CHEMPHYSCHEM

Supporting Information

Integration of Organic Fluorophores in the Surface of Polymer-Coated Colloidal Nanoparticles for Sensing the Local Polarity of the Environment

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cphc_201100901_sm_miscellaneous_information.pdf

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Integration of Organic Fluorophores in the Surface of Polymer Coated Colloidal Nanoparticles for Sensing the Local Polarity of the Environment

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I) Synthesis

I.1) General scheme

The different compounds used in this study and the way they were synthesized are sketched in Figure SI-I.1.1. All sketches are not drawn to scale and have to be understood as idealized model structures.

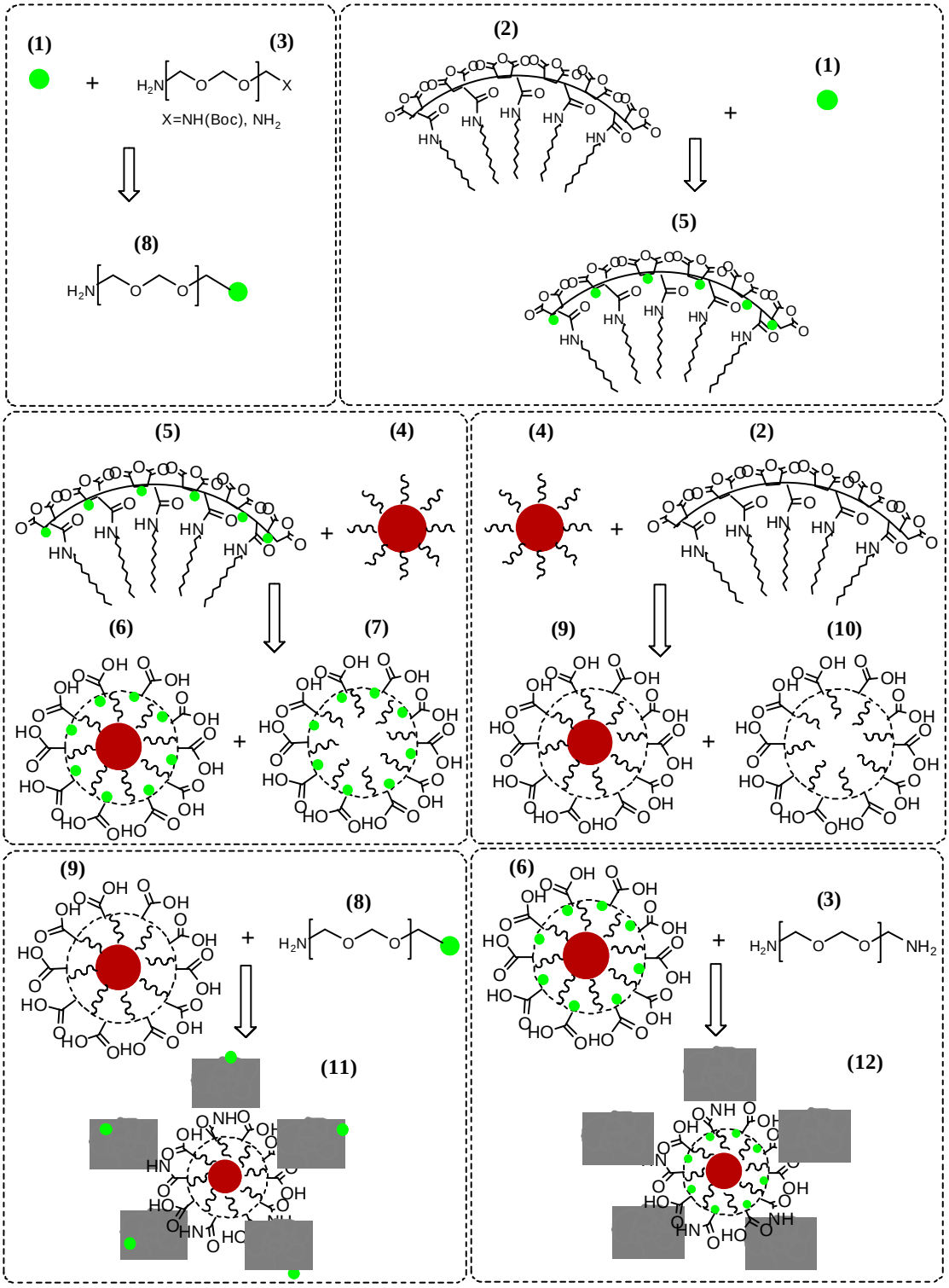

Figure SI-I.1.1: Schematic representation of the measured compounds: ***1****: NH_2-modified FE (for structure see Fig. SI-I.7.1);* ***2****: Amphiphilic polymer (PMA);* ***3****: Diamino or Boc-protected and NHS-modified PEG;* ***4****: Hydrophobic dodecanethiol-capped Au NPs (Au NPs);* ***5****: FE modified amphiphilic polymer (FE-PMA);* ***6****: FE modified polymer-coated Au NPs (APFE);* ***7****: Empty polymer micelles made out of FE-modified amphiphilic polymer (PFE, FE-PMA micelles);* ***8****: FE conjugated Boc-protected NHS-PEG (FE-PEG);* ***9****: Polymer-coated Au NPs (AP);* ***10****: Empty polymer micelles made out of amphiphilic polymer (PMA micelles);* ***11****: FE-PEG-modified polymer-coated Au NPs, whereby the PEG was attached to the polymer after the polymer coating;* ***12****: FE-PMA coated Au NPs saturated with PEG on the surface, whereby the PEG was attached after polymer coating with FE-PMA. The samples* ***1****,* ***6****,* ***7****,* ***8****,* ***11****,* ***12*** *were spectroscopically investigated.*

Some basic characterization of FE-dye is shown in Figures SI-I.1.2 -SI-I.1.4.

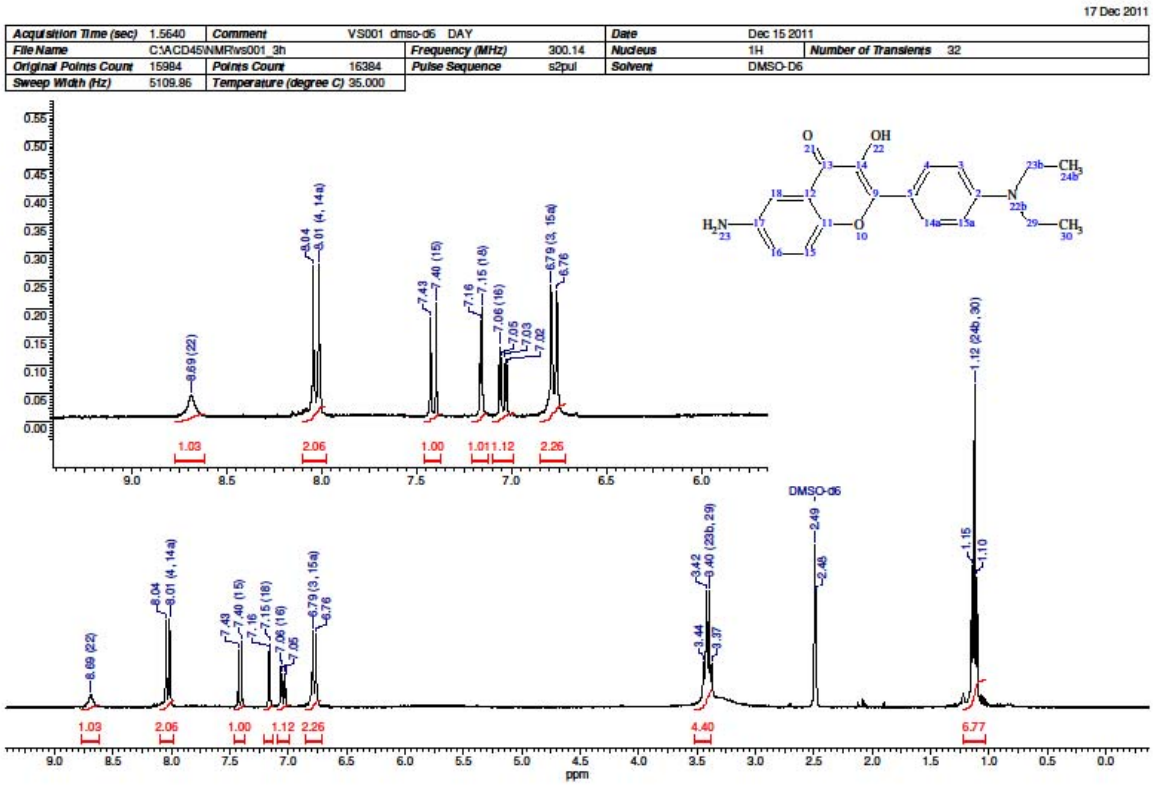

Figure SI-I.1.2: ^{1}H-NMR spectra of FE dye.

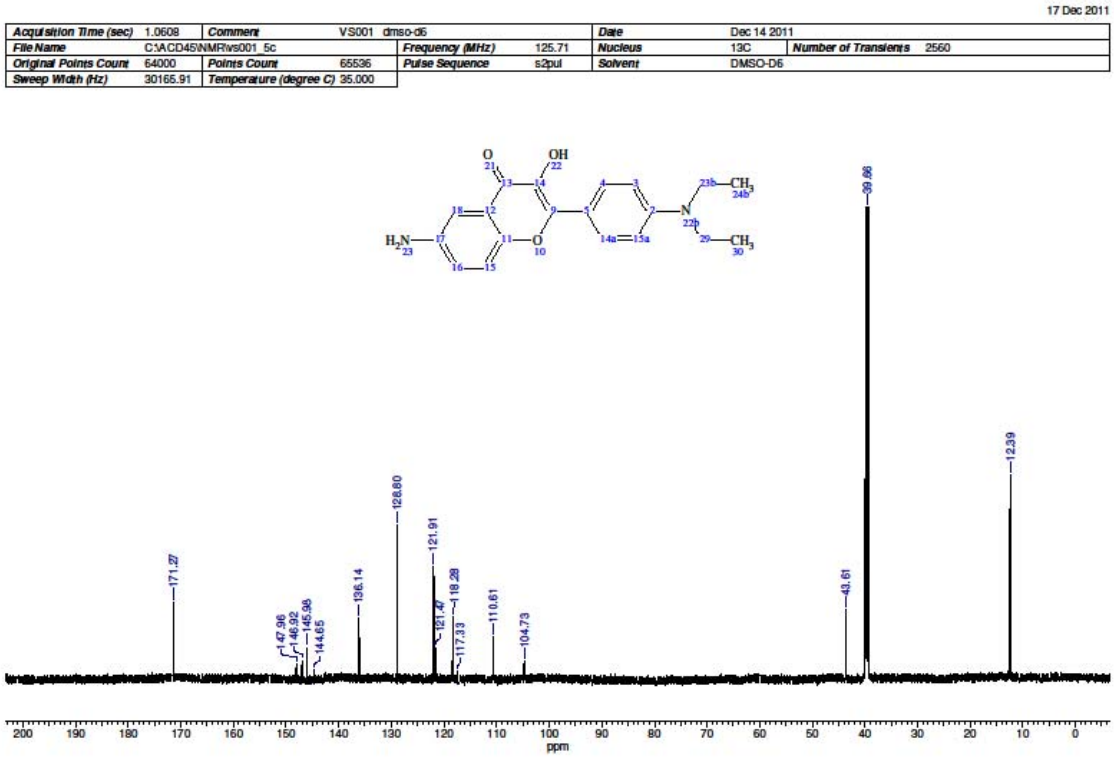

Figure SI-I.1.3: ^{13}C-NMR spectra of FE dye.

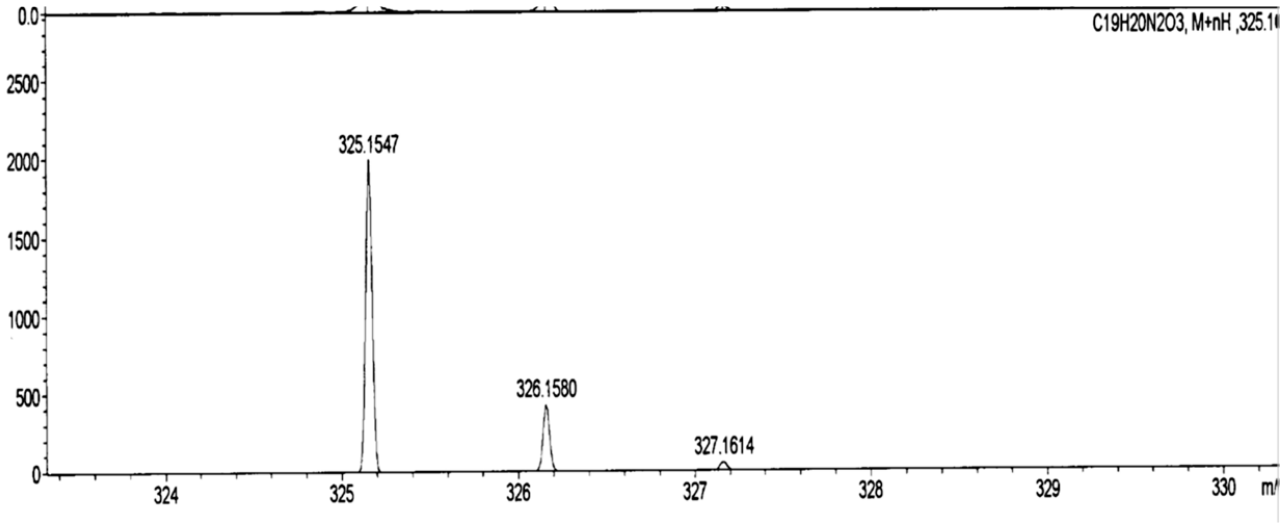

Figure SI-I.1.4: High Resolution Mass Spectrum of FE dye.

I.2) Synthesis of amphiphilic polymer (PMA, compound 2)

The amphiphilic polymer was synthesized according to previous reports [1-3]. In a 250 mL round bottom flask, poly(isobutylene-*alt*-maleic anhydride (3.084 g, 20 mmol monomer[1]; average M_w ~6,000 g/mol, Sigma, #531278) were placed. Then, dodecylamine (2.70 g, 15 mmol; ≥ 98%, Fluka, #44170) were dissolved in 100 mL anhydrous tetrahydrofuran (THF, ≥ 99.9%, Aldrich, #186562). The solution was added over the poly(isobutylene-alt-maleic anhydride) and the mixture was sonicated for about 20 seconds, followed by heating to 55-60°C for 1 hour under stirring. The solution was then concentrated to 30-40 mL by evaporation of THF[2] and kept stirring overnight. The reaction mixture was then completely dried by evaporation and the polymer was redissolved afterwards in 40 mL anhydrous chloroform (≥99%, Sigma, #372978), so that the final monomer concentration was 0.5 M[3]. It is important to note that all the organic solvents used here were purchased in anhydrous quality in order to keep the maleic anhydride rings intact. After the synthesis, the amphiphilic polymer still had at least[4] 25% active maleic anhydride rings which could be used for further functionalization of NPs.

