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ARTICLE

## Fluorescent core-shell nanoparticles and nanocapsules using comb-like macromolecular RAFT agents: synthesis and functionalization thereof

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Fluorescent nanoparticles and nanocapsules (FNPs) were synthesized *via* a one-pot RAFT miniemulsion process copolymerizing BODIPY-methacrylate and styrene in water. Ultra-bright sub-100 nm core-shell nanoparticles could be obtained with BODIPY covalently linked in the core, and possessing various shells. The nature and architecture of the particle shells could be tuned by using different macromolecular RAFT (macro-RAFT) agents in the miniemulsion polymerization process. The macro-RAFTs agents were composed of poly(ethylene oxide) acrylate (PEOA) and/or acrylic acid (AA), for their biocompatibility and functionality respectively, in different proportions. Interestingly, with comb-like macro-RAFT agents comprising a high number of PEOA, nanocapsules were formed, while with linear macro-RAFT agents or with those exhibiting a high number of AA, full core-shell nanoparticles were obtained. For all the structures the control over the polymerization, and the physico-chemical properties, such as size and morphology, zeta-potential and photophysical properties were measured and compared with FNPs exhibiting a linear PEO-*b*-PAA block copolymer shell structure (C. Grazon, J. Rieger, R. Méallet-Renault, G. Clavier, B. Charleux, *Macromol. Rapid Commun.* 2011, 32, 699). Regardless of the shell structures, the brightness of the formed nanoparticles was estimated to be 100-1000 times higher than that of quantum dots. Ultimately, the shell of the different FNPs was functionalized with a second fluorophore *via* the AA's carboxyl groups. Thus, water-soluble ultra-bright FNPs with two fluorophores in distinct environments (water and in polystyrene) were obtained. They should have great potential for bioimaging applications.

### Introduction

In the last decade, fluorescent molecules and nano-objects have received increasing interest for their high potential in biology and biochemistry. They are especially attractive for sensing, bioimaging and biomedical applications.<sup>1,2</sup> Two main types of fluorescent nanoparticles (FNPs) can be distinguished<sup>3,4</sup>: (1) inorganic intrinsically fluorescent nanoparticles, such as quantum dots<sup>5</sup>, and (2) non-fluorescent particles, mostly silica<sup>6</sup> or polymer<sup>7</sup> particles, that are doped with organic fluorophores. Usually, the second type of FNPs is less toxic and more easily functionalizable than the first type. Nevertheless, one of its problems is that the fluorophores can leak out of the particles with time. In order to avoid this shortcoming, the best solution is to covalently link the fluorophores to the polymer backbone. This can be achieved either by post-modifying the polymer with reactive fluorophores or by copolymerizing fluorescent

monomers with a comonomer.<sup>8-10</sup> When bio-imaging applications are targeted, it is crucial to control the nanoparticles' surface properties in order to control their interaction with cells or other bio-surfaces<sup>11</sup>, and to functionalize the nanoparticles with specific biomolecules relevant for targeting.<sup>12-16</sup> Fluorescent core-shell polymeric nanoparticles composed of a hydrophobic fluorescent core and a hydrophilic shell can be prepared using Reversible Deactivation Radical Polymerization (RDRP, formerly termed Controlled Radical Polymerization), in aqueous dispersed media.<sup>17,18</sup> Recent progress in RDRP in aqueous dispersed media, particularly in RAFT (Reversible Addition Fragmentation chain Transfer) polymerization, has allowed the synthesis of block copolymers particles with multi-functionality in water. Indeed, when reactive soluble polymers instead of surfactants are used in such a process, amphiphilic copolymers are formed that assemble *in-situ* during their synthesis into core-shell nanoparticles. This latter approach was named polymerization-induced self-assembly (PISA).<sup>19,20,21,22,23</sup> It was recently applied to the synthesis of fluorescent core-shell nanoparticles where a free fluorescent dye (Nile Red) was encapsulated in a dispersion polymerization approach performed in methanol.<sup>24</sup> In another study, reactive ketone functions were introduced in core-shell nanoparticles synthesized by PISA in water, and fluorescein was grafted post-polymerization *via* hydrazone chemistry.<sup>25</sup> In our previous study<sup>26</sup>, we have developed a simple surfactant-free

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one-pot miniemulsion polymerization process in water based on the RAFT mechanism. It allows the controlled synthesis of fluorescent nanoparticles with a hydrophobic core made of styrene copolymerized with a BODIPY monomer, and a hydrophilic shell based on a linear poly(ethylene oxide)-*block*-poly(acrylic acid) (PEO-*b*-PAA) diblock copolymer. BODIPY was chosen as fluorophore for its attractive spectroscopic features such as an emission spectrum tunable from green to red and a high fluorescence quantum yield. Thanks to its polymerizable methacrylate function, it was covalently linked to the polymer chains to avoid leaking of the fluorophore out of the particle. Poly(ethylene oxide) was selected as steric stabilizer for its biocompatibility and stealth properties<sup>27</sup>, and acrylic acids provide carboxyl groups available for post-functionalization. A miniemulsion process was mandatory because of the very low solubility of the fluorescent BODIPY monomer ( $\pi$ ) in water, inhibiting diffusion through the water phase as would be required in emulsion or dispersion polymerization conditions.<sup>26,29</sup> To stabilize the droplets of the miniemulsion, amphiphilic triblock copolymers had to be synthesized by chain extension with styrene/ $\pi$  in the bulk prior to the real miniemulsion polymerization step.

In the current study, we were interested in tuning the surface chemistry of the fluorescent nanoparticles, by coating them with either hydrosoluble homopolymers, linear PAA or comb-like poly(poly(ethylene glycol) methyl ether acrylate) (PPEOA), or their random copolymers, P(AA-*co*-PEOA). Compared to our former study using PEO-*b*-PAA diblock copolymers made in three synthetic steps, those can be obtained more readily thanks to a more straightforward synthetic pathway (economy of one synthesis step). However, the change in the macromolecular RAFT (macro-RAFT) agents' architecture made the adjustment of the polymerization conditions necessary. In the past, Hawkett *et al.*<sup>30,31</sup> had indeed studied the miniemulsion polymerization of styrene and butyl acrylate using PAA-based amphiphilic macro-RAFT agents and they concluded that the molar mass of the hydrophilic PAA and the hydrophobic segment must be precisely adjusted in order to reach control over the miniemulsion polymerization system. Whereas, PEO<sup>32,33</sup>, PAA<sup>34,35</sup>, P(AA-*co*-PEOA)<sup>36,37</sup>, PMAA<sup>38</sup> or P(MAA-*co*-PEOMA)<sup>39,40</sup>-based macro-RAFT agents have already been successfully used in RAFT *emulsion* polymerization, to our knowledge, in *miniemulsion* conditions, *linear* PAA<sup>30,31</sup> and PEO<sup>29,41</sup>-based macro-RAFT agent have been used, but *comb-like* structures, based on PEOA or/and AA have not been studied yet.

In the first part of the present study, conditions using comb-like PPEOA or P(AA-*co*-PEOA)-based RAFT agents for stabilizing and controlling the miniemulsion polymerization of styrene have been searched. After robust conditions were found, the fluorescent BODIPY phenyl methacrylate ( $\pi$ ) was added to the process in order to prepare aqueous dispersions of fluorescent nanoparticles at high solids contents. The second part of the study was then dedicated to the physico-chemical characterization of the particles: the particles' aggregation number, hydrodynamic diameter and morphology, zeta potential and the photophysical properties were determined

and compared to the former nanoparticles prepared using a linear PEO-*b*-PAA diblock copolymer as a stabilizing block. Finally, we explored the possibility to post-functionalize the NP's shell by grafting a second fluorophore in order to design nanoparticles which emit at two distinct wavelengths.

## Experimental section

### Instrumentation

<sup>1</sup>H NMR spectra were recorded in CDCl<sub>3</sub> on a JEOL ECS (400 MHz) spectrometer. All chemical shifts are referenced to Me<sub>4</sub>Si (TMS) used as internal standard.

