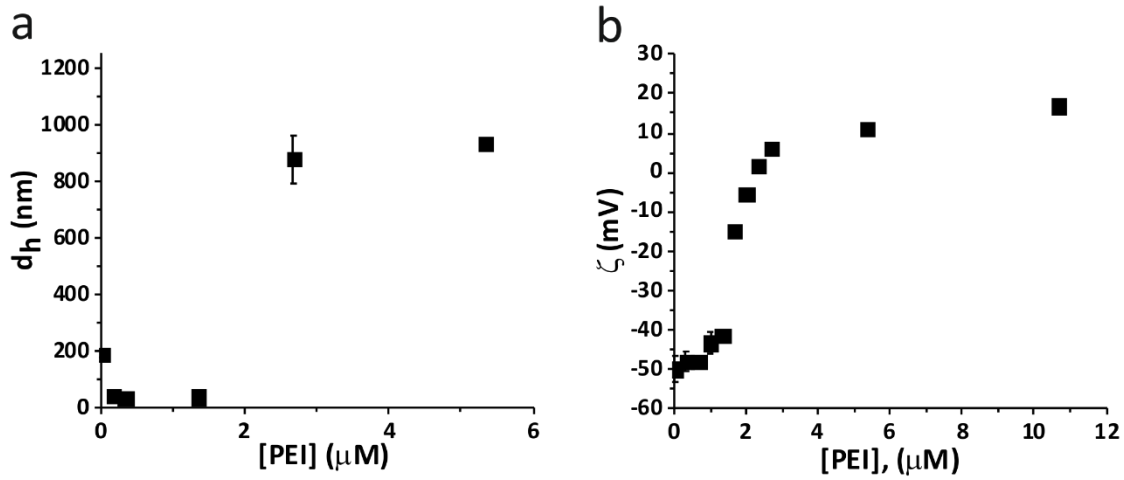

Figure S3. (a) Hydrodynamic diameters from DLS and (b) zeta potential (ζ) data of the SNPs upon addition of increasing amounts of PEI-FL.

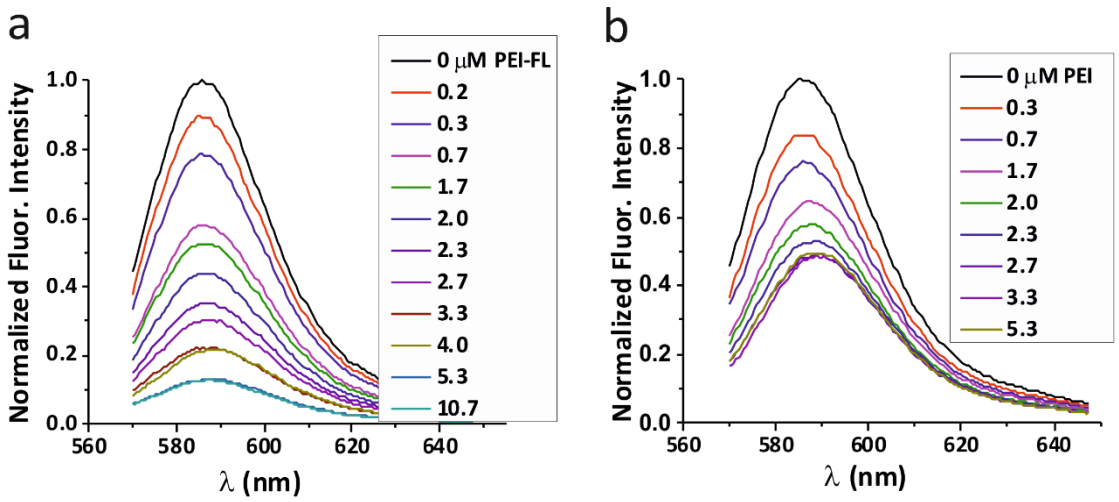

Figure S4. Normalized fluorescence spectra after excitation at 560 nm of SNPs composed of PiBMA-CD-RhB (3.3 μM CD, 1 μM RhB), PiBMA-CD (11.7 μM CD) and PiBMA-TBP (15 μM TBP) upon addition of increasing amounts of PEI-FL (a) or PEI (b).