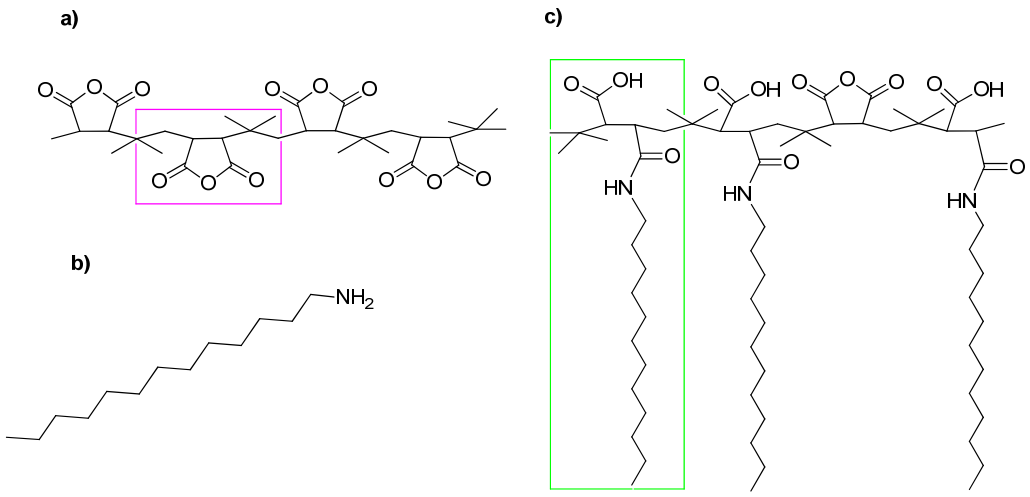

*Figure SI-I.2.1: a) Poly(isobutylene-alt-maleic anhydride) is used as hydrophilic backbone. The purple box shows one monomer unit with a molecular weight of 154 g/mol. There are about 39 anhydride monomers in each polymer chain. b) Dodecylamine is used as hydrophobic side chains. Amphiphilic polymer was obtained by reaction of the hydrophilic backbone with hydrophobic side chains. c) Structure of the amphiphilic polymer (PMA compound **2**). The green box shows the second monomer unit.*

I.3) Synthesis of FE modified amphiphilic polymer (FE-PMA, compound 5)

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[1] The polymer has an average molecular weight of $\langle M_w \rangle$ ~ 6,000 g/mol. Each polymer molecule comprises around 39 monomer units, whereby the molecular weight of one monomer unit is approximately M_w = 154 g/mol. Therefore 3.084g polymer corresponds to 3.084g / 154 $g \cdot mol^{-1}$ = 20 mmol monomer units. See Figure SI-I.2.1.

[2] To enhance the reaction between the maleic anhydride rings and the amino groups.

[3] For the definition of the monomer unit see Figure SI-I.2.1.

[4] For our calculations we assume 100% reaction efficiency between the amino-bearing hydrocarbon chains and the anhydride rings, being fully aware that this value will not be reached in practice.

The synthesis of the FE-modified amphiphilic polymer in organic solvent was carried out by linking the NH_2-FE dye (compound **1**, NH_2-modified hydroxyflavone) to the polymer's hydrophilic backbone through the formation of an amide bond between the unreacted maleic anhydride rings and the amino group of the dye. This synthesis is based on the same procedure employed for linking the hydrophobic side-chains to the hydrophilic polymer backbone (*cf.* Chapter I.2, Figure SI-I.2.1).

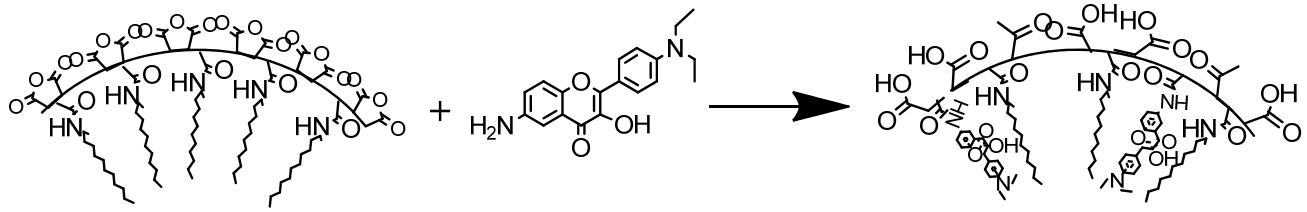

*Figure SI-I.1.3.1: FE modified amphiphilic polymer (compound **5**) is synthesized by linking amino-FE dye (compound **1**) to the amphiphilic polymer (compound **2**).*

For the synthesis of FE modified polymer we have taken a molar mixing ratio of FE / monomers of amphiphilic polymer of 2/100, *i.e.* 2% of the initial amount of anhydride rings were reacted with dye. Synthesis was performed in one step in anhydrous $CHCl_3$: NH_2-FE (compound **1**, 2.4 mL of 1 mM solution in $CHCl_3$) and amphiphilic polymer (compound **2**, 0.24 mL of 0.5 M monomer units solution in $CHCl_3$) were mixed together and the mixture was reacted overnight on a shaker (Sartorius, Certomat® MO II). After overnight incubation the modified polymer (compound **5**, FE-PMA) was used without further purification for subsequent polymer coating of Au NPs similar to the use of the plain polymer (PMA). A final monomer concentration of 45 mM of the modified polymer was estimated from the final volume of the mixture.

1.4) Synthesis of dodecanethiol stabilized Au nanoparticles (Au NP, compound 4)

Dodecanethiol protected gold NPs (Au NPs) were synthesized using a two-phase (organic-aqueous) method based on previously published protocols [4-7]. This method is based on the transfer of gold tetrachloroaurate from aqueous phase to organic phase using tetraoctylammonium bromide as phase transfer catalyst. Reduction of tetrachloroaurate with sodium borohydride, followed with a ligand exchange step using dodecanethiol yields the desired product **4**. Hydrogen tetrachloroaurate (III) hydrate (0.30 g, 0.9 mmol; 99.9 %, Alfa Aesar, #12325) was weighted in a 40 mL glass vial. The powder was then dissolved in 25 mL of Milli-Q water. Then, tetraoctylammonium bromide (TOAB, 2.17g, 4.0 mmol; Sigma-Aldrich, #294136) was dissolved in 80 mL of Toluene (>99.5%, Carl Roth GmbH - Co. KG., #9558.3) on a 250 mL Erlenmeyer flask. The tetraoctylammonium bromide solution was then transferred to a 250 mL separation funnel. The hydrogen tetrachloroaurate solution was then poured into the separation funnel. The mixture was vigorously shaken for 5 minutes to transfer the $AuCl_4^-$ to the organic phase through formation of the ionic pair ($NR_4^+AuCl_4^-$). The initially transparent organic phase turns into red while the light yellow colored aqueous phase becomes transparent. This indicates the transfer of tetrachloroaurate from the aqueous phase to the organic phase. The aqueous phase was then discarded and the solution was transferred into a 250 mL round bottom flask. The mixture is stirred at room temperature. Then sodium borohydride (0.334g, 8.8 mmol; Sigma-Aldrich, #452882) was dissolved in 25 mL Milli-Q water in a 40 mL glass vial. Note: this solution should be prepared freshly before use due to the hydrolysis of sodium borohydride. The sodium borohydride solution was quickly added dropwise within one minute to the gold solution in toluene. The abrupt change in color from red

to deep violet and the hydrogen gas bubbles observed until the whole $NaBH_4$ solution was poured in the flask indicate the nucleation of the gold clusters. The solution was then left on stirring for 60 minutes at room temperature to allow the NPs to stabilize.

The mixture was then transferred again to a separation funnel and the aqueous phase was discarded. 25 mL of Hydrochloric acid (10 mM, Merck KGaA, #1.09057.1000) was then added to the solution and the solution was shaken gently for 1 minute. Note: Vigorous shaking at this stage can cause formation of emulsion, which should be avoided. The aqueous phase was discarded and the organics were washed with 25 mL of sodium hydroxide (10 mM, Carl Roth GmbH - Co. KG., #K021.1) to remove the excess of acid by shaken gently for 1 minute. The aqueous phase was then discarded the organics were then washed three times with 25 mL of Milli-Q. In each step black aggregates were observed in the aqueous phase. The washing process was repeated until the aqueous phase was clear. The solution was then transferred from the separation funnel into a 250 mL round flask and the solution was left stirring overnight at room temperature to allow for Ostwald ripening.

Then 10 mL of 1-dodecanethiol (98 %, Sigma-Aldrich, #471364) were added to the NPs solution and the mixture was heated to 65 ^{0}C for 2 hours. The strong affinity of the thiol group towards gold permits the exchange of the tetraoctylammonium present on the NP surface for dodecanethiol. Then, the NP solution was poured into several 40 mL vials and centrifuged at 2000 rpm for 5 minutes. In this way one can get rid of agglomerates. The supernatant was collected in a 100 mL beaker and the agglomerates were discarded. The NPs were then precipitated using methanol (99.8%, Sigma, #32,241-5) to get rid of excess dodecanethiol. For this, about 20 mL of the NPs solution was poured each in new 40 mL glass vials and each vial was filled with methanol. The NPs were again centrifuged at 2000 rpm for 5 minutes. Note: after the centrifugation the supernatant should be colorless and the NPs should be all precipitated on the bottom of the vials. Otherwise additional methanol should be added in order to increase the polarity of the medium promoting the precipitation of the NPs. The supernatant was discarded and the NPs at the bottom of the vial were resuspended in chloroform. After all these steps hydrophobic dodecanethiol protected gold NPs with an average core diameter of 4 nm were obtained (compound **4**, see Figure SI-I.4.1). A TEM image of the resulting NPs is shown in Figure SI-I.4.2.

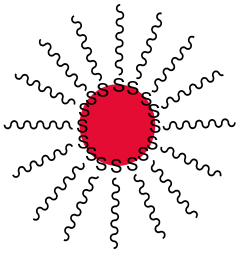

*Figure SI-I.4.1: Hydrophobic Au NPs (compound **4**). The surface of the inorganic Au cores (drawn in red) is coated by hydrophobic dodecanethiol (drawn in black).*

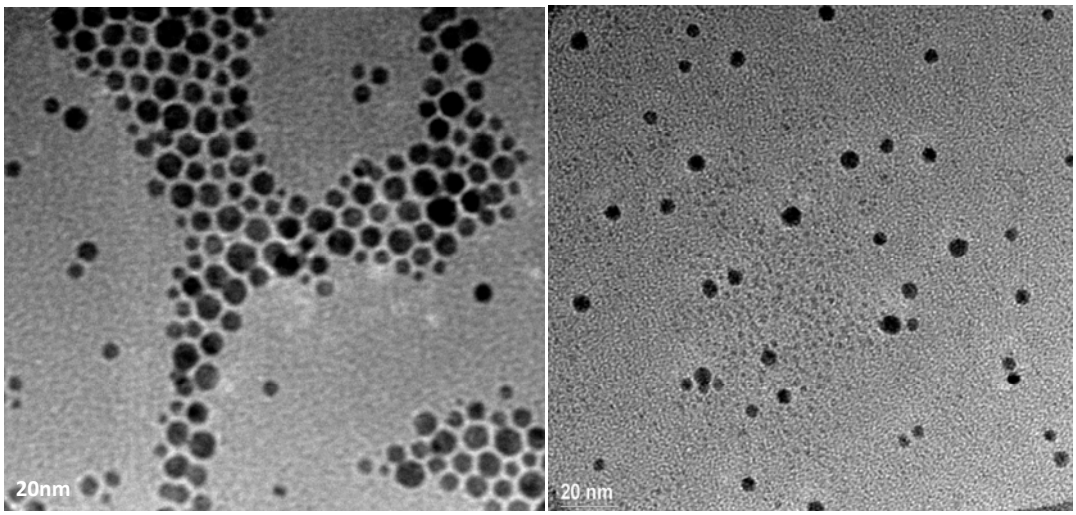

*Figure SI-I.4.2: TEM image of 4 nm Au NPs. a) Au NPs stabilized in chloroform (compound **4**). b) Amphiphilic polymer coated Au NPs in water (compound **9**). The scale bar corresponds to 20 nm.*

I.5) Polymer coating of AuNPs with PMA (AP, compound 9) and FE-PMA (APFE, compound 6)

The polymer coating procedure was performed according to previous reports [1-3, 6, 7]. In this work we mixed the FE-PMA solution with Au NPs by a molar ratio of 100 monomer units of polymer per nm^2 of effective NP surface area[5]. The amphiphilic polymer (FE-PMA) hereby is wrapped around the surface of the Au NPs (see Figure SI-I.5.1) [6]. Immediately after mixing the Au NPs with FE-PMA solution, the solvent was slowly evaporated under reduced pressure until the sample was completely dry. The process (addition of chloroform and subsequent evaporation) was repeated for three times to get homogeneous coating. The remaining solid film in the flask was redissolved in SBB pH12 buffer (sodium borate 50 mM, pH 12) under vigorous stirring until the solution turned clear. The obtained solution was a mixture of two species; polymer coated Au NPs and empty polymer micelles (see Fig SI-I.5-1). Since the polymer is added in excess to get better polymer coating excess, polymer units can self-assemble to hollow structures resembling empty micelles. The polymer micelles are transparent in nature; however fluorescent dye labeled polymer micelles can be visualized on gels. Empty micelles are slightly smaller than the NPs and can be separated as shown in Figure SI-I.6-1.