The number-average molar mass ( $M_n$ ), the weight-average molar mass ( $M_w$ ), and the molar mass dispersity ( $M_w/M_n$ ,  $\mathcal{D}$ ) were determined by size exclusion chromatography (SEC -  $M_{n,SEC}$ ) using THF as an eluent at a flow rate of 1 mL min<sup>-1</sup>. For analytical purposes, the carboxyl groups of the hydrophilic block or statistical copolymers were transformed into methyl esters. Therefore, the copolymers were recovered by drying the aqueous dispersions. After dissolution in a THF/H<sub>2</sub>O mixture and acidification of the medium with a 1M HCl solution, they were methylated using an excess of trimethylsilyldiazomethane.<sup>42</sup> Polymers were analyzed at a concentration of 5 mg mL<sup>-1</sup> in THF after filtration through 0.45  $\mu$ m pore size membrane. The SEC apparatus was equipped with a sample delivery module (GPCmax from Malvern Instruments) and two columns thermostated at 40°C (PLgel Mixed C (7.5 mm  $\times$  300 mm), bead diameter: 5  $\mu$ m). Detection was made with a differential refractive index detector (Viscotek VE 3580 RI detector) and a UV-vis. detector (Waters 486 Tunable Absorbance Detector). The Viscotek OmniSEC software (v 4.6.2) was used for data analysis and the relative  $M_n$  and  $M_w/M_n$  were calculated with a calibration curve based on polystyrene standards (from Polymer Laboratories).

The z-average particle diameter (named  $D_z$ ) and the particle size distribution (dispersity factor, named  $\sigma$ ), were determined by dynamic light scattering (DLS) of the diluted aqueous dispersions, at an angle of 90° at 20°C, with a Zetasizer Nano S90 from Malvern, using a 4 mW He-Ne laser at 633 nm. A value of  $\sigma$  below 0.1 is characteristic of a narrow particle size distribution. All calculations were performed using the Nano DTS software.

Zeta potentials ( $\zeta$ ) were performed on a Zetasizer Nanoseries (Malvern) apparatus at the Institut Curie, Paris, France. Samples were prepared at a concentration of 0.005 wt% diluted with 14mM NaCl water, buffered with 1mM phosphate/citrate salts (pH values varied from 4 to 8). Samples were analyzed in DTS 1060 plastic cells, at 25°C. Three measurements of at least ten scans were performed for each sample.

UV-vis. spectra were recorded on a Varian Cary 5000E (Palo Alto, CA USA) double beam spectrometer using a 10 mm path quartz cell from Thuet (Bodelsheim, France). Fluorescence emission spectra were measured on a SPEX Fluoromax-3 (Horiba Jobin-Yvon). A right-angle configuration was used. Optical density of the samples was checked to be less than 0.1 to avoid reabsorption artifacts. The fluorescence quantum

yields  $\Phi_F$  were determined using Rhodamine 590 ( $\Phi_F = 0.95$  in ethanol) as a reference (error of 5 %).<sup>43</sup> The fluorescence decay curves were obtained with a time-correlated single-photon-counting method using a titanium-sapphire laser (82 MHz, repetition rate lowered to 4 MHz thanks to a pulse-peaker, 1 ps pulse width, a doubling crystals is used to reach 495 nm excitation) pumped by an argon ion laser from Spectra Physics (Mountain View, CA USA). For the fluorescent BODIPY-based monomer,  $\pi$ , the Levenberg-Marquardt algorithm was used for non-linear least square fit as implemented in the Globals software (Globals Unlimited, Villa Grove, USA). Lifetimes are given with an error of  $\pm 0.05$  ns. In order to estimate the quality of the fit, the weighted residuals were calculated. In the case of single photon counting, they are defined as the residuals, *i.e.* the difference between the measured value and the fit, divided by the square root of the fit.  $\chi^2$  is equal to the variance of the weighted residuals. A fit was said appropriate for  $\chi^2$  values between 0.8 and 1.2.

For multi-exponential fluorescent decays (nanoparticles), no fit was attempted and the average fluorescence lifetimes were calculated by integrating the area below the decay curve as:<sup>44</sup>

$$\langle \tau \rangle = \frac{\int_0^{\infty} tI(t)dt}{\int_0^{\infty} I(t)dt} \quad \text{Equation 1}$$

Conventional transmission electron microscopy (TEM) was performed on a JEOL JEM CX II UHR microscope operating at 100 keV and equipped with a Keen View CCD camera from Soft Imaging System (Olympus) calibrated with three polystyrene particle samples (PELCO 610-SET - 91, 300, and 482 nm, Ted Pella Inc.). The acquisition was done with the iTEM software from Soft Imaging System (Olympus). The samples were diluted in water prior to analysis and then deposited on a carbon-coated copper grid.

## Materials

Poly(ethylene glycol) methyl ether acrylate (Sigma-Aldrich,  $M_n = 454 \text{ g mol}^{-1}$ ), acrylic acid (99 %, Aldrich), 2-methyl-2-[[dodecylsulfanylthiocarbonyl]sulfanyl]propanoic acid (97%, Strem, TTCA), (trimethylsilyl)diazomethane (2.0 M in diethyl ether, Aldrich), 4,4'-azobis(4-cyanopentanoic acid) (Aldrich, ACPA), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (Sigma, EDC), fluoresceinamine (Sigma, FA), ethanolamine (Sigma-Aldrich, EtOA) and ethylenediamine (Sigma-Aldrich, Et(NH<sub>2</sub>)<sub>2</sub>) were used as received. Solvents (Carlo Erba) were of synthetic grade and purified according to standard procedures. Styrene was distilled under reduced pressure. 2,2'-Azobis(2-methylpropionitrile) (98%, Sigma, AIBN) was recrystallized from chloroform containing a few drops of petroleum ether. The synthesis of BODIPY-based fluorescent monomer with a phenyl methacrylate function ( $\pi$ , **Figure SI-1**) was performed as previously described.<sup>26</sup>

## Synthesis of fluorescent nanoparticles by RAFT miniemulsion polymerization

The RAFT copolymerization of styrene and  $\pi$  was performed in a one-pot phase inversion process<sup>26</sup>, in the presence of a stabilizing P(PEOA-co-AA), PPEOA or PAA macro-RAFT agent.

Those macro-RAFT agents were synthesised according to our previous study<sup>36</sup>, using different amounts of PEOA, AA and the trithiocarbonate-type RAFT agent, TTCA (**Scheme SI-1**). In a typical macro-RAFT synthesis, P(PEOA<sub>11</sub>-co-AA<sub>11</sub>), Table SI-1, entry 3), the RAFT agent, TTCA, (0.80 mmol, 291 mg, MW = 364 g mol<sup>-1</sup>), acrylic acid (AA, 10 mmol, 720 mg), PEOA (10 mmol, 4.54 g) and DMF (as an internal reference for the <sup>1</sup>H NMR determination of the monomer consumption in deuterated chloroform) (4 mmol, 292 mg) were dissolved in 9.9 mL of 1,4-dioxane at room temperature. Then, 0.1 mL of a 0.53 M solution of ACPA in 1,4-dioxane were added. The mixture was purged with nitrogen for 30 min in an ice bath, and then placed in an oil bath thermostated at 80°C to initiate the polymerization. After 90 min, the reaction was stopped by immersion of the flask in iced water. The monomer conversion was determined by <sup>1</sup>H NMR in CDCl<sub>3</sub>. The copolymer was precipitated twice in cold *n*-pentane in order to remove the monomers and dried under reduced pressure.

Then, for a typical experiment of the nanoparticles synthesis (**FNPS**), 197 mg of P(PEOA<sub>11</sub>-co-AA<sub>11</sub>) macro-RAFT agent ( $3.2 \times 10^{-5} \text{ mol}$ ,  $M_n = 6.2 \text{ kg mol}^{-1}$ ) were dissolved in a mixture of 660 mg of styrene ( $6.3 \times 10^{-3} \text{ mol}$ ), 2.0 mg of AIBN ( $1.2 \times 10^{-5} \text{ mol}$ ) and 29 mg of monomer  $\pi$  ( $6.3 \times 10^{-5} \text{ mol}$ ), in a septum-sealed flask. The mixture was purged with argon for 30 min in an ice bath, and then placed in an oil bath thermostatically controlled at 80°C to initiate polymerization. After 70 min, the reaction was stopped by immersion of the flask in iced water. The conversion of the monomers (styrene and  $\pi$ ) was determined by gravimetry and SEC, respectively (for details see below). Then, 5 mL of a 0.1 M NaOH solution (pH = 12.5) were added to this cold organic mixture. An ultrasonic horn (Bandelin electronics, Sonoplus HD 2200) was then placed in the biphasic mixture cooled down in an ice bath and powered at 130W for 10 minutes to form the metastable miniemulsion.