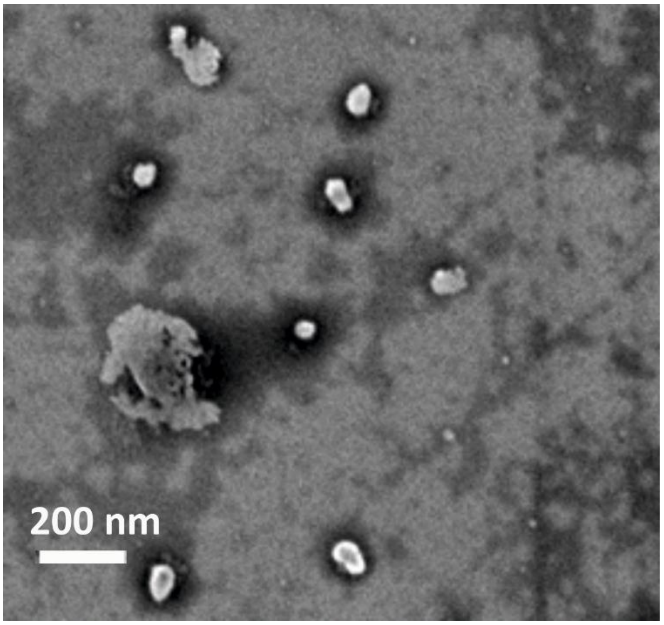

Figure S5. SEM of original SNPs.

As a control, SNPs of unlabelled PiBMA-CD were prepared and free RhB was added at the same dye concentration as for the dyelabelled SNPs shown in Fig. 3, after which free fluorescein with or without PEI was added. In both cases, quenching of RhB was no longer observed (Fig. S6a). This indicates that RhB must be incorporated inside the SNPs, by covalent attachment to one of its constituents, to be able to observe quenching due to the loading of cargo. Similarly, when free FL was added to the RhB-labelled SNPs prepared with PiBMA-CD-RhB, quenching of RhB was also not observed (Fig. S6b). However, quenching did take place once PEI was also added, which indicates that the quenching of RhB originates from the charge neutralization induced by the cationic cargo.