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[5] The core diameter of the hydrophobic Au NPs is 4 nm which was determined by TEM analysis. The effective diameter d_{eff}= 6.4 nm includes the dodecanethiol molecules around the inorganic core which are estimated to contribute around 1.2 nm in film thickness. Then the effective surface area per NP (A_{eff}) was estimated to be 128.7 nm^2 by using the equation $A_{eff} = 4\pi\times(d_{eff}/2)^2$. To give an example, we calculate how much volume $V_{polymer}$ (in mL) of polymer with a monomer-concentration (*cf.* Figure SI-I.2.1) $c_{polymer}$ = 0.5 M is needed for the polymer-coating of $V_{particle}$ = 1 mL Au NPs with a concentration $c_{particle}$ = 0.01 mM. First, the total number of Au NPs $N_{total\ particle}$ equals to the molar amount of NPs multiplied with the Avogadro number N_A (6.02×10^{23} M^{-1}): $N_{total\ particle}= c_{particle}\times V_{particle}\times N_A$= 0.01 mmol/L ×1 mL ×$6.02\times10^{23}$ mol^{-1} = 6.02×10^{15}. Since each NP has an effective surface area $A_{eff} = 4\pi\times (d_{eff}/2)^2 = 4\pi\times(6.4\ nm/2)^2$ = 128.7 nm^2 the total effective surface area is $A_{eff\ total} = A_{eff}\times N_{total\ particle}$ = 128.7 $nm^2\times6.02\times10^{15}$ = 7.75×10^{17} nm^2. In this paper we used 100 monomers of polymer per nm^2 of A_{eff}. Therefore the total amount of monomer required for the coating is $N_{total,polmer}$ = 100 $nm^{-2}\times A_{eff,total}$ = 100 $nm^{-2}\times7.75\times10^{17}$ nm^2 =7.75×10^{19}. By using Avogadros number finally the required volume of polymer solution which has to be added can be calculated: $V_{polymer}= N_{total,polmer}/N_A/c_{polymer}$ = $7.75\times10^{19}/(6.02\times10^{23}\ mol^{-1})/(0.5\ M)$ = 0.2574 mL.

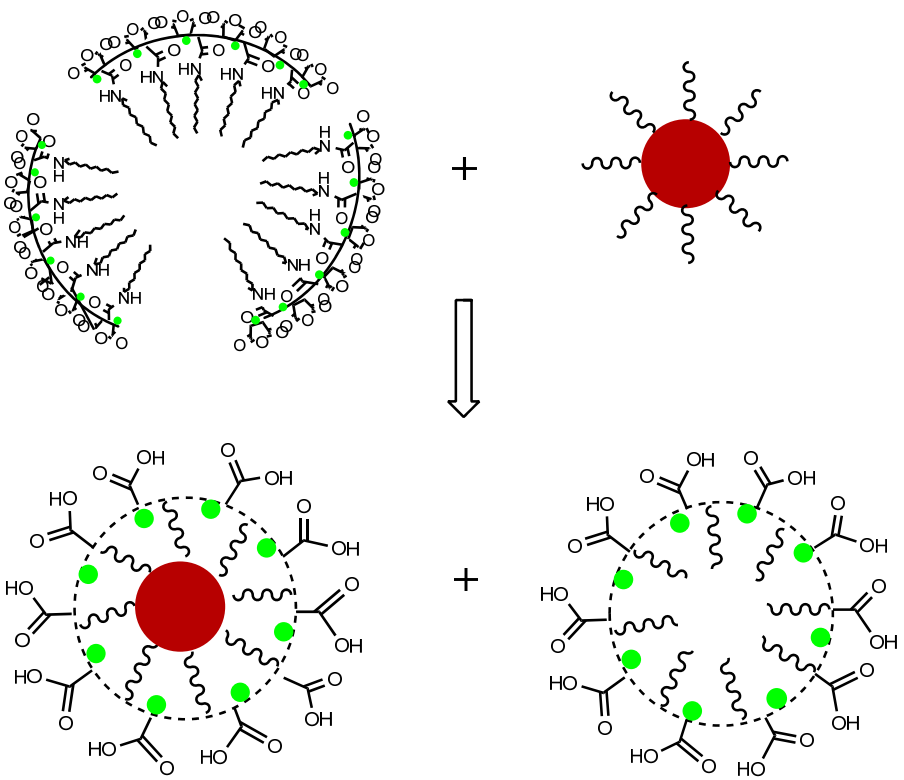


Figure SI-I.5.1: Hydrophobic Au NPs capped by hydrophobic dodecanethiol (drawn in black) wrapped into an amphiphilic polymer (FE-PMA). As the exact structure of the polymer around the Au NPs is unknown this image has to be understood as idealized plausible sketch.

I.6) Purification of APFE (compound 6)

Polymer coating of Au NPs results in different species in the solution besides the actual polymer coated NPs, e.g. NP agglomerates of bigger sizes, free polymer in the form of empty micelles, free unbound dye molecules, etc. Therefore, the Au NPs need to be purified. Several processes can be involved for purification such as filtration, ultracentrifugation, and chromatography. In the present case the Au NPs were purified by gel electrophoresis. Firstly, the polymer coated Au NPs were passed through a 0.22 µm filter to remove bigger aggregates, in case any of them were present. The NPs were then run on 2 % agarose gels for 100 minutes under the following conditions: 100 V, 500 mA, 250 W, 0.5 × TBE buffer (44.5 mM Tris-borate and 1 mM EDTA, pH = 8.3, Sigma-Aldrich, #T3913). The separation between NPs and micelles can be seen in the picture of the gel shown below (Figure SI-I.6.1). The respective bands of NPs and micelles were extracted by cutting the gel bands out of the gel, putting them into 50 kDa dialysis membrane tubes (Spectra/Pro 6 dialysis tubing, 50 kDa MWCO, 34 mm flat width, Spectralabs, #132544) and applying again voltage (100 V) in the gel electrophoresis set-up until the NPs had migrated out of the gel. In this way the collected polymer-coated Au NPs were dissolved in 0.5 × TBE buffer. After purification with gel electrophoresis, the polymer coated Au NPs (compound **6** or **9**) were concentrated with centrifuge filters (membrane: 100 kDa M_w cut off PES, Sartorius Stedim, #VS2042) with a speed of 3000 rpm. The concentration of the NPs in the final concentrated sample was determined by UV/vis spectroscopy using the Beert-Lambert law, ($A = \varepsilon \cdot L \cdot c$). The extinction coefficient

(ε) of the 4 nm metallic core Au NPs have a value of $\varepsilon_{plasmon}$= $8.63 \cdot 10^6$ $M^{-1}cm^{-1}$[8] at the plasmon peak, located around 520 nm, see Figure SI-I.6.2. In this work we used polymer-coated Au NPs with a concentration of about ~ 2.7 µM in TBE 0.5X buffer as stock solution for the following experiments.

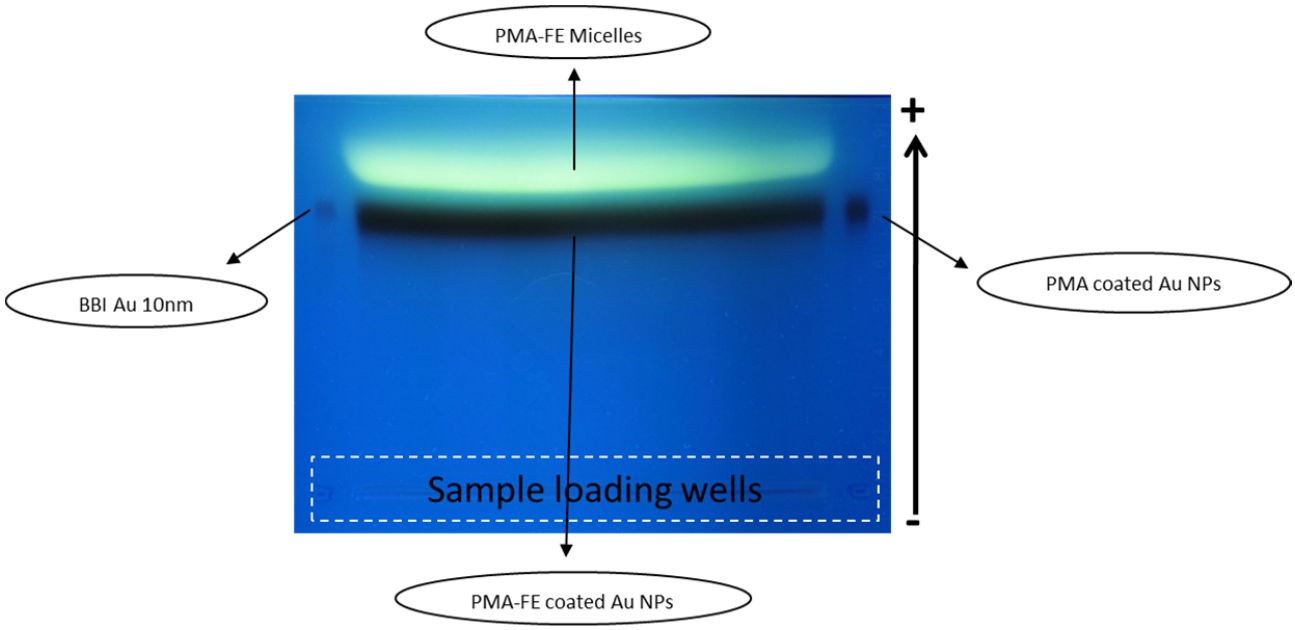


Figure SI-I.6.1: Electrophoretic purification of polymer coated Au NPs. The fluorescent band corresponds to empty micelles and the dark band corresponds to PMA-FE coated Au NPs. As reference 10nm phosphine coated Au [9] was used as a control to estimate the size of resulting NPs.

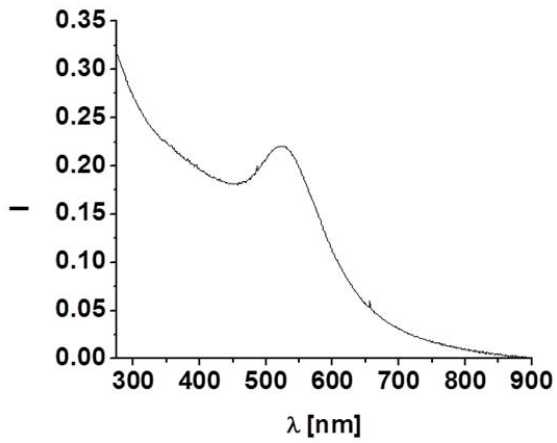


Figure SI-I.6.2: UV/vis spectrum of polymer-coated Au NPs (compound **9***). The NP concentration $c_{particle}$ is estimated from the absorption spectrum I [λ] by using the Beer Lambert law: $A = c_{particle} \times \varepsilon_{plasmon} \times L$, where A is the absorption value at the plasmon peak (520 nm), and $\varepsilon_{plasmon}$ is the extinction coefficient of Au NPs at the plasmon peak position. Here we used the value $\varepsilon_{Plasmon} = 8.63 \cdot 10^6$ M^{-1}-cm^{-1}, which is based on calculations by Eric Dulkeith for different sizes of Au NPs [8]. L is the light path length. Here we used 1 cm quartz cuvettes.*

I.7) Conjugation of FE to Boc-protected-amino NHS-PEG (Boc-protected FE-PEG)

7 mg (0.02 mmol) of NH_2-FE (compound **1**, see Figure SI-I.7.1, M_W = 324.38 g/mol) were dissolved in 21.5 mL ethanol (≥ 99.8%, p.a. Roth, #9065.2) to get a solution of 1 mM concentration. The color of the solution was yellowish-green. Tert-butoxycarbonyl (abbreviated as Boc-protected as illustrated in Figure SI-I.7.2) polyethylene glycol was used as spacer to separate the dye molecules from the NP surface. To link the FE dye to Boc-protected poly(ethylene glycol) (10 kDa: Rapp Polymere, #13 10000-20-21) a solution of Boc-protected PEG was prepared to obtain a concentration of 3 mM. FE-PEG conjugates were then prepared by mixing the two constituents (FE in ethanol, PEG in chloroform) in a glass vial with molecular ratios of FE / PEG of 3 / 1. The mixture (Boc-protected FE-PEG) was left stirring overnight. The reaction is depicted in Figure SI-I.7.3.

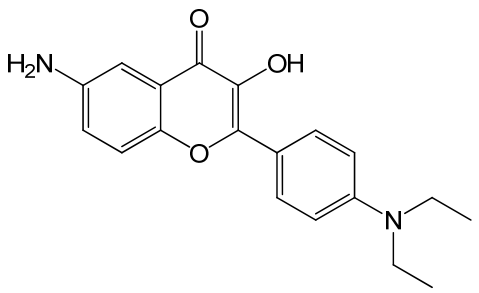


*Figure SI-I.7.1: Molecular structure of NH_2-FE (compound **1**).*

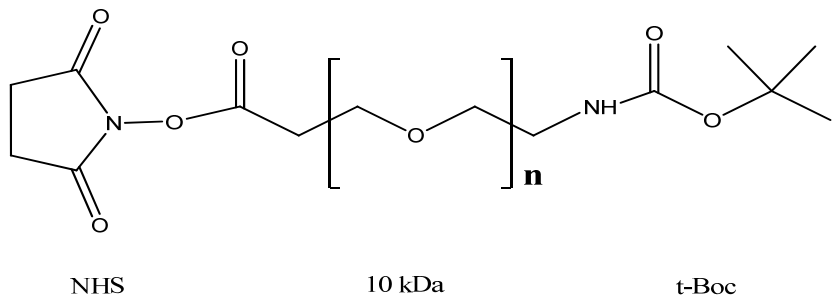


*Figure SI-I.7.2: Molecular representation of Boc-protected NH_2-NHS PEG molecules (compound **2**).*

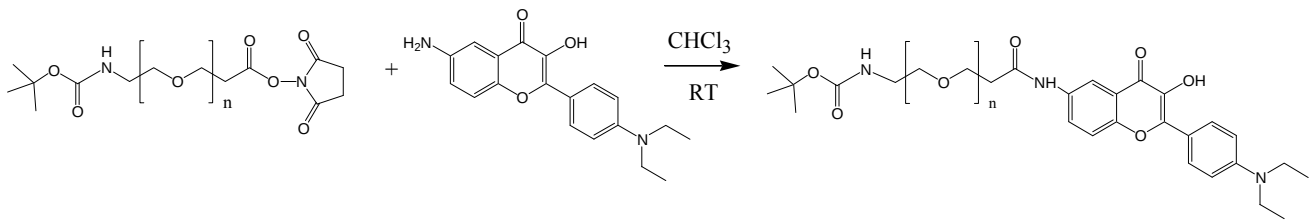


*Figure SI-I.7.3: Reaction of NHS-Boc protected PEG (compound **2**) and NH_2-FE dye (compound **1**). The NHS group of PEG is replaced by the amino-moiety of the dye.*

I.8) Purification of FE-PEG conjugates:

The mixture of NH_2–FE and NHS-PEG (Boc) was reacted overnight to ensure maximum efficiency of reaction. The solvent ($CHCl_3$) was then evaporated and product was redissolved in 1 mL of water. PD-10 desalting column (Sigma, order number: 54805) was then used to get rid of free dye. The eluted product is not simply FE-PEG but also contains other species. The product was then purified through HPLC using Sephacryl S300 stationary phase (N,N'-methylene bisacrylamide; Sephacryl™ High Resolution (HR) S-300, GE Healthcare, #17-0599-10) and mobile phase SBBS pH 9.0 (0.5 M Boric acid, 5.0 M NaCl). The sample was eluted for 120 minutes. The collection phase was kept between 40 minutes and 100 minutes. The eluted fractions were collected as Peak 1, Peak 2, Peak 3, whereas Peak 4 was just labeled in the chromatogram and not collected. The three peak fractions were analyzed separately. Peak 1 and Peak 4 can be ruled out for the case of FE-PEG conjugate therefore the focus was on Peak 2 and Peak 3.