After the miniemulsion formation, the pH decreased to 11. The miniemulsion was purged with argon for 30 min in an ice bath, and then placed in an oil bath thermostatically regulated at 80°C to reinitiate the polymerization. Sampling was performed at regular time intervals and monomer conversions were determined by gravimetric analysis for styrene corrected from the styrene loss by evaporation during the sonication process (25 wt% calculated by comparison of <sup>1</sup>H NMR spectra in CDCl<sub>3</sub> and gravimetric analysis<sup>45</sup>) and by SEC using UV-visible detection at 528 nm for  $\pi$ .

Nanoparticles chains' theoretical number-average molar mass ( $M_{n,th}$ ) were calculated as  $M_{n,th} = M_{CTA} + 1/n_{CTA} \times (\chi_S \times m_S + \chi_{\pi} \times m_{\pi})$ , where  $\chi_i$  stands for the individual molar conversion of monomer *i*,  $m_i$  stands for the mass of monomer *i* used in the synthesis and CTA refers to the chain transfer agent.

## Functionalization of the nanoparticles (e.g. with fluoresceinamine)

In a typical experiment, 0.3 mL of the pristine nanoparticles aqueous dispersion batch (0.1 g mL<sup>-1</sup>) was diluted to 4 mL of water in the dark (final concentration = 8 mg mL<sup>-1</sup>). In parallel, ~ 5 mg of fluoresceinamine (FA, 0.01 mmol) was dissolved in 0.4



mL of ethanol. Once the FA was dissolved, the solution was added to the nanoparticle dispersion and the mixture was vigorously stirred at 4°C. Then, a solution of EDC (15 mg, 0.1 mmol) in water (1 mL) was added to the previous mixture. To quench the reaction, ethanolamine (2.3  $\mu$ L, 0.04 mmol) was added 2 hours later. The reaction solution was stirred at 4°C for further 12 hours in the dark. Finally, the mixture was transferred in a cellulose ester dialysis membrane (MWCO: 300 kDa, Spectrapor) and dialyzed against water for 7 days in the dark. The grafting efficiency of FA was quantified by UV-vis spectrometry by comparison of the number of FA with the number of  $\pi$  per FNP. To do so, an absorption spectra of the FNP was recorded in water at pH = 8 at room temperature and then the maximum absorption band of the BODIPY (at 528 nm,  $\epsilon_{\pi} = 73 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ <sup>[26]</sup>) was compared with the one of the FA (at 494 nm,  $\epsilon_{\text{FA, grafted}} = 88 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  at pH 8<sup>[46]</sup>).

## Results & discussion

### Synthesis of core-shell polystyrene nanoparticles

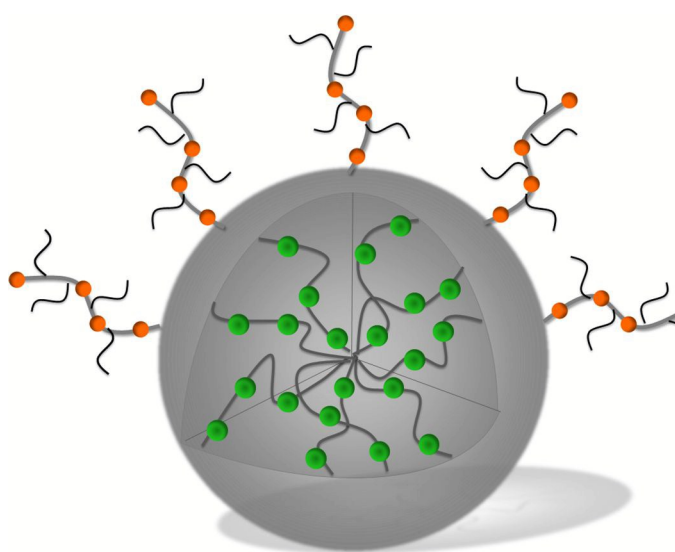
Firstly, different water-soluble macro-RAFT agents containing AA and/or PEOA (with molar ratios of AA/PEOA = 100/0, 50/50 and 0/100) were synthesized in dioxane solution in the presence of TTCA as a chain transfer agent (Scheme SI-1, Table SI-1). They were named P(AA<sub>x</sub>-co-PEOA<sub>y</sub>). In this way, PPEOA and PAA homopolymers, and three P(AA<sub>50%</sub>-co-PEOA<sub>50%</sub>) copolymers with AA/PEOA = 50/50 of different molar mass were obtained.

According to our previously published process, the core-shell nanoparticles (Figure 1) were then synthesized *via* a one-pot miniemulsion polymerization process in two steps (Scheme SI-2). In the first synthesis step, an amphiphilic macro-RAFT agent P(AA<sub>x</sub>-co-PEOA<sub>y</sub>)-b-PS<sub>0</sub> is prepared through the bulk polymerization of styrene in the presence of the water-soluble

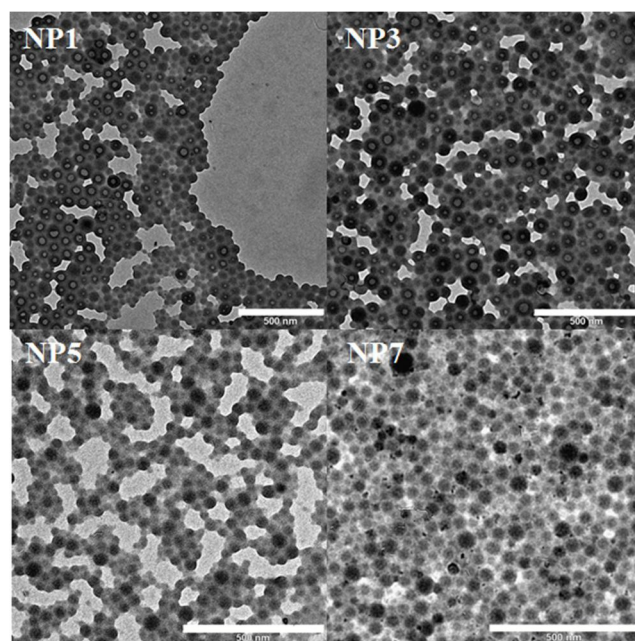
macro-RAFT agent. The polymerization is stopped at low monomer conversion and a viscous solution of the amphiphilic diblock copolymer in styrene is obtained. Then, basic water is added and the mixture is sonicated in order to form a miniemulsion of droplets of the remaining styrene that are stabilized by the amphiphilic macro-RAFT agents formed in the previous step.

Core-shell polymeric nanoparticles composed of P(AA<sub>x</sub>-co-PEOA<sub>y</sub>)-b-PS<sub>0</sub>-PS<sub>1</sub> are finally obtained after 4 hours of polymerization at 80°C, and quasi complete monomer conversion is generally reached. For all experiments, the global molar ratio of styrene to macro-RAFT agent was kept constant and equal to 200, so that the final polymers should all possess polystyrene segments of the same molar mass.