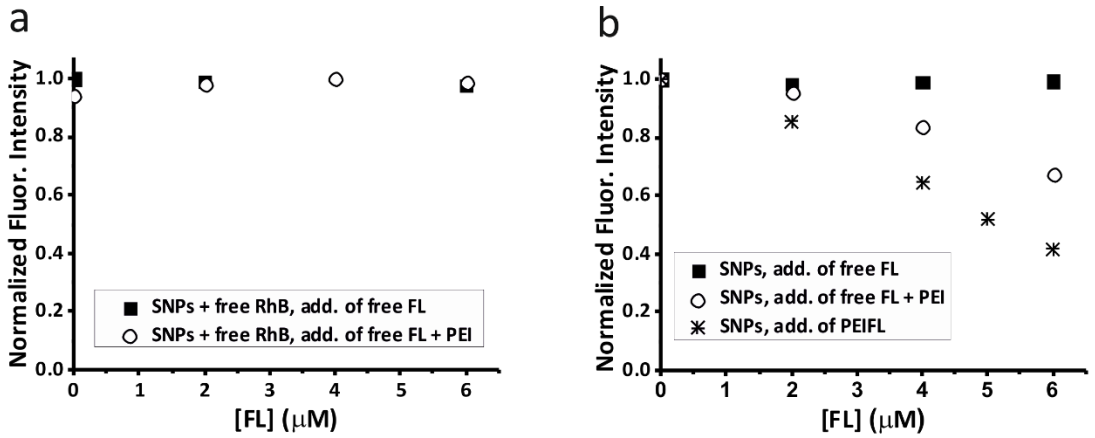

Figure S6. Fluorescence emission intensity (excitation 560 nm, emission 585 nm) of the controls. The difference in the x axis with respect to previous graphs is caused by the fact that here it concerns the concentration of FL, which is 3 times higher than the concentration of PEI (Table S1) . When using free FL together with PEI, a ratio of 3:1 = Fl:PEI is maintaned. (a) SNPs prepared with free RhB maintaining the same concentration of RhB in the particles as with the labelled ones, and adding free FL or free FL and PEI. (b) SNPs prepared with labelled RhB and adding free FL or free FL and PEI. For comparison, the values with PEIFL are also shown (as in Fig. 5b). The SNPs with labelled RhB were composed of PiBMA-CD-RhB (3.3 μM CD, 1 μM RhB), PiBMA-CD (11.7 μM CD), and PiBMA-TBP (15 μM TBP). The ones with free RhB were composed of PiBMA-CD (15 μM CD) and PiBMA-TBP (15 μM TBP).

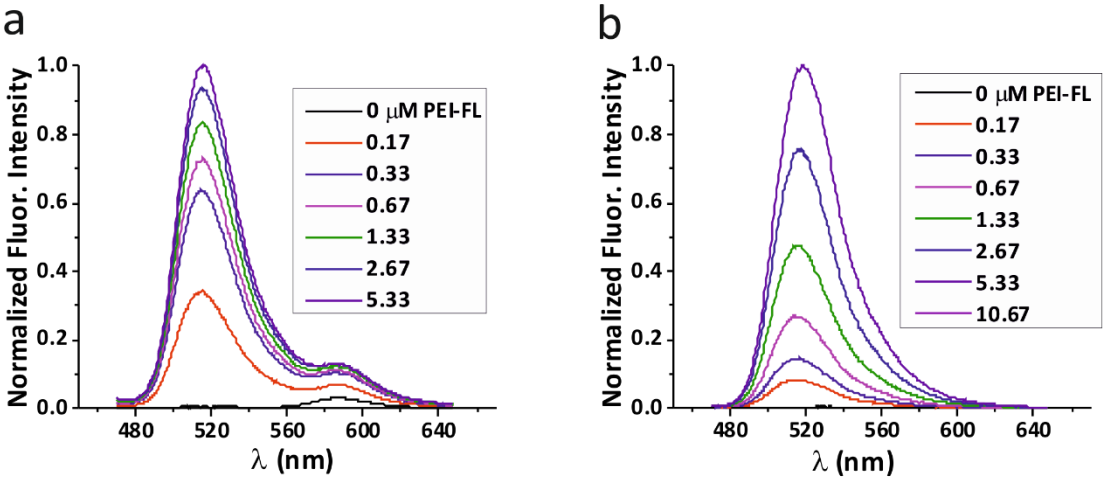

Figure S7. Normalized fluorescence spectra after excitation at 450 nm of a) the SNPs composed of PiBMA-CD-RhB (3.3 μM CD, 1 μM RhB), PiBMA-CD (11.7 μM CD) and PiBMA-TBP (15 μM TBP) upon addition of PEI-FL and b) buffer upon addition of PEI-FL.

References

1 L. Grana Suarez, W. Verboom and J. Huskens, *Chem. Commun*., 2014, **50**, 7280.
2 H. Law, M. Benito, M. Garcia Fernandez , L. Jicsinszky, S. Crouzy and Defaye, *J. Phys. Chem. B*, 2011, **115**, 7524.
3 X. Li, Z. Zhou, W. Zhou, L. Dai and Z. Li, *Analyst*, 2011, **136**, 5017.
4 A. Kulkarni, K. DeFrees, S.-H. Hyun and D. H. Thompson, *J. Am. Chem. Soc.*, 2012, **134**, 7596.
5 R. V. Benjaminsen, M. A. Mattebjerg, J. R. Henriksen, S. M. Moghimi and T. L. Andresen, *Mol. Ther.*, 2013, **21**, 149.
6 J. D. Ziebarth and Y. Wang, *Biomacromolecules*, 2009, **11**, 29.