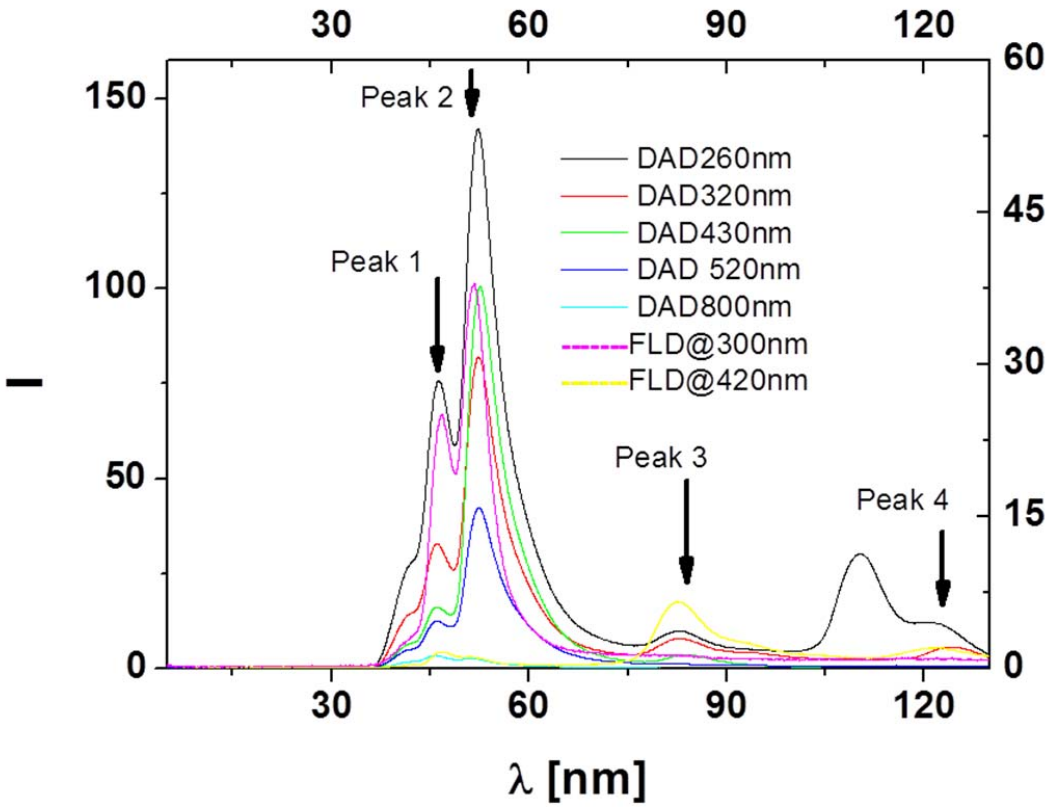


Figure SI-I.8.1: Purification of FE-PEG conjugates via HPLC. The four peaks refer to the different products collected on the basis of absorbance and fluorescence of mixture. DAD and FLD are aborption and fluorescence intensities, respectively.

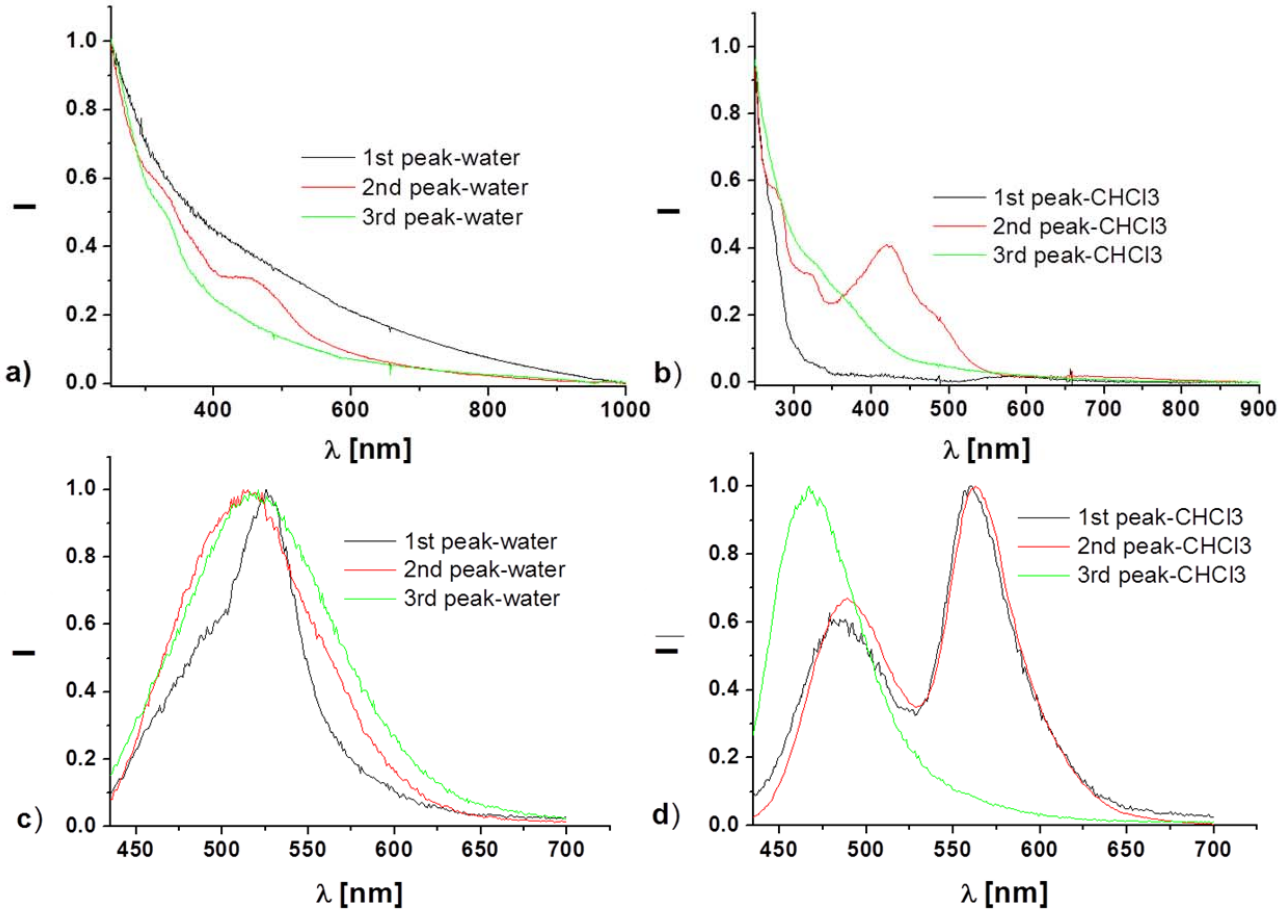


Figure SI-I.8.2: Absorption (in water (a) and chloroform (b)) and emission (in water (c) and chloform (d)) spectra of all the three fractions (Peak1, Peak2, Peak3 of FigureSI-I.8.1). The third peak is referred to as FE-PEG and further analyzed. The gel electrophoresis of FE-PEG shows a blue colored band moving towards negative pole (cf. Figure SI-I.8.1).

Boc-protected FE-PEG was also purified by gel electrophoresis. For this purpose the solvent (chloroform) was first evaporated and the product was then redissolved in Milli-Q water. The product was run on a TBE 0.5X buffered, 2% Agarose gel (under the conditions 100 V, 250 mA, 500 W) for 150 minutes until the two bands (FE dye and FE-PEG (Boc-protected; here description for 10 kDa PEG)) were clearly separated as shown in Figure SI-I.8.2. The light blue colored FE-PEG band moving towards negative terminal was cut out. The gel band was put into a dialysis membrane (Spectra/Pro 6 dialysis tubing, 3500 Da MWCO, 18mm flat width, Spectralabs #132590). The membrane was put in the gel electrophoresis set-up and voltage was applied for 20 minutes until the FE-PEG came out of the gel. The solution was then collected into a flask and water was evaporated in order to concentrate the sample to 2 mL. Then, the sample was collected and passed through the PD 10 desalting column which has been equilibrated with Milli-Q water to get rid of salt rich TBE buffer[6]. In this way, purified FE-PEG dissolved in Milli-Q water was obtained.

[6] Note: This is important as the presence of salt can complicate the assessment of the final amount of the product.

Figure SI-I.8.3: Electrophoretic separation of FE-PEG conjugates and FE dye in solution. The fraction running towards negative terminal was extracted by using membrane of MWCO 3.5 kDa.

The Peak 3 was also blue fluorescent upon excitation with UV light.

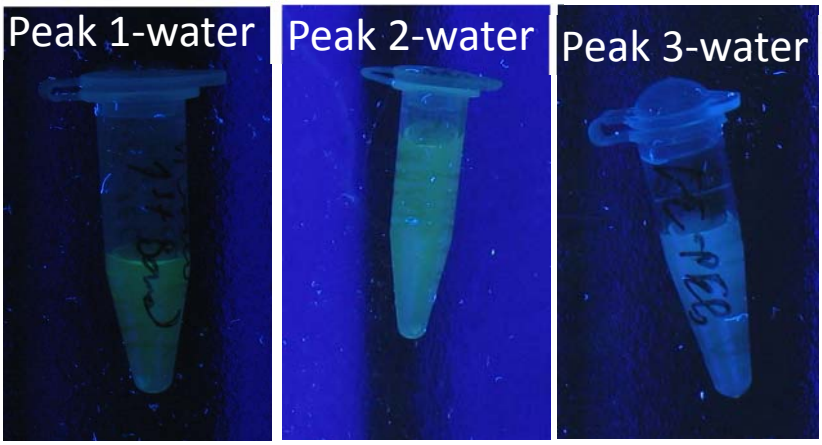


Figure SI-I.8.4: The three collected fraction in water under UV light (λ = 365 nm). The difference in color is visible for the 3rd fraction.

The assays of conjugation of Peak 3 fraction yielded retarded NPs on the gel, showing the presence of PEG whereas the conjugation of Peak 2 fraction doesn't retard the particles on gel (Figure SI-I.8.5). In order to further verify the hypothesis, NMR study of the two fractions was carried out. The spectra showed the presence of PEG in Peak 3. However, the aromatic rings signals appearance was not obvious. The spectral profile of Peak 2 lacked the presence of PEG (Figure SI-I.8.6a).

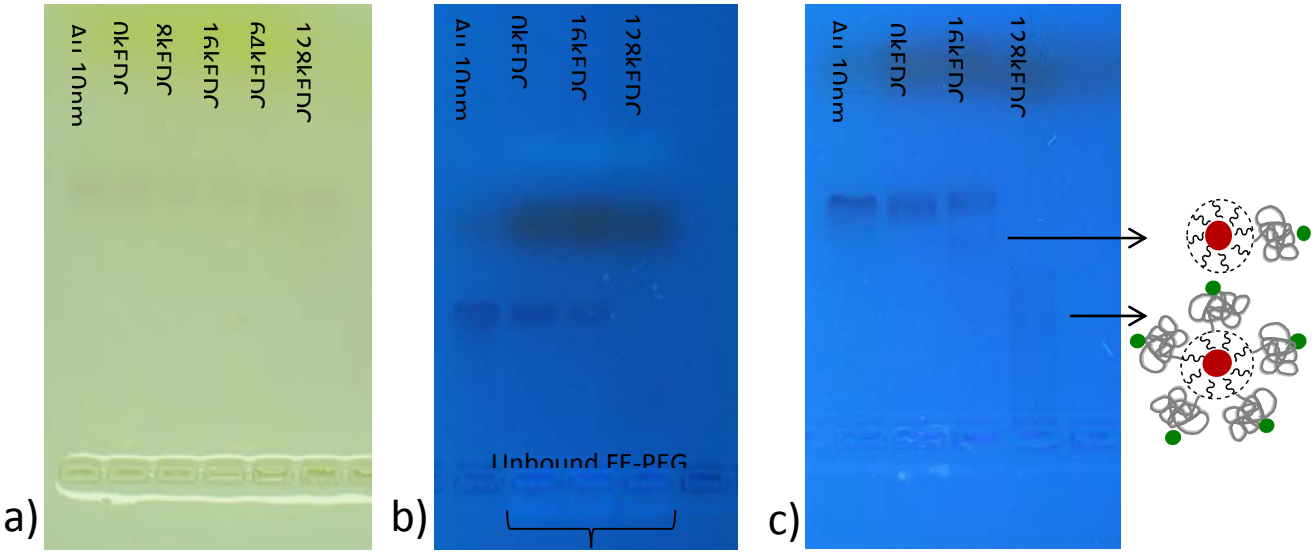


Figure SI-I.8.5: a) Conjugation of Peak 2 to the PMA-coated Au-NPs via EDC. No retardation was found even at an EDC ratio of 128,000/NP (see section I.10). b) Conjugation of Peak 3 to PMA-coated Au-NPs. The NPs were found to retard at higher EDC ratio > 16 kDa. c) Reverse UV view of picture b).