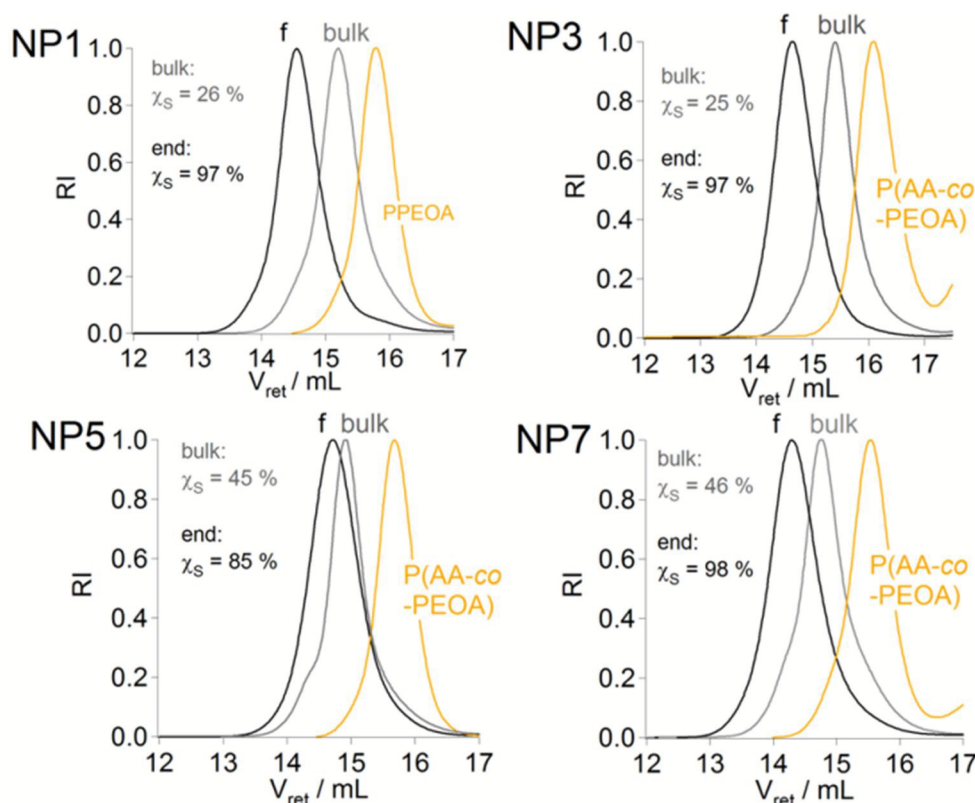
However, by changing the time of the first polymerization step, *i.e.* the bulk step, (while keeping the styrene to macro-RAFT ratio constant), the molar mass of the polystyrene segments PS<sub>0</sub> allowing for the stabilization of the monomer droplets in the miniemulsion polymerization step could be varied. It was thus possible to change the hydrophilic/lipophilic balance of the macro-RAFT without changing the total amount of styrene in the final nanoparticles. It has already been demonstrated<sup>30,31,47</sup> that the hydrophobic segment in the amphiphilic stabilizer has a crucial impact on the particle formation in heterogeneous polymerizations and particularly on the control over the polymerization.



**Fig. 1** Schematic representation of the core-shell fluorescent nanoparticles stabilized by P(AA-co-PEOA) comb-like polymer.



**Fig. 2** Transmission electron microscopy images of polystyrene nanoparticles with PPEOA (NP1) or P(AA-co-PEOA) (NP3, NP5, NP7) comb-like shells. Scale bar: 500 nm.



**Fig. 3** Size exclusion chromatograms in THF (RI detection) for polymer chains of **NP1**, **NP3**, **NP5**, **NP7** at the beginning of the polymerization (corresponding to the isolated macro-RAFT agent) (— yellow), at the end of the bulk polymerization PS0 (— grey) and at the end of miniemulsion polymerization (— black).

**Table 1** summarizes the syntheses of various polystyrene nanoparticles stabilized by either a PPEOA, P(AA-co-PEOA) or PAA, shell. First, the PPEOA macro-RAFT agent with a molar mass of  $5.4 \text{ kg mol}^{-1}$  was used. This macro-RAFT agent is readily soluble in styrene, which allows performing the above described one-pot miniemulsion process. Stopping the first, bulk polymerization step at different conversion rates, amphiphilic stabilizing PPEOA-*b*-PS0 copolymers possessing  $DP_{n,s}$  of PS0 equal to 40 and 65 were formed (syntheses of **NP1** and **NP2**, respectively). After the miniemulsion polymerization, for both experiments spherical nanoparticles with a hydrodynamic diameter around 90-95 nm containing a hole in their center were obtained (**NP1** in **Figure 2** and **NP2** in **Figure SI-2**). They resemble nano-sized vesicles or capsules.

In both cases, the polymerizations were well controlled with molar mass dispersities  $\mathcal{D}$  below 1.37 and molar mass that shifted completely with monomer conversion (SEC traces in **Figure 3**, **NP1** and **Figure SI-3**, **NP2**).

Next, the three comb-like P(AA<sub>50%-CO</sub>-PEOA<sub>50%</sub>) macro-RAFT agents with a random distribution of equimolar amounts of AA and PEOA<sup>36</sup> differing in their molar mass ( $M_n = 3.5, 6.2$  and  $9.2 \text{ kg mol}^{-1}$ , cf. Table SI-1) were used. With the shortest macromolecular RAFT agent P(AA<sub>6</sub>-CO-PEOA<sub>6</sub>) ( $M_n = 3.5 \text{ kg mol}^{-1}$ ) and a polystyrene extension of 38 monomer units in the bulk polymerization step, again spherical particles with a diameter of 90 nm and a void in their center were obtained (**NP3**, **Figure 2**), similar to the experiments using the PPEOA macro-RAFT agents. The polymerization was well controlled with a low molar mass dispersity of 1.29 and no traces of remaining macro-RAFT agent

were detected by SEC (**NP3**, **Figure 3**). With the second, longer macro-RAFT agent P(AA<sub>11</sub>-CO-PEOA<sub>11</sub>) ( $M_n = 6.2 \text{ kg mol}^{-1}$ ), spherical particles (**NP5**) were also obtained, but they were smaller in size ( $D_z = 70 \text{ nm}$ ) than **NP3** and without any void (Figure 2). The polymerization was well-controlled with a dispersity  $\mathcal{D}$  of 1.35, and SEC traces (**NP5**, **Figure 3**) proved again good efficiency using the P(AA<sub>50%-CO</sub>-PEOA<sub>50%</sub>) macro-RAFT agent. The third, longest macro-RAFT agent possessing the highest amount of PEO grafts P(AA<sub>16</sub>-CO-PEOA<sub>17</sub>) ( $M_n = 9.2 \text{ kg mol}^{-1}$ ) was then tested: with a chain extension of 35 styrene units, the nanoparticles obtained after the miniemulsion polymerization (**NP6**) were polydisperse in size, bigger than the previous ones with a z-average diameter,  $D_z$ , of 100 nm. Again, they did not present any holes (**NP6**, **Figure SI-2**). The chain extension took place during polymerization as asserted by SEC, but the molar mass dispersity was higher, in the range of 1.6 (Figure SI-3). A former study had related the heterogeneity in particle size to an insufficient length of the hydrophobic PS segment necessary to durably localize the amphiphilic stabilizer at the styrene droplet/water interface.<sup>30,31</sup> We thus performed a second experiment with P(AA<sub>16</sub>-CO-PEOA<sub>17</sub>), in which a longer hydrophobic, anchoring PS0 segment was prepared in the bulk polymerization step. Indeed, using a macro-RAFT agent chain extended by 70 styrene units in average (instead of 35, **NP7** vs. **NP6**), the resulting nanoparticles were much more homogeneous in size (**NP7**, **Figure 2**) and the polymerization was well controlled as indicated by the lower molar mass dispersity ( $\mathcal{D} = 1.36$ ). No remaining macro-RAFT agent was detectable in the SEC traces (**NP7**, **Figure 3**). Finally, we

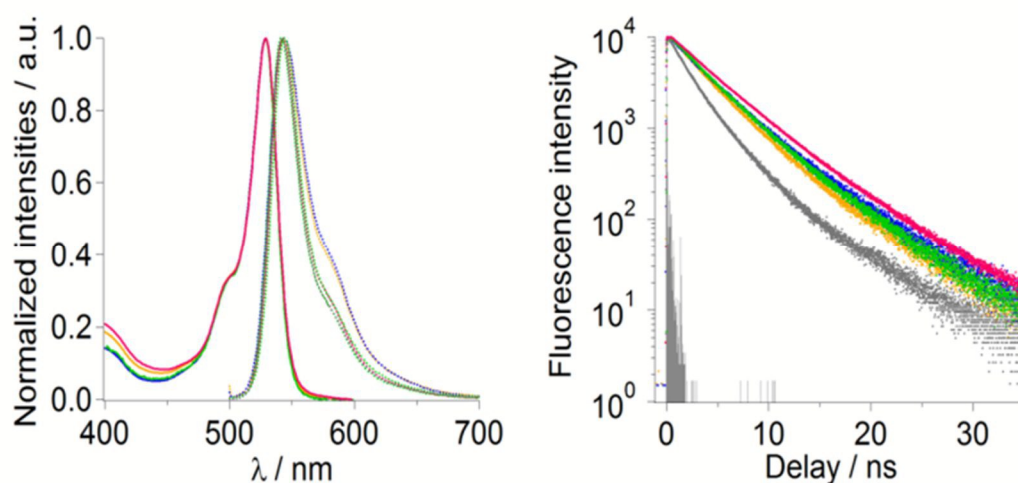
**Table 1** Experimental results for the core-shell (fluorescent) nanoparticle syntheses. Nanoparticles were synthesized at 80°C using a one-pot procedure, with different macro-RAFT agents and stopped after 4h ( $[S]_0 = 1.3 \text{ mol L}^{-1} \text{ H}_2\text{O}$ ,  $[S]_0/[\text{RAFT}]_0 = 200$ ,  $[\text{RAFT}]_0/[\text{AIBN}]_0 = 3$ ,  $[\text{NaOH}]/[\text{AA}] = 1.3$ ,  $\text{pH} \approx 12.5$ ).

NP	macroRAFT agent	$n_{\pi}^a$	$t_0^b$ , min	$\chi_{s,0}^{b,c}$ , %	$DP_n$ (PS <sub>0</sub> ) <sup>b</sup> , %	$\chi_{s,f}^{c,d}$ , %	$\chi_{\pi,f}^{d,e}$ , %	$M_{n,th}$ , kg mol <sup>-1</sup>	$M_{n,SEC}$ , kg mol <sup>-1</sup>	$\mathcal{D} = M_w/M_n$	$D_z$ (σ), nm	Void?
NP1	PPEOA <sub>11</sub>	0	90	20	40	97	-	20.6	21.5	1.29	95 (0.03)	✓
NP2	PPEOA <sub>11</sub>	0	150	33	65	75	-	17.0	15.7	1.37	90 (0.10)	✓
<b>FNP2</b>	PPEOA <sub>11</sub>	1.3	150	26	51	84	98	19.4	18.3	1.43	110 (0.18)	✓
NP3	P(AA <sub>6</sub> -CO-PEOA <sub>6</sub> )	0	70	24	38	97	-	18.9	18.7	1.29	90 (0.07)	✓
<b>FNP3</b>	P(AA <sub>6</sub> -CO-PEOA <sub>6</sub> )	1.8	80	14	27	100	97	18.2	19.6	1.29	85 (0.19)	✓
NP5	P(AA <sub>11</sub> -CO-PEOA <sub>11</sub> )	0	150	45	69	85	-	19.7	17.1	1.35	70 (0.12)	0
<b>FNP5</b>	P(AA <sub>11</sub> -CO-PEOA <sub>11</sub> )	1.9	150	41	66	94	97	22.4	18.5	1.40	85 (0.10)	0
NP6*	P(AA <sub>16</sub> -CO-PEOA <sub>17</sub> )	0	75	18	35	99	-	30.0	27.7	1.56	100 (poly)	0
NP7	P(AA <sub>16</sub> -CO-PEOA <sub>17</sub> )	0	150	46	70	98	-	26.8	24.4	1.36	110 (0.14)	0
NP8	PAA <sub>3</sub>	0	90	20	15	100	-	14.0	16.3	1.36	95 (0.10)	0
NP9**	PAA <sub>35</sub> -b-PS <sub>19</sub>	0	-	-	19	90	-	20.4	27.8	1.84	65 (0.06)	0

\* This experiment was conducted with a ratio  $[S]_0/[\text{RAFT}]_0 = 240$ . \*\* For this experiment the one-pot procedure could not be applied, since PAA<sub>35</sub>-TTC is not soluble in styrene. The amphiphilic macro-RAFT agent was thus synthesized in *solution* and isolated before being used in the RAFT miniemulsion polymerization. <sup>a</sup> Average number of BODIPY monomer per polymer chain. <sup>b</sup> Time 0 refers to the end of the bulk polymerization and the start of the miniemulsion polymerization ( $t_0$ ), conversion of styrene at  $t = 0$  ( $\chi_{s,0}$ ) and degree of polymerization,  $DP_n$ , of PS block at  $T = 0$  (PS<sub>0</sub>). <sup>c</sup> Styrene conversion determined by gravimetry.<sup>26</sup> <sup>d</sup> time f stands for the end of miniemulsion polymerization (final sample). <sup>e</sup> BODIPY monomer ( $\pi$ ) conversion determined by SEC using the UV-vis. detection.

intended synthesizing nanoparticles with a pure linear poly(acrylic acid) shell using the same procedure. A hydrophilic macro-RAFT agent (PAA<sub>35</sub>,  $M_n = 2.9 \text{ kg mol}^{-1}$ ) possessing a total number of monomer units similar to P(AA<sub>16</sub>-CO-PEOA<sub>17</sub>,  $DP_n = 33$ ) was first synthesized to stabilize the particles during the miniemulsion polymerization. Nevertheless, this PAA macro-RAFT agent was not soluble in the styrene phase and the developed two steps bulk polymerization-phase inversion process could thus not be applied. Because of the insolubility of PAA<sub>35</sub> in styrene, we performed the chain extension of polystyrene (19 units) in solution (in 1,4-dioxane at 80°C), isolated the amphiphilic macro-RAFT agent and then used it as a stabilizer and control agent in the miniemulsion polymerization. This macro-RAFT agent possesses a large number of acrylic acid units (35) and should *a priori* provide very efficient electrostatic repulsion at the miniemulsion polymerization pH of 12. Actually, spherical, full nanoparticles (**NP9**) could be obtained, which possessed the smallest average

diameter of the series (65 nm) (**NP9**, Figure SI-2). However, the control of the polymerization was limited, with higher molar mass dispersities and final molar masses slightly higher than the theoretically expected values. In order to overcome the problem of solubility of the PAA<sub>35</sub> macro-RAFT agent, and to avoid the synthesis in dioxane solution and purification step of the amphiphilic macro-RAFT agent, a small oligo-RAFT agent of only 3 units of acrylic acid was also prepared. This macro-RAFT agent was readily soluble in styrene so that the polymerization of styrene could be performed in bulk. At the end of the bulk step, a PAA<sub>3</sub>-b-PS<sub>15</sub> macro-RAFT agent was obtained and used in the miniemulsion polymerization step. Full particles (**NP8**) were again obtained, which were however quite heterogeneous in size (**NP8**, Figure SI-2). Nevertheless the polymerization was well controlled ( $\mathcal{D} = 1.36$ ) and no macro-RAFT agent was detected in the SEC chromatograms (**NP8**, Figure SI-3). *Partial Discussion.* As described above, various core-shell nanoparticles with comb-like hydrophilic shells composed of



**Fig. 4** Spectra of the fluorescent nanoparticles with different hydrophilic shells (**FNP2** (— pink), **FNP3** (— yellow), **FNP5** (— blue), **FNP10** (— gray), **FNP11** (— green)), recorded in water. Left: absorption (full lines) and emission (dotted lines,  $\lambda_{exc} = 495 \text{ nm}$ ) spectra. Right: fluorescence decays (light grey line is the instrument response function,  $\lambda_{exc} = 495 \text{ nm}$ ,  $\lambda_f = 543 \text{ nm}$ ).