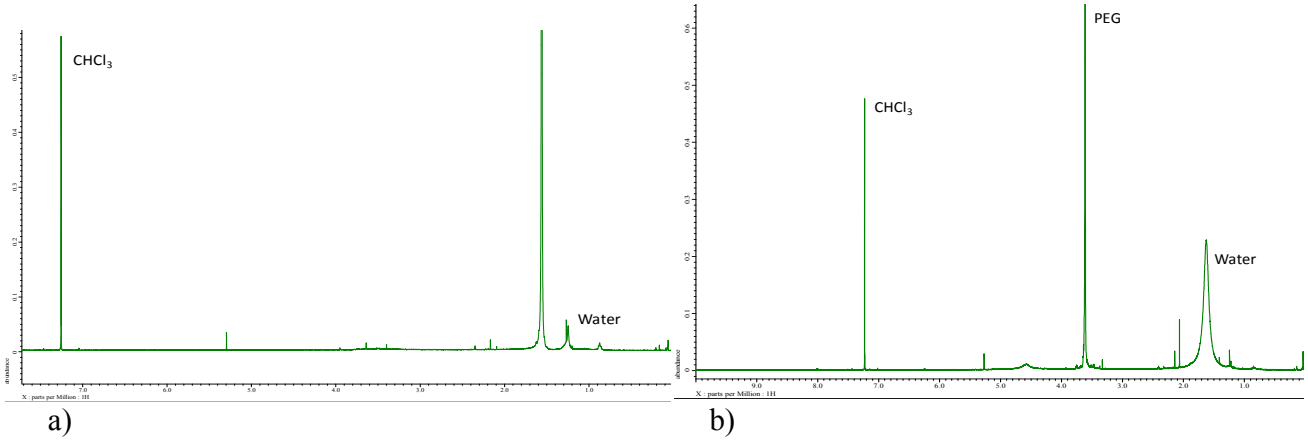


Figure SI-I.8.6: NMR spectra of a) 2nd eluted fraction from HPLC b) 3rd eluted sample from HPLC. No trace of PEG was found in 2nd peak whereas a PEG trace was present at position 3.6 in spectra.

I.9) Deprotection of Boc-protected FE-PEG (FE-PEG, compound 8)

The purified product in the aqueous phase was then mounted onto a rotary evaporator system (Laborota 4003 Control Evaporator, Heidolph®) and the water was evaporated. Then, the product was redissolved in 2 mL dichlomethane. 0.1 mL (1.3 mmol) of trifluoroacetic acid (≥99.0%, Sigma-Aldrich, Trifluoroacetic acid-CHROMASOLV®, for HPLC #302031) was then added as a deprotection reagent. Deprotection from Boc was carried out following a preexisting protocol with some modifications [10]. To the solution of Boc-protected FE-PEG in dichloromethane, 1 mL of trifluoroacetic acid was added and the solution was heated under reflux overnight. The solvent was then evaporated and the product (FE-PEG) was redissolved in SBB 9.0 buffer. The reaction scheme is depicted in Figure SI-I.9.1. Deprotection was verified with NMR, *cf.* Figure SI-I.9.2.

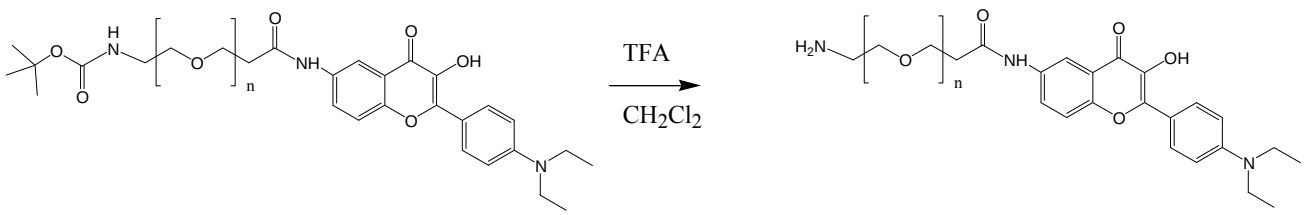


Figure SI-I.9.1: The deprotection scheme of Boc-protected FE-PEG to NH_2-FE-PEG.

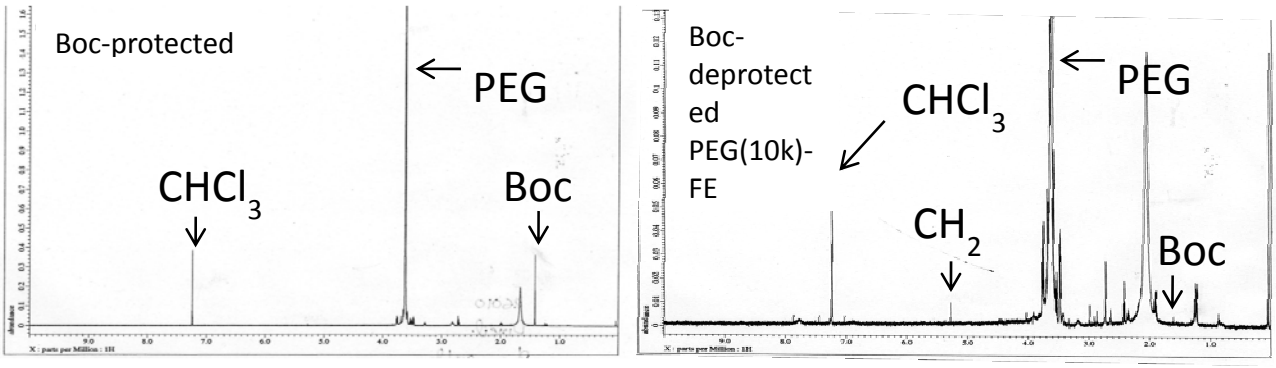
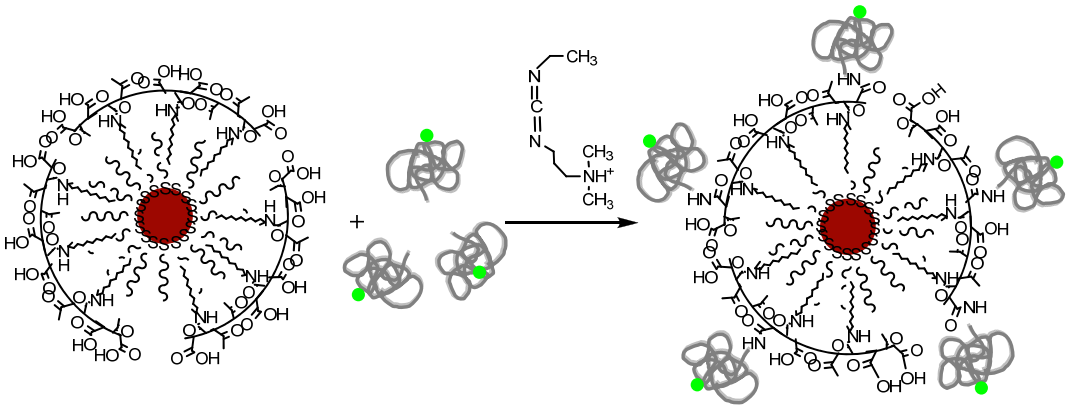


Figure SI-I.9.2: NMR spectra for the confirmation of Boc deprotection via TFA. The comparison between the starting BocNH-PEG-NHS and the reaction product shows the disappearance of the Boc tert-butyl group signal aroung 1.4 ppm.

I.10) Conjugation of FE-PEG to AP (compound 11)

In order to know the amount of yielded FE-PEG product, the solution was poured in a round flask and the buffer was removed by evaporation. The dried product (FE-PEG), was weighted to get the amount of FE-PEG. The FE-PEG film was then dissolved in SBB 9.0 buffer. In order to get PEG-conjugated Au NPs, polymer coated Au NPs (AP) were mixed with the FE-PEG molecules whereby the concentrations were adjusted in a way that 500 FE-PEG molecules were added per Au NP. The conjugation was mediated through a coupling agent (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, EDC, Sigma-Aldrich, #E7750). Freshly prepared EDC solution with a concentration of 0.768 M in SBB 9.0 buffer (sodium borate, 50 mM, pH 9) was added into the PEG / AP mixture. The amount of added

EDC was adjusted to the ratio 32000 EDC molecules per Au NP. The EDC activates the carboxylic groups on the polymer surface promoting the reaction with the amino terminals of the PEG[7] (Figure SI-I.10.1). The resulting mixture was left at room temperature for 2 hours without agitation. The ratio of EDC was chosen based on the series of experiments performed.



*FigureSI-I.10.1: FE-PEG (compound **8**) was attached to the surface of negatively charged polymer-coated Au NPs (AP, compound **9**) with EDC chemistry, resulting in FE-PEG-modified polymer-coated Au NPs (compound **11**).*

I.11) Purification of FE-PEG modified AP

FE-PEG conjugated Au NPs synthesized as described above (compound **11**) were purified by using HPLC (Agilent 1100 series). The cross-linked copolymer of allyl dextran and N,N'-methylene bisacrylamide (Sephacryl™ High Resolution (HR) S-300, GE Healthcare #17-0599-10) was used as stationary phase for column chromatography. The S-300 gel was packed in a glass column (Omnifit Glass Columns, 006CC-15-75-AA, 15 mm dia x 750 mm long; Omnifit). SBBS (sodium borate, 50 mM, Sodium chloride, 100 mM, pH 9) was used as eluent. The flow rate of solvent was fixed to 1 mL/min. The fraction containing the NPs was then concentrated again and fluorescence measurements were made.

[7] Appropriate reaction conditions help to minimize interparticle crosslinking.

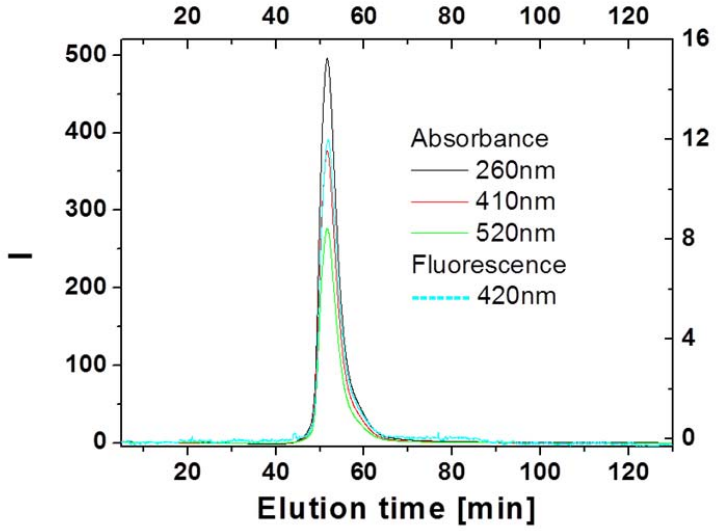


Figure SI-I.11.1: Purification of FE-PEG (10K PEG) conjugated Au NPs with HPLC. No free FE-PEG was detected depicting that reaction efficiency was high enough. Black, red and green lines correspond to the absorption spectra at 260 nm, 420 nm and 517 nm, whereas cyan line depicts the fluorescence spectra recorded on the eluted samples.

I.12) Conjugation of PEG to APFE (compound 12)

FE-PMA coated Au NPs (APFE), were synthesized as already described in Chapters SI-I.5 and SI-I.6. EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, Sigma-Aldrich #E7750) was then used to further functionalize the NP surface with PEG molecules. 10 kDa Bis-amino-PEG with two amino terminals (compound **3**) was weighted and dissolved in Milli-Q water to make a 15 mM stock.

In order to choose an appropriate amount of PEG to be added to Au NPs for conjugation first the ratio of PEG to NP had to be optimized. For this purpose solutions with FE-PMA coated Au NPs (compound **6**) of 2 μM concentration were mixed with the diamine-PEG molecules (compound **3**), whereby the concentrations were adjusted in a way that 500 diamine-PEG molecules were added per Au NP. Freshly prepared EDC solutions with a concentration of 0.768 M in SBB9 buffer (sodium borate, 50 mM, pH 9) were added into the PEG / Au NP mixture, whereby the amount of added EDC was varied in a way that 8000 to 128000 EDC molecules were added per Au NP [7]. EDC activates the carboxyl groups on the polymer surface leading to the linkage with the amino terminals of the PEG. The resulting mixtures were left at room temperature for 2 hours without agitation. In order to analyze the amount of PEG per Au NP the mixtures were run on 2% agarose gels at 100 V for 1 hour, see Figure SI-I.12.1a. The more PEG is attached per NP, the more retarded the bands are [7]. Based on the results shown in Figure SI-I.12.1a we used 8000 EDC molecules per Au NPs in all following conjugation experiments (Figure SI-I.12.1b).

In this way diamino-PEG (compound **3**) was linked with EDC to FE-PMA-coated Au NPs (compound **9**) using the following ratios in the reaction mixtures: 500 diamino-PEG molecules and 8000 EDC molecules per Au NP. After incubation the reaction mixtures were run for purification on 2% agarose gels in 0.5 × TBE buffer. The Au NPs were extracted from the gel by putting the gel pieces containing the NPs inside dialysis membrane tubes (50 kDa M_w cut off, Spectrum labs, #132544) and applying again voltage with the gel electrophoresis set-up until the NPs had migrated out of the gel. The NPs were then concentrated in centrifuge filters (membrane: 100 kDa M_w cut off PES, Sartorius Stedim, #VS2042) and transferred to Milli-Q water by running them through PD-10 Desalting Column. In Figure SI-I.12.1b, a gel picture of PEG-Au conjugates with EDC ratio 8000/NP (compound **11**) is shown.

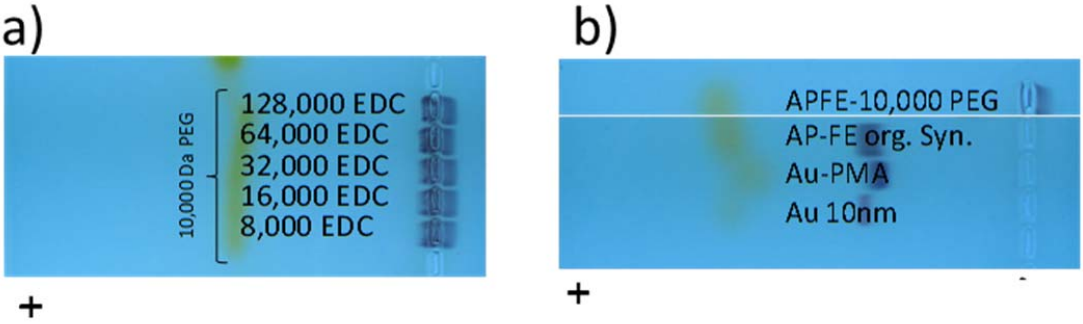


Figure SI-I.12.1: a) Test-series for finding the appropriate EDC to NP ratio for all the three samples with different PEG lengths. The ratio of PEG molecules was kept constant at 500 PEG molecules per NP. The image shows an agarose gel on which the conjugates had been run. Due to their negative charge they migrate towards the plus pole (i.e. from right to left in the image). b) From the test experiment the appropriate ratio EDC / NP to get sufficient amount of PEG on the NP surface was chosen to be 8000 EDC molecules per NP. "Big" batches were prepared with this ratio and purified with gel electrophoresis[8]. In the image a gel with conjugates of different lengths of PEG is shown. From left to right: phosphine stabilized Au of 10 nm diameter as marker [9], PMA coated Au NPs (AP), APFE conjugated with no PEG, APFE.