PEOA and/or AA were synthesized using a one-pot surfactant-free miniemulsion process. It appeared that the nature of the corona and the length of the polystyrene block *PS*<sub>0</sub> of the amphiphilic macro-RAFT agent (used as both a control agent and a stabilizer in the miniemulsion step) had an influence on the control over the polymerization and on the morphology<sup>48</sup> of the objects. With the long, highly hydrophilic macro-RAFT agent P(AA<sub>16-co</sub>-PEOA<sub>17</sub>) comprising a short polystyrene *PS*<sub>0</sub> segment formed in the bulk polymerization step (**NP6**), particles were heterogeneous in size and the polymerization was not well controlled. In contrast, the experiment conducted with the same P(AA-co-PEOA) macro-RAFT agent, but possessing a longer polystyrene *PS*<sub>0</sub> segment showed a good control over the polymerization (**NP7**). Good control over polymerization was also obtained using the macro-RAFT agents with lower molar masses, PPEOA<sub>11</sub>, P(AA<sub>11-co</sub>-PEOA<sub>11</sub>) and P(AA<sub>6-co</sub>-PEOA<sub>6</sub>). A plausible explanation for the loss of control in experiment **NP6** exhibiting a short *PS*<sub>1</sub> segment relative to P(AA-co-PEOA), might be that in this case the localization of the macro-RAFT agent at the monomer droplets/water interphase is rather dynamic. Thus, this macro-RAFT agent should exhibit a preference for the water phase compared to the interface of the styrene droplets/particles. Consequently, particle stabilization is less efficient, yielding higher particle size dispersity, and a loss of control over the polymerization.<sup>20,49</sup> In conclusion, with respect to the molar mass of the hydrophilic segment, a sufficiently long polystyrene block *PS*<sub>0</sub> must be attached in the bulk polymerization step in order to reach control over polymerization. Similarly, Hawke *et al.* had used amphiphilic PAA<sub>5-b</sub>-PS<sub>γ</sub>-TTC-C<sub>4</sub> macro-RAFT agents with polystyrene blocks of variable lengths in the miniemulsion polymerization of styrene.<sup>30,31</sup> They observed that the polymerization was best controlled with the macro-RAFT agents possessing the longest hydrophobic PS segment ( $\gamma = 24$ ). They explained that only the most hydrophobic PAA<sub>5-b</sub>-PS<sub>γ</sub>-TTC-C<sub>4</sub> remained efficiently anchored at the styrene droplets/water interface, thus preventing droplets from coalescence and Ostwald ripening.

Another parameter which apparently affected the polymerization mechanisms is the presence or absence of PEOA in the macro-RAFT agent: it seems responsible for changes in particle morphology. Particles prepared without PEOA in the macro-RAFT agents (**NP8** and **NP9**) do not possess any voids. In

contrast, particles obtained with homopolymer PPEOA (**NP1**) possess one hole in the center, just as nanocapsules do. In the past, multiple holes have already been observed in PS particles when PEO was present in the macro-RAFT agent used in the emulsion<sup>50</sup> and miniemulsion<sup>31</sup> polymerization of styrene. PPEOA or other PEO-based polymers are soluble in both the water phase and the styrene phase, while deprotonated PAA is only soluble in the basic water phase. As such, PPEOA-*b*-PS might be located both at the droplet interface and inside the monomer-swollen particles. Similarly, nanoparticles formed with the *comb-like* P(AA<sub>x-co</sub>-PEOA<sub>y</sub>)-*b*-PS macro-RAFT agent containing the lowest number of AA possess a hole in their center, while those formed with 11 or 16 AA units do not. In our previous study, it had already been observed that a small fraction of the polystyrene particles synthesized using a PEO<sub>45-b</sub>-PAA<sub>15-b</sub>-PS<sub>25</sub> macro-RAFT agent possessed holes in their center.<sup>26</sup> Again, this might be explained by the differences in solubility: for low weight fractions of acrylic acid, the macro-RAFT agent is soluble in both water and the organic styrene phase, which should favor the formation of double emulsions. At the high pH of the miniemulsion (pH 12), the increase of the AA fraction certainly reduces the solubility of the macro-RAFT agents in the styrene phase, and therefore prevents the formation of double emulsions resulting in the formation of plain full particles.

#### Fluorescent nanoparticles

Nanoparticles **NP2**, **NP3** and **NP5** were homogenous in size and polymerizations were well controlled (experimental molar masses that correspond to the theoretical ones and  $\mathcal{D} < 1.4$ ). Moreover, **NP3** and **NP5** possess multiple carboxyl groups in their P(PEOA-co-AA) shell that might be used for further grafting of small molecules. Their synthesis conditions were therefore selected as starting points for the synthesis of the corresponding fluorescent particles. In order to prepare fluorescent nanoparticles of the same structure (**FNP2**, **FNP3** and **FNP5**), BODIPY methacrylate ( $\pi$ ), the fluorescent monomer of choice (Figure SI-1), was simply added to styrene in the bulk polymerization step, and then the miniemulsion polymerization was performed according to the protocols established for the non-fluorescent nanoparticles.

**Table 2** Physico-chemical properties of FNP with different shells, recorded in water at RT.

Shell	FNP	[ $\pi$ ] <sup>b</sup>			Fluorescence properties				DLS		TEM	
		$n_{\pi}$ <sup>a</sup>	mol L <sup>-1</sup>	N <sub>π</sub> <sup>c</sup>	$\langle \tau \rangle$ <sup>d</sup>	$\Delta_{1/2}$ <sup>e</sup>	$B \times 10^7$ <sup>f</sup>	$D_z(\sigma)$ <sup>g</sup>	D <sub>TEM</sub> <sup>h</sup>	N <sub>Agg</sub> <sup>i</sup>	D <sub>j</sub>	chains nm <sup>-2</sup>
PPEOA <sub>11</sub>	FNP2	1.3	0.08	-	0.58	4.9	970	-	110 (0.18)	-	-	-
P(AA <sub>6-co</sub> -PEOA <sub>6</sub> )	FNP3	1.8	0.13	-	0.44	4.2	1230	-	85 (0.19)	-	-	-
P(AA <sub>11-co</sub> -PEOA <sub>11</sub> )	FNP5	1.9	0.10	1960	0.52	4.5	1300	7.4	85 (0.10)	37	1030	0.24
PEO <sub>45-b</sub> -PAA <sub>15</sub> <sup>*</sup>	FNP10	2.1	0.17	3680	0.24	3.2	980	6.4	80 (0.15)	40	1750	0.35
PEO <sub>45-b</sub> -PAA <sub>15</sub> <sup>*</sup>	FNP11	1.1	0.08	1930	0.39	4.3	980	5.5	75 (0.12)	40	1750	0.35

<sup>a</sup> Average number of BODIPY monomers ( $\pi$ ) per polymer chain. <sup>b</sup> Molar concentration of BODIPY monomer ( $\pi$ ) per liter of styrene. <sup>c</sup> Average number of  $\pi$  per nanoparticles determined using equation SI-3. <sup>d</sup> Average decay time, determined using equation 1 ( $\lambda_{exc} = 495$  nm,  $\lambda_F = 543$  nm). <sup>e</sup> Fluorescence spectra full width at half maximum ( $\Delta_{1/2}$ ). <sup>f</sup> estimated nanoparticle brightness (eq. 2). <sup>g</sup> z-average particle diameter measured by DLS. <sup>h</sup> FNPs' core diameter estimated by TEM. <sup>i</sup> Aggregation number calculated with Equation SI-4 based on TEM diameters. <sup>j</sup> density of the polymer chains on the FNPs' surface (Equation SI-4). \* Already published results<sup>26,52</sup>



**Table 3** Physico-chemical characteristics of pristine or grafted (fluorescent) nanoparticles recorded in water. Zeta potentials ( $\zeta$ ) were recorded in phosphate/citrate buffers ([phosphate/citrate] = 1mM, [NaCl] = 14mM).

Exp	NP	Shell structure	Amine <sup>a</sup>	$\zeta$ / mV					FA/FNP <sup>c</sup>
				pH 4	pH 5	pH 6	pH 7	pH 8	
-	NP5		-	-12		-26	-28	-24	-
NP5- NH <sub>2</sub> -	NP5		Et(NH <sub>2</sub> ) <sub>2</sub>	+10	+2	-15		-16	-
NP5- FA	NP5	comb-like	FA + EtOA	-1		-18		-21	-
-	FNP5		-	-16		-29	-36	-30	-
FNP5-FA	FNP5		FA + EtOA	+5		-12	-16	-21	520
-	NP11		-	n.d. <sup>b</sup>	n.d. <sup>b</sup>	-16	-17	-17	-
NP11- NH <sub>2</sub>	NP11		Et(NH <sub>2</sub> ) <sub>2</sub>	+5	-2	-11	-15	-14	-
NP11- FA	NP11	linear	FA + EtOA	-9		-14		-14	-
-	FNP11		-	n.d. <sup>b</sup>	n.d. <sup>b</sup>	-17	-14	-16	-
FNP11- FA	FNP11		FA + EtOA	-11			-15	-15	1230

<sup>a</sup> Type of amine grafted on the (F)NP shell. <sup>b</sup> n.d. = not determined (The measurement of the zeta potential at that pH was impossible since the nanoparticles precipitated). <sup>c</sup> Average number of fluoresceinamine (FA) grafted per fluorescent nanoparticles determined by absorption using the relative intensity of FA and  $\pi$  ( $\epsilon_{\pi} = 73 \times 10^3 \text{M}^{-1} \text{cm}^{-1[26]}$ ,  $\epsilon_{\text{FA, grafted}} = 88 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$  at pH 8<sup>[46]</sup>).