I.13) Size determination of NPs

Size determination of NPs: Particle size determination is of exquisite importance in nanomedicine and drug delivery. The size resemblance of NPs to the biological moieties is believed to impart their various properties. The size of NPs was measured by using different techniques such as Dynamic Light Scattering (DLS), Electrophoretic migration and Size Exclusion Chromatography (SEC) of FE-NPs [11]. For DLS measurements the Nanosizer ZS from Malvern was used. Four different samples were measured including two control samples such as (AP and AP-PEG(10k)). The samples were diluted down to 200 nM concentration. The hydrodynamic size of FE-NP and NP-PEG-FE was 13.2 nm and 24.6 nm respectively.

[8] All chemical from the reaction mixture run at different speed on the gel in comparison to the PEG-modified polymer-coated NPs. In this way excess reactants can be separated from the NPs.

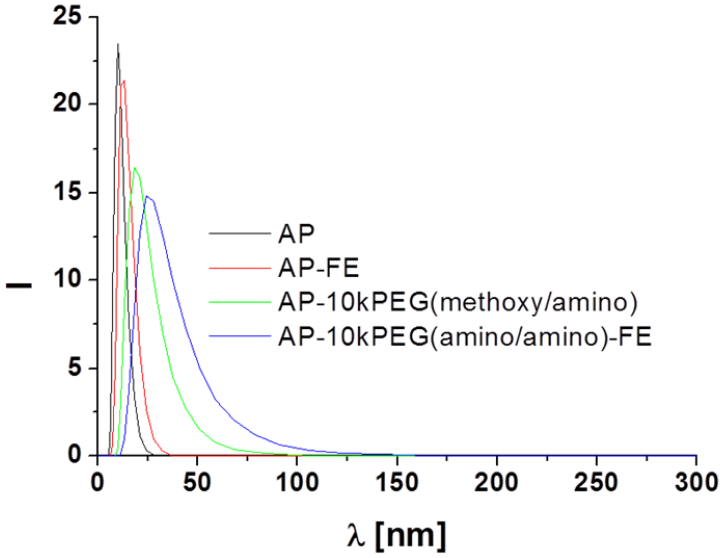


Figure SI-I.13.1: Determination of NP size by dynamic light scatteing. The Au NPs with FE dye incorporated in the polymer shell were measured and compared with the Au NPs without incorporated dye. The NP-PEG-FE system was measured and compared with NP-PEG of same molecular weight but without terminal FE dye. In both the cases the dye loaded NPs were found to be bigger as compared to the respective reference samples.

II) Spectroscopic analysis

II.1) Absorption and emission spectra of FE

General properties of FE:

Environment sensitive molecular sensors and probes exhibiting dual fluorescence response are subjected to intensive research due to their wide applications in biology and chemistry [12]. 3-Hydroxyflavones and their derivatives belonging to the wide family of flavonoids are important representatives of such sensors [13]. Upon excitation these organic compounds exhibit intramolecular proton transfer (ESIPT) reaction. The proton exchange is carried out between the hydroxyl group at position 3 and the carbonyl group at position 4 of the phenol ring [14]. This proton transfer gives rise to the tautomeric state of the molecules as shown in Figure SI-II.1.1. In aprotic environments, the two bands in the emission spectrum of FE dye molecules are attributed to the normal excited state (N*) and tautomeric excited (T*) state species. The ratio of the intensities of the two bands I_{N*}/I_{T*} is a function of the polarity of the dye environment. In protic environments, FE may be solvated (hydrated) to form so called H-bonded form. In this case upon excitation FE gives only one excited state that corresponds to H-N* form which leads to a single emission band in the fluorescence spectrum of H-bonded form (Figure SI-II.1.1). Such difference in emission spectra of hydrated and non-hydrated forms makes FE a good sensor for hydration of the dye environment. The level of hydration may be estimated from the formula: $I_{H\text{-}N*}/(I_{N*}+0.5\times I_{T*})$ [15], where $I_{H\text{-}N*}$, I_{N*} ,and I_{T*} are the intensities of H-N*, N* and T* bands consequently. In the present study we used FE dye, 4´-dialkylamino-3-Hydroxyflavone, for studying the solvatochromic effects of surrounding media upon the surface properties of water soluble colloidal Au NPs. Such nanometric probe could be used as potential sensor of polarity, ions and electric fields, and also as probe to study polymers, reverse micelles, lipid membranes and proteins. The advantage of these probes is their strong ratiometric response to interactions with the environment, as provided by changes in the relative intensities of these bands.

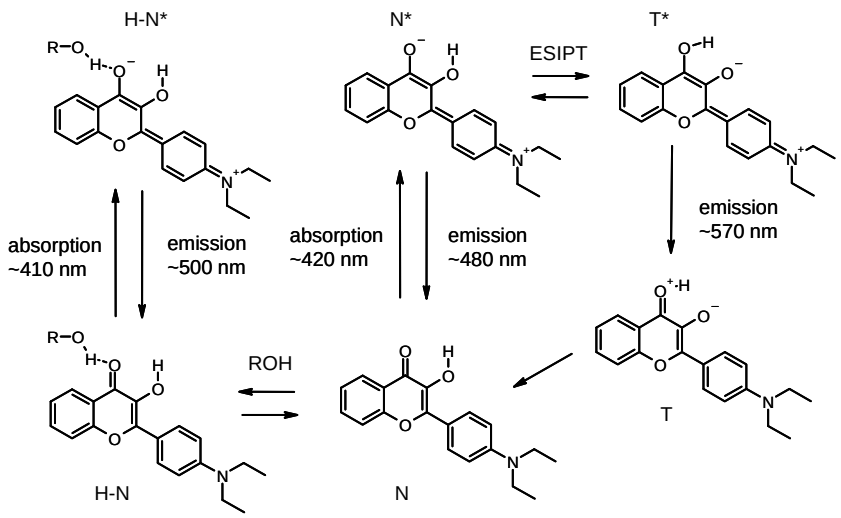


*Figure SI-II.1.1: The physical significance of possible excitable states of FE dye (compound **1**). N* and T* states exist in equilibrium in non-hydrated media. In H-bonding environment the third state that corresponds to solvated (hydrated) form of FE H-N* appears. Thus three states co-exist in an H-bonding media where often (esp. in water) the H-N* band dominates in the spectra.*

Absorption spectra of FE dependent on pH:

An UV/vis spectrometer (Agilent 8453) was used to measure the absorption spectra of FE dye and their pH dependence. To determine the pH dependence, the dye was dissolved in ethanol and then diluted with TBE 0.5X (pH 8.3) buffer. TBE 0.5X was used since the same solvent has to be used for later measurements with FE-NP conjugates. The samples were then mixed with standard pH buffers (Fixanal, Fluka / Sigma, # 38745-9-1EA-R) of different pH as indicated in the following spectra. The mixture sample in TBE 0.5x / pH-buffer was made in a ratio so that the final pH was not influenced by the TBE buffer. It was observed that FE absorption is independent of pH changes of the surrounding medium in moderately basic medium. A slight shift was observed for pH 10.

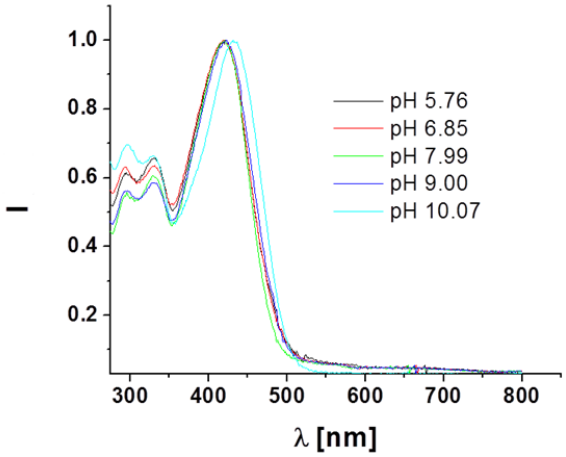


Figure SI-II.1.2: Absorption spectra I(λ) of FE dye at different pH. The spectra were normalized to the peak at 410nm. The same wavelength was later used for concentration measurements of all compounds.

Fluorescence spectra of FE dye in solvents of different polarity:

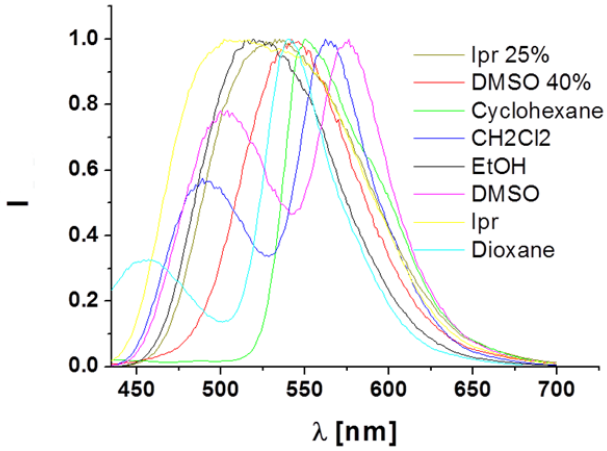


Figure SI-II.1.2: Fluorescence spectra I(λ) of FE dye in different solvents. The spectra were normalized to their peak amplitude. The solvents have been chosen as model representatives of a) non-polar b) aprotic polar and c) protic polar solvents. The dual emission results only in case of aprotic solvents. In both other cases the FE dye has a single emission peak from the hydrophobic (in case of non-polar solvents) or hydrated (in case of protic polar solvents) conformation.

Excitation and Emission spectra of FE dye:

Excitation and emission spectra were measured by using Fluorolog-3 spectrophotometer (HORIBA Jobin Yvon).

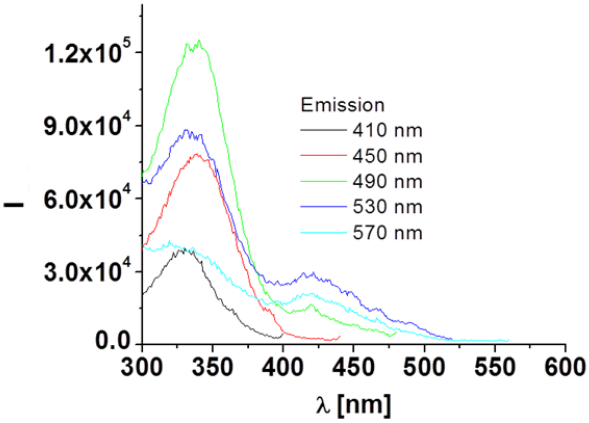


Figure SI-II.1.3: Excitation spectra of FE dye at different emission wavelengths measured in TBE 0.5X buffer.

The excitation spectra of FE dye (Figure SI-II.1.3) reveal two peaks at wavelengths 350 nm and 420 nm. Both of these peaks were used to study the emission of FE dye. The later peak was also used to carry out all the fluorescence measurements. In Figure SI-II.1.4 emission spectra of FE dye under presence of different solvents and molecules in solution are shown.

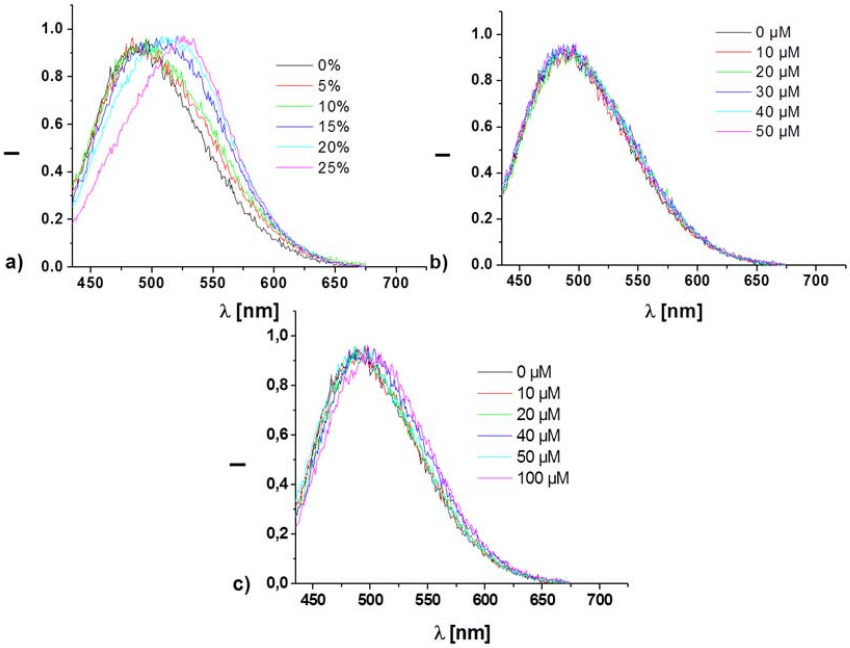


Figure SI-II.1.4: Emission spectra of FE dye at excitation at 350 nm in TBE 0.5X buffer with addition of a) isopropanol (Ipr), b) spermine, and c) Cetyltrimethylammonium bromide (CTAB) at different concentrations. All spectra are normalized to the emission peak at 500 nm. The less intense sharp peak at 400 nm is the water raman peak which can be neglected.

II.2) Absorption and emission spectra of APFE

Emission and excitation spectra of APFE:

Emission and excitation spectra of FE-PMA coated Au NPs (APFE) are shown in Figures SI-II.2.1 and SI-II.2.2. Emission spectra are deconvoluted into distinct peaks in Figure SI-II.2.3. The ratio of the intensities of the two emission bands I_{N*}/I_{T*} depends on the polarity of the environment of the FE dye. The polarity f is a function of the dielectric constant (ε) and can be represented as $f(\varepsilon) = (\varepsilon-1)/(2\varepsilon+1)$ [14]. In case of APFE dissolved in TBE buffer the emission intensity ratio of the FE dye was found to be $I_{N*}/I_{T*} = 2.89$. This corresponds to a microenvironment of the FE dye of $f(\varepsilon) \approx 0.4854$ or $\varepsilon \approx 50$, as had been determined from the dependence of $\log(I_{N*}/I_{T*})$ versus $f(\varepsilon)$ as shown in Fig. 2b of Klymchenko et al. [14] or Fig. 7 in Caarls et al. [16]. This means that the polarity of the microenvironment of FE dye in APFE is higher than the polarity of DMSO (ε = 46.8), but less than the polarity of water (ε = 80). Deconvolution of the spectra also helps to estimate of the level of hydration of the dye in APFE. The ratio of hydrated to non-hydrated of FE may be calculated as $I_{H-N*}/(I_{N*}+0.5xI_{T*})$ and for APFE is equal to 0.6. That means that only ~ 30% of FE dye molecules in APFE shell are hydrated.