**FNP2**, **FNP3** and **FNP5** were synthesized with the same molar concentration of  $\pi$  (about 1% relative to styrene) (Table 1). At the end of the miniemulsion polymerization, for all FNPs, the molar conversion of  $\pi$  was higher than 97%. SEC analyses (Figure 5, Figure SI-4) revealed that the addition of  $\pi$  did not perturb the polymerization control: molar mass control and low molar mass dispersities  $\mathcal{D}$  were maintained for all fluorescent NP (**FNP2**, **FNP3** and **FNP5**). Furthermore, the UV-vis. SEC detection ( $\lambda = 528 \text{ nm}$ ) superposed with the RI signal, which means that the whole polymer distribution contained BODIPY. Both types of particles (with or without  $\pi$ ) had the same morphology (Figure 5, Figure SI-5) and were similar in size (only the diameter of **FNP2** increased by 20% compared to the non-fluorescent particles **NP2**). So, including approximately 1 mol% (i.e. 5 wt%) of  $\pi$  with respect to styrene in the miniemulsion process did not disturb the polymerization mechanism, independently of the macro-RAFT agent used. The developed process is thus robust enough for the synthesis of fluorescent nanoparticles with various hydrophilic shell structures. UV-vis. absorption spectra of FNPs shows the same absorption band as a single  $\pi$  monomer in toluene (Figure 4), which is another proof for the incorporation of  $\pi$  in the FNPs and the chemical integrity of the fluorophore after the radical polymerization.

However, the fluorescence emission spectra of the different FNP structures revealed differences. For all FNPs, the full width at half maximum (FWHM) in the absorption spectra is the same whatever the FNP ( $\Delta_{1/2 \text{ abs}} = 937 \text{ cm}^{-1}$ ) while the FWHM in emission ( $\Delta_{1/2}$ ) is larger (Table 2). Usually such effect is attributed to a decreased rigidity in the excited state.<sup>51</sup> FWHM in emission are higher for **FNP3** and **FNP5**, FNP with a comb-like P(AA-co-PEOA) copolymer shell structure, compared to **FNP2**, with a PPEOA homopolymer shell. This might be interpreted in terms of environment (structure of the shell, density of the polystyrene-based core) since the parent monomer dye is the same whatever the FNP. In other words, BODIPY dyes in **FNP3** and **FNP5** should sense little constraint and may have the possibility to evolve in the somewhat loose polystyrene matrix. Thus once in the excited states they might relax to lower energy

states which could be responsible for the observed band broadening.

In our previous studies using linear PEO<sub>45</sub>-*b*-PAA<sub>15</sub> (Scheme SI-3) and approximately 1.1  $\pi$  per polymer chain (Table 2, **FNP11**)<sup>26,52</sup>, nanoparticles with a fluorescence quantum yield of 0.39 could be obtained in water (while the quantum yield of the  $\pi$  monomer is 0.69 in toluene). It has also been observed that an increase in the  $\pi$  concentration from one fluorophore (**FNP11**) to two fluorophores  $\pi$  (**FNP10**) per polymer chain significantly decreased the fluorescence quantum yield  $\Phi_F$  from 0.39 to 0.24 and the lifetime from 4.3 ns to 3.2 ns. Moreover, we had demonstrated the greater reactivity of  $\pi$  compared to styrene, leading to a gradient in composition in the polymer chain with a higher density of the fluorophore  $\pi$  close to the hydrophilic shell and a lower density in the middle of the hydrophobic core.<sup>45</sup> The observed decrease of  $\Phi_F$  was thus attributed to the formation of non-fluorescent aggregates between polymer chains, preferentially present at the periphery of the particle core.<sup>52</sup> In the present study using a comb-like stabilizing corona, **FNP2**, **FNP3** and **FNP5** contain between 1.3 and 1.9  $\pi$  fluorophores per polymer chain. Compared to our previous study, they exhibit higher  $\Phi_F$  of 0.58, 0.44 and 0.52, respectively, and longer fluorescent lifetimes of 4.9, 4.2 and 4.5 ns (Table 2, Figure 4). This interesting result can only be the result of the change in macromolecular architecture of the stabilizing shell, as both types of fluorescent particles are composed of the same polymers (PEO, PAA, PS) and contain  $\pi$  as a fluorophore. It is well-known that the presence of carboxyl groups close to BODIPY fluorophores can decrease their fluorescence efficiency.<sup>53</sup> In the former linear PEO<sub>45</sub>-*b*-PAA<sub>15</sub> block copolymer particles (**FNP10** and **FNP11**), carboxyl groups adjoined the hydrophobic PS core of the nanoparticles and thus the fluorescent monomer units. In contrast, in **FNP2**, **FNP3** and **FNP5** the hydrophilic shell is made of *comb-like* copolymers of acrylic acid and PEOA, and consequently the  $\pi$  monomers (in the core) should be spatially separated (by the PEO copolymer brush) from most of the carboxylic acids groups. Moreover, the packing of the polymer chains in the particles might be considered: for the FNPs without any voids the aggregation

number ( $N_{agg}$ , Equation SI-1) and the density of the polymer chains on the FNPs' surface ( $d$ , Equation SI-4) can be estimated. The results are displayed in Table 2. Interestingly, for the FNPs with a P(AA<sub>11</sub>-co-PEOA<sub>11</sub>) shell (**FNP5**),  $N_{agg}$  and  $d$  were respectively 40% and 30% times lower than for the FNPs with a shell of PEO<sub>45</sub>-*b*-PAA<sub>15</sub> (**FNP10**). This result might be explained considering the bulkiness of the PEOA macromonomer, which makes the hydrophilic shell a cumbersome brush type polymer compared to the tight linear PEO-*b*-PAA copolymer. Consequently, the polymer chains in **FNP10** should be more tightly packed than in **FNP5**, which might favor the formation of  $\pi$  inter-chains aggregates at the periphery of the hydrophobic core. This fits the fact that the fluorescence emission spectra ( $\Delta_{1/2}$ ) of "linear shell" FNP are narrower than the ones of "comb-like shell" FNPs, showing that the environment of the  $\pi$  monomer in the first type of FNP is denser. In conclusion, we thus believe that it is for these two reasons (steric hindrance of the shell due to a difference in the macromolecular architecture and the presence of the carboxylic acids near the surface) that the fluorescence efficiency changes with the chemical nature and especially the macromolecular architecture of the particle shell.

Thanks to the aggregation number, the number of fluorophores  $\pi$  per particle ( $N_{\pi}$ , Equation SI-3) could be estimated. For **FNP5** and **FNP10** with a PS core of about 40 nm in diameter<sup>‡</sup>, the number of  $\pi$  is respectively 1960 and 3680. Ultimately, the important parameter for fluorescent bio-imaging is the total brightness of the FNP which is given by:

$$B = N_{\pi} \times \epsilon_{\pi} \times \Phi_F \quad \text{Equation 2}$$

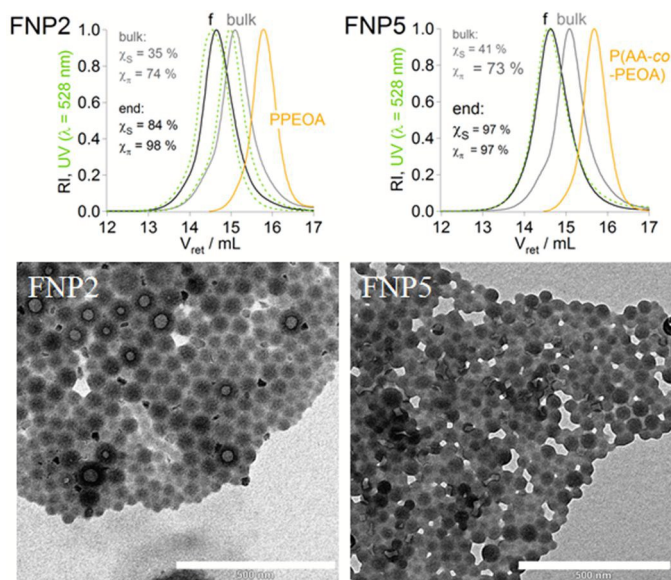
where  $N_{\pi}$  is the number of  $\pi$  per particle,  $\epsilon_{\pi}$  the molar coefficient extinction of  $\pi$  at 528 nm, and  $\Phi_F$  the quantum yield of the FNPs.