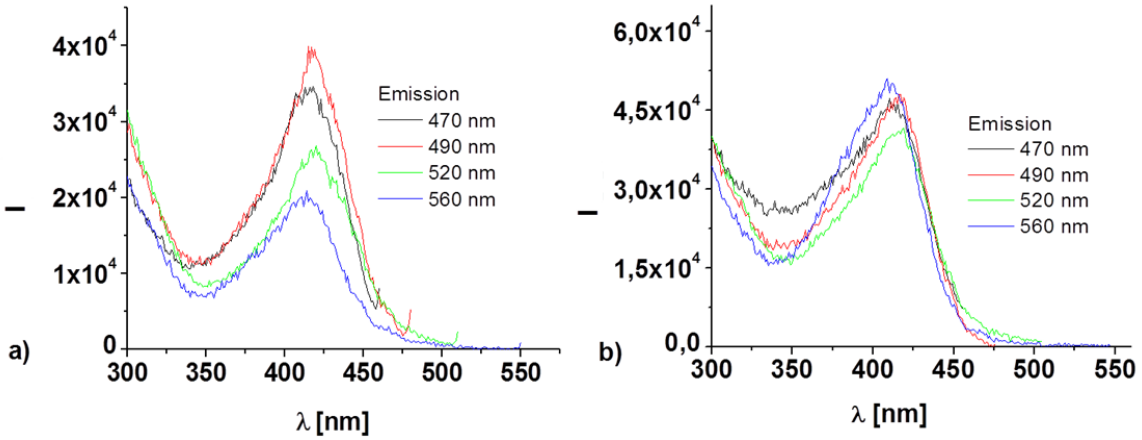


Figure SI-II.2.1: Excitation spectra of APFE recorded for emission at 470, 490, 520, and 560 nm. a) in TBE 0.5x, b) in a 25%/75% isopropanol/water mixture. Emission at longer wavelengths upon excitation at 420 nm is reduced in case of TBE buffer, whereas in the case of isopropanol/water mixtures the emission is enhanced, showing the enhancement of the hydrophobic peak with low polarity solvents.

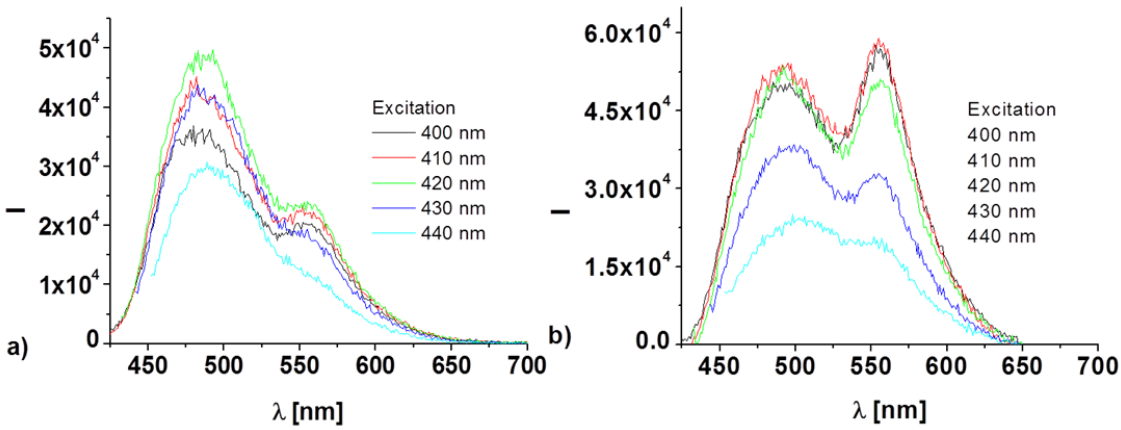


Figure SI-II.2.2: Emission spectra of APFE recorded at excitation at 400, 410, 420, 430, and 440 nm. a) in TBE 0.5X buffer, and b) in a 25%/75% isopropanol/water mixture.

Deconvolution of emission spectra of APFE:

The emission spectra of FE dye was deconvoluted into three bands according to the algorithm described by Caarls et al [16]. Each spectral band was approximated by a Siano-Metzler asymmetric log-normal function (alogn), whereby ν_0 is the peak position in wavenumber, H is the full width at half maximum, ρ is asymmetry and I_0 is the height:

$$\text{alogn}(\nu_0, H, \rho, I_0, \nu) = I_0 e^{-(\frac{\ln 2}{\ln \rho^2})(\ln[(\frac{\nu-\nu_0}{H})(\frac{\rho^2-1}{\rho}+1)])^2}$$

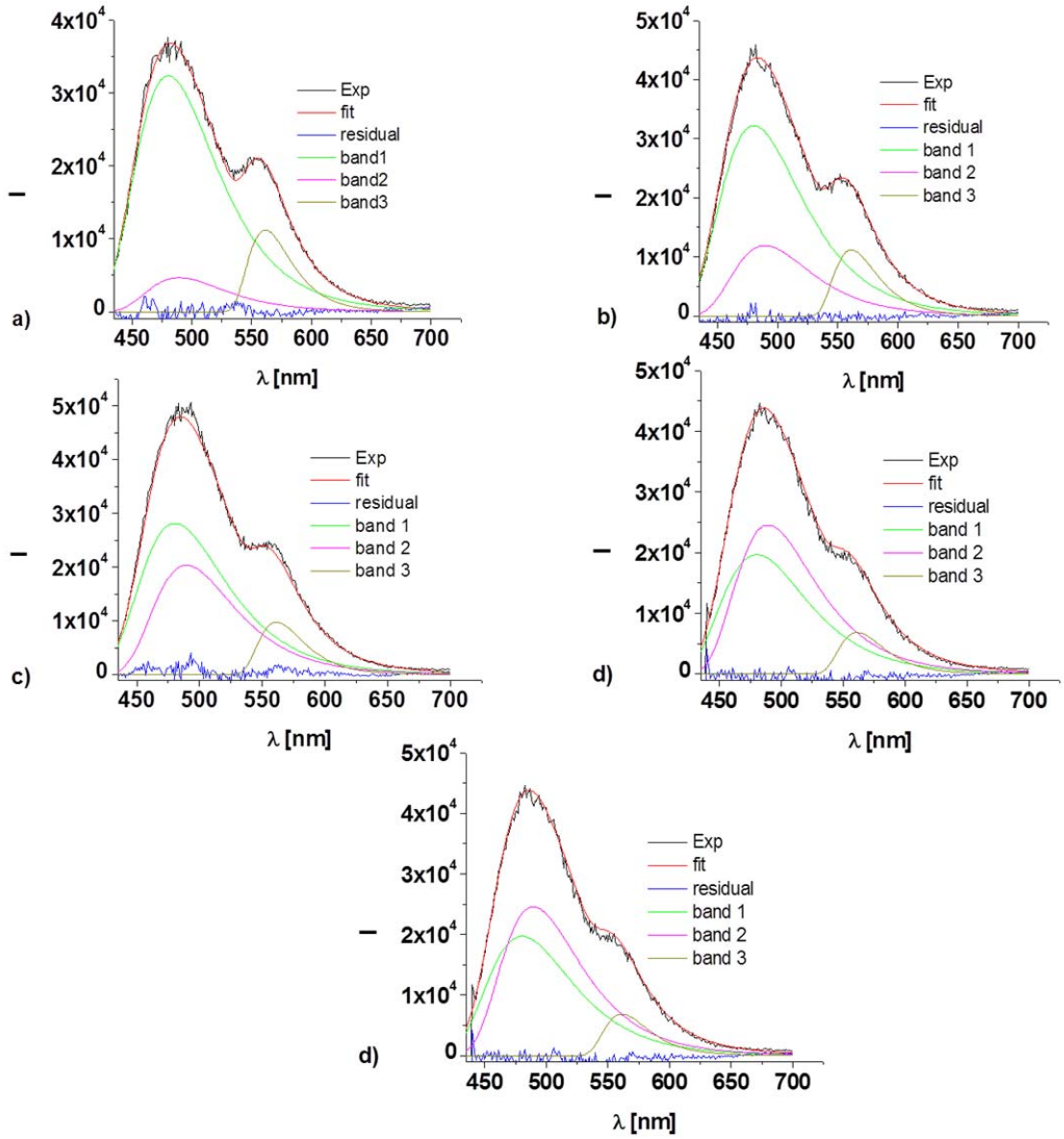


Figure SI-II.2.3: Deconvolution of emission spectra of APFE, which had been recorded in TBE 0.5X at different excitation wavelengths of a) 400 nm, b) 410 nm, c) 420 nm, d) 430 nm, and e) 440 nm. The black curves correspond to the experimental data. The red curves correspond to a fit of the experimental data composed out of 3 different emission peaks (green, pink and yellow). The green curve shows the residual between the experimental data and the fit. The blue, light blue, and pink emission peaks correspond to the normal (N), tautomeric (T*), and hydrated (H-N*) species of FE (cf. Chapter II.1). The hydrated species gets stronger at longer excitation wavelength.*

Emission spectra of APFE and PFE in solvents of different polarity and ionic strength:

In order to tune the polarity of the solvent, mixture of water and 2-propanol (Roth, ROTISOLV® HPLC, #7343.1) was used in different proportions. In this way the polarity index could be tuned by mixing the two constituent 2-propanol (polarity Index: p.I. 3.9) and water (p.I. 9.0). The corresponding spectra are shown in Figure SI-II.2.4. The effect of variable salt (KCl) concentration is shown in Figure SI-II.2.5

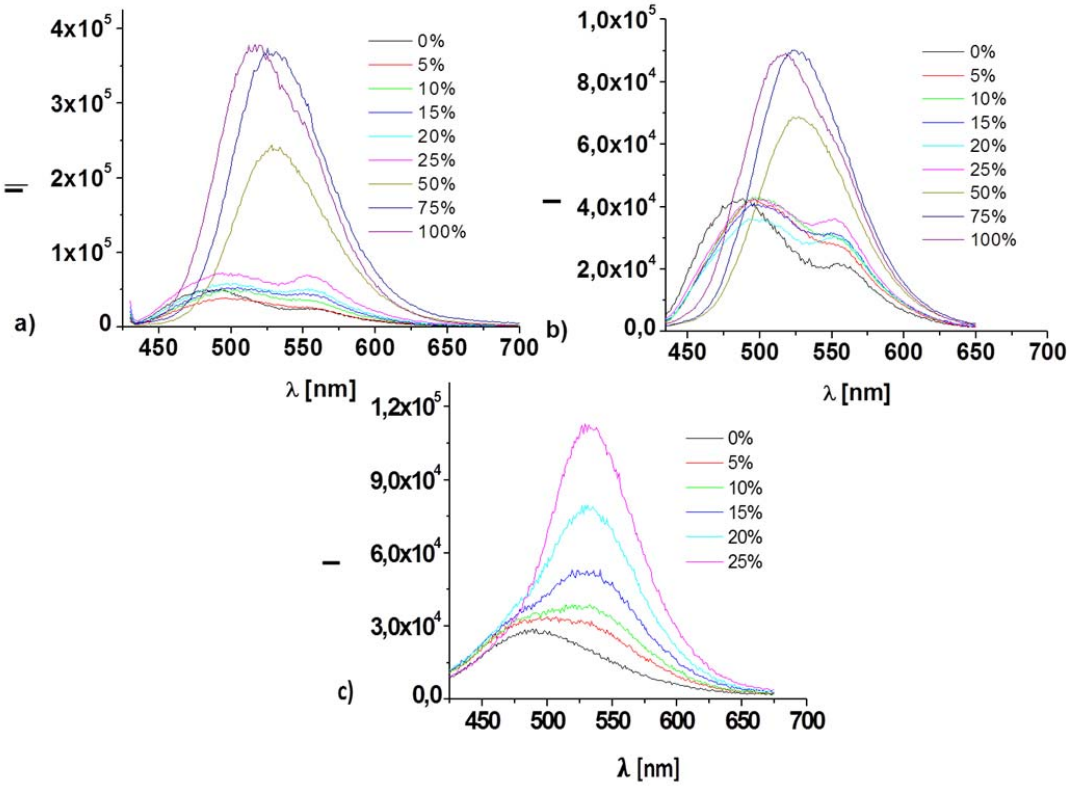


Figure SI-II.2.4: Effect of isopropanol titration on the emission properties of a) APFE, b) PFE (FE-PMA micelles), and c) FE at excitation at 420 nm. NOTE: The emission spectra shown in figure SI-II.1.4 (a) has an excitation wavelength of 350nm while here we have an excitation wavelength of 420nm. Therefore the two spectra should not be confused.

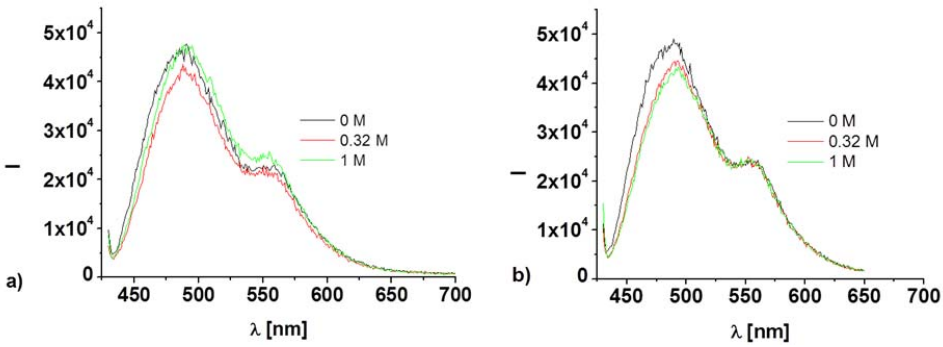
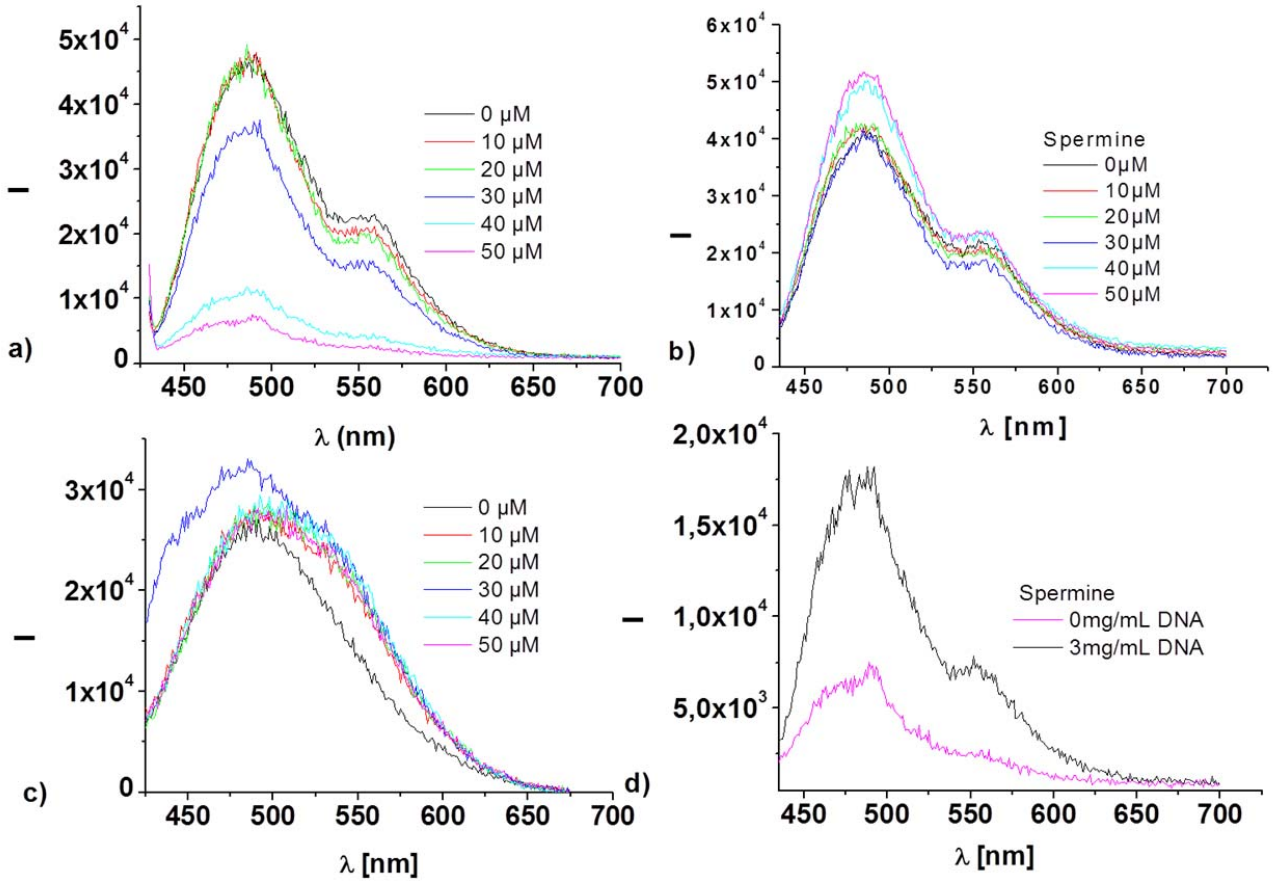


Figure SI-II.2.5: The effect of ionic strength on the emission properties of A) APFE (Au NPs coated with FE-PMA), and b) PFE (micelles out of FE-PMA). The KCl concentration was not increased beyond 1 M due to the fact that NPs can precipitate at higher salt concentration due to reduction of surface charge. Spectra were recorded at an excitation of 420 nm.

Emission spectra of APFE and PFE upon presence of spermine and CTAB:

Spermine (≥ 97%, Sigma, #S3256) was used to study the behavior of NP surface upon interaction with polyamines. Spermine solutions were prepared in TBE 0.5X buffer and then the NP solutions were titrated with the spermine solution to vary the concentration of polyamines (see Figure SI-II.2.6).



FigureSI-II.2.6: Effect of polyamine (spermine) on the emission spectra of a) APFE, b) PFE, and c) FE at Excitation of 420 nm. In case of APFE, at 40 μM of spermine concentration, the emission intensity is reduced about 5X compared to that of APFE without spermine. In contrast no comparable effect of spermine on the emission of PFE and FE was found, even not at sufficiently high concentrations such as 10 mM spermine. d) Quenching of APFE fluorescence due to the presence of 50 μM spermine can be retrieved upon addition of ct-DNA which complexes the spermine.

Cetyl-trimethylammonium Bromide (CTAB, ≥ 99.0% Fluka #52369) is a cationic surfactant and is known to assemble to micelles in aqueous solution after having reached a certain concentration known as critical micelle concentration (CMC). The effect of CTAB on the emission of APFE and PFE was studied. Figure SI-II.2.7 indicates that APFE is more sensitive to the presence of detergent than PFE

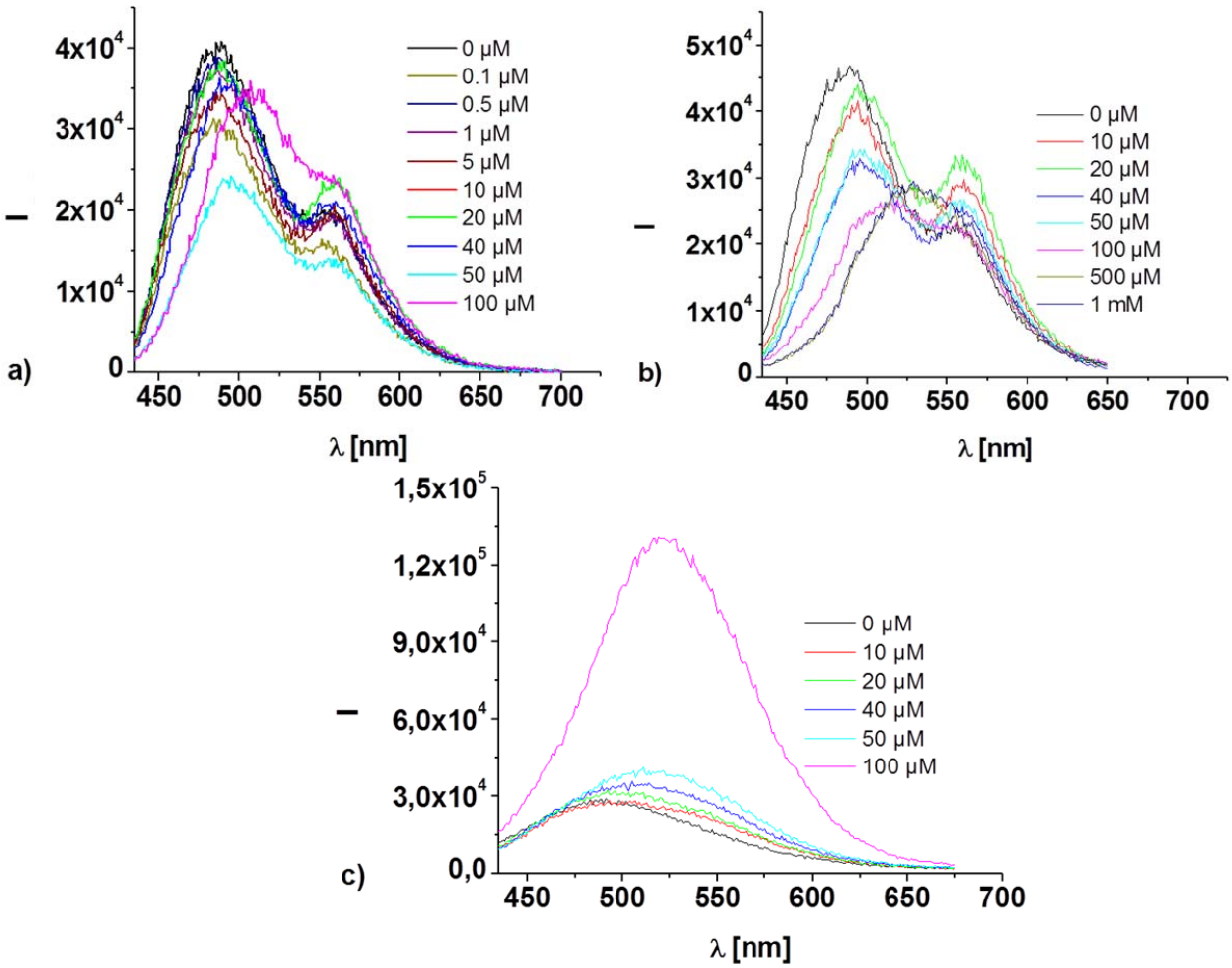


Figure SI-II.2.7: Effect of the zwitterionic detergent CTAB on the emission of a) APFE, b) PFE, and c) FE at excitation of 420 nm. The relative ratio of the two peaks (hydrophilic and hydrophobic) increases upon addition of CTAB and leads to hydration at a concentration of 100 μM of detergent.

II.3) Absorption and emission spectra of FE-PEG and AP-PEG-FE:

Absorption and Emission spectra of FE-PEG (10 kDa):

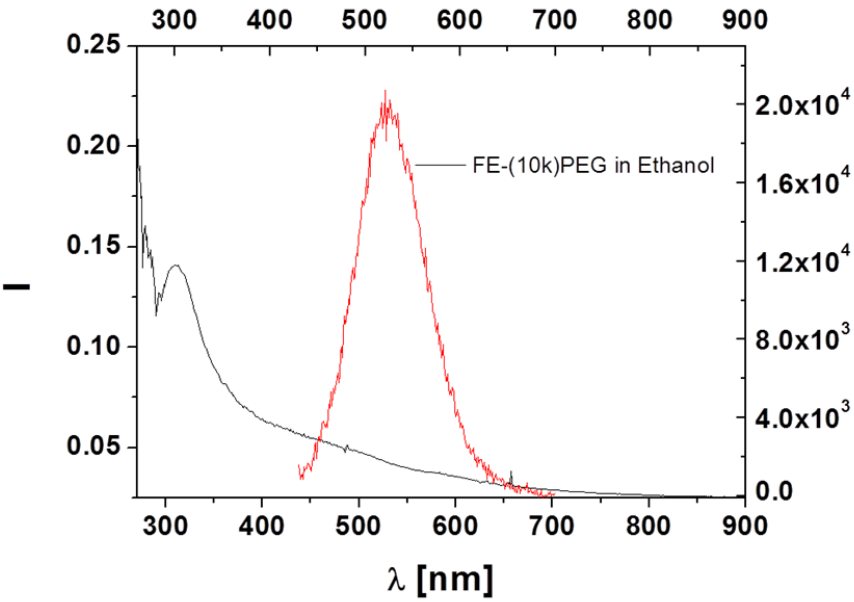


Figure SI-II.3.1: The absorption (black) and emission (red) spectra of FE-PEG in ethanol. The blue shifted absorption maximum is visible.

Emission spectra of FE-PEG (10 kDa) in different solvents:

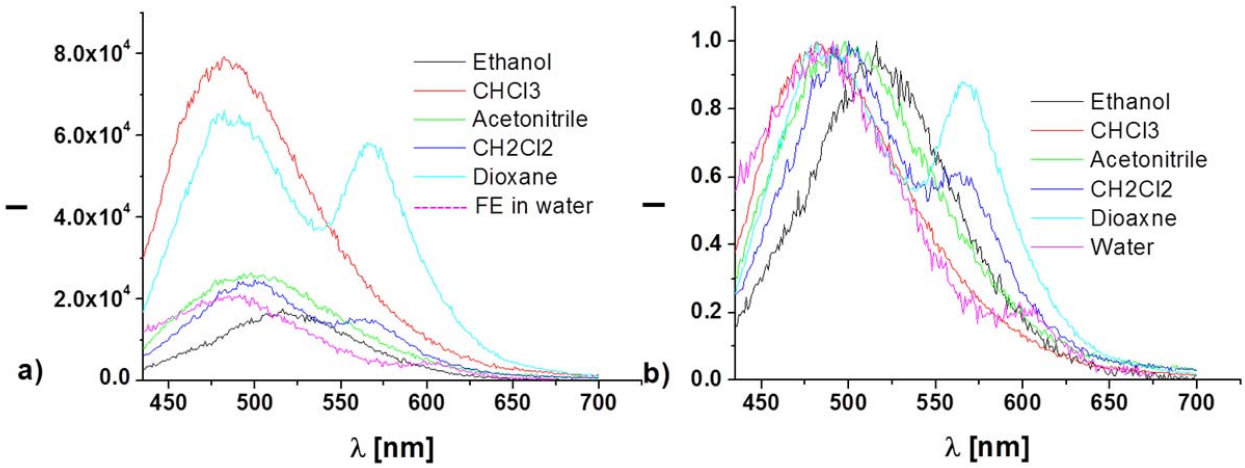


Figure SI-II.3.2: a) Emission spectra of FE-PEG were measured in different polarity solvents. It was found that the dual emission of FE can be retrieved in aprotic sovents (Dichloromethane, Dioxane) where the H-N band remains dominant in case of protic (Ethanol, Aceonitrile, water) and apolar (Chloroform) solvents. B) Normalization of Figure SI-II.3.2a.*

Absorption and Emission spectra of AP-PEG-FE:

AP was coated with FE-PEG to result in FE-PEG modified AP. PEG of different molecular weight was used. Absorbance and fluorescence spectra are shown in Figure SI-II.3.3.

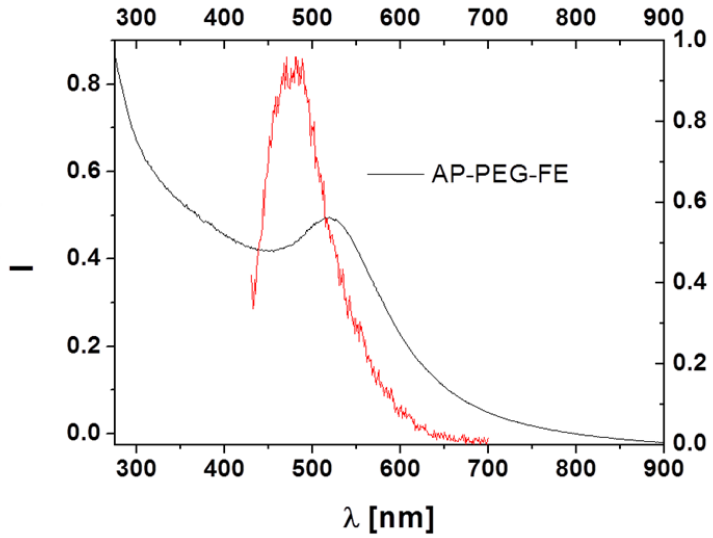


Figure SI-II.3.3: The absorption (black) and emission (red) spectra of AP-PEG-FE in water.

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