For a single  $\pi$  in toluene, the brightness is equal to  $5.0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ . In our previous studies, we found that the brightness of **FNP10** (Table 2) was 1300 times higher than a single BODIPY monomer  $\pi$ . Here, the brightness of the new **FNP5** is 1500 times higher than one  $\pi$ . Thus, even if **FNP5** contains around twice less  $\pi$  per nanoparticle, they are brighter than **FNP11** (which is due to the less dense morphology of the FNP leading to a higher  $\Phi_F$ ). Generally, quantum dots with a core made of CdSe, CdS, or CdTe emitting between 370 and 750 nm have a brightness between  $6 \times 10^4$  and  $6 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .<sup>3</sup> Based on the former calculations ( $B = 7.4 \times 10^7 \text{ M}^{-1} \text{ cm}^{-1}$ ), the synthesized FNPs are approximately 100-1000 times brighter than usual quantum dots. As such, those FNPs are among the brightest polymer-based fluorescent nanoparticles synthesized at the day according to Klymchenko *et al.* study<sup>10</sup>, which makes them very powerful candidates for imaging.

### Functionalization of the FNPs

Functionalized fluorescent nanoparticles<sup>13-15</sup> are of particular interest for biomedical applications. Generally, nanoparticles are functionalized thanks to reactive groups, such as thiols, amines, and acids, present on the NP surface. The fluorescent nanoparticles and their non-fluorescent models ((**F**)NP2, (**F**)NP3, (**F**)NP5 and (**F**)NP11) all possess carboxylic acids units in their shell, but they are differently distributed along the polymer chain, *i.e.* in the shell. (**F**)NP2 exhibits a single carboxylic acid at its outmost  $\alpha$ -chain extremity originating from the RAFT agent's (TTCA) R leaving group (Scheme SI-1). In contrast, (**F**)NP3 and (**F**)NP5 possess carboxylic acids randomly distributed in the bulky P(AA-co-APEO) shell. Finally, (**F**)NP11 possess a block of poly(acrylic acid) located between the PEO segment and the hydrophobic core of the nanoparticles.

In order to evaluate the impact of the shell architecture on grafting efficiencies, ethylenediamine (Et(NH<sub>2</sub>)<sub>2</sub>) was first grafted as a model molecule (via the formation of activated esters of AA with EDC) (Table 3).<sup>54,55</sup> NP5 and NP11 were selected for those preliminary grafting experiments since they possess a similar number of carboxyl groups, but a *comb-like* and *linear* architecture, respectively. In order to rapidly evaluate if the grafting took place or not, the zeta potentials ( $\xi$ , Table 3) of the nanoparticles were measured at different pH (pH = 4, 5, 6, 7 and 8). Whatever the pH, for the pristine NP5 and NP11 dispersions, negative zeta potentials were measured. Actually, above pH = 5, carboxylic acids are at least partially deprotonated, and PEO-coated nanoparticles give generally negative zeta potentials<sup>55</sup>. Interestingly, NP11 nanoparticle dispersions prepared with the *linear* PEO-*b*-PAA diblock copolymer precipitated below pH = 5<sup>26</sup>, whereas the corresponding *comb-like* NP5 did not. Again, the shells' macromolecular bulky architecture, where AAs are randomly distributed between bulky PEO grafts, should be responsible of the observed differences. Indeed, it is well known that PAA (in its protonated form, *i.e.* generally at pH below the pK<sub>a</sub> around 5) can form hydrogen-bonded complexes with PEO segments.<sup>57</sup>



**Fig. 5** Top: Size exclusion chromatograms in THF (plain lines: RI detection, broken — green lines: UV-vis. detection at  $\lambda = 528$  nm) for polymer chains of **FNP2** and **FNP5** at the beginning of the polymerization (corresponding to the isolated macro-RAFT agent) (— yellow), end of mass polymerization PS0 (— grey) and end of miniemulsion polymerization (— black). Bottom: Transmission electron microscopy images of polystyrene copolymerized with  $\pi$  nanoparticles with PPEOA (**FNP2**) or P(AA<sub>x</sub>-co-PEOA<sub>y</sub>) (**FNP5**) comb-like shells. scale bar: 500 nm.

Thus, at pH = 5, the PAA intermediate block of **NP11** can form hydrogen bonds with the PEO block and lead to particle aggregation. In contrast, in **NP5** the carboxyl groups are randomly distributed in the polymer chains of the shells, and the evenly distributed PEO grafts may then prevent particle destabilization by steric stabilization. Interestingly, after grafting with ethylenediamine, Et(NH<sub>2</sub>)<sub>2</sub>, *linear NP11* (**NP11-NH<sub>2</sub>**) particle dispersions remained colloidally stable at pH below pH 5, which is a first proof of grafting. Moreover, their zeta potential became positive at pH 4, an indirect proof of the introduction of amino groups in the shell. *Comb-like NP5-NH<sub>2</sub>* nanoparticles had a less negative zeta potential compared to **NP5**. As for **NP11** the zeta potential became positive for low pH (pH = 4 and 5), evidencing the grafting of the diamine. The variation of the zeta potential before and after grafting and also at different pH is more pronounced for *comb-like NP5* than for *linear NP11*, which might be explained by the more efficient screening of the PAA part by the dense linear PEO outer shell in **NP11** (Scheme SI-3).

Following these encouraging preliminary results with ethylenediamine, fluoresceinamine (FA) was grafted on **FNP2**, **FNP5** and **FNP11** under the same conditions. The number of FA per nanoparticle could be easily calculated after grafting by comparing the absorption band of the BODIPY (maximum at 528 nm) with the one of the FA (maximum 494 at nm) in basic conditions ( $\epsilon_{\pi} = 73 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ <sup>[26]</sup>,  $\epsilon_{\text{FA, grafted}} = 88 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  at pH 8<sup>[46]</sup>), knowing the number of BODIPY fluorophores per particle,  $N_{\pi}$ . It was determined that *comb-like FNP5-FA* possessed in average 520 FA per nanoparticles, while *linear FNP11-FA* were functionalized by 1230 FA in average (Table 3). Those results indicate that for both **FNP5** and **FNP11**, 5 % of the carboxyl groups were functionalized. Similar grafting efficiencies were also measured for **NP5** and **NP11** when the grafting was performed with ethanolamine, EtOA (respectively  $56 \pm 3\%$  and  $62 \pm 4\%$  of the acid functionalized, determined by elementary analysis, using O and N on 15 mg of dried NPs).<sup>58</sup> The zeta potential of *comb-like FNP5-FA* is strongly dependent on pH (positive at pH = 4), while the zeta potential of *linear FA-FNP11* is negative over the whole range of pH, probably because the fluorescein and the remaining carboxyl groups are screened by the outer linear PEO segment.<sup>56</sup> These results demonstrate that the two types of nanoparticles have the same reactivity towards amines regardless of the shell architecture. The overall poor grafting efficiency with fluoresceinamine may be due to its steric hindrance and the electrostatic repulsion between the charged acid groups and FA (both negatively charged at the pH of the reaction).

With the same strategy, it was not possible to graft FA on **FNP2** particles, which possess only one carboxylic acid located at the chain end of the corona polymers. It may be due to the hindered accessibility of this  $\alpha$ -end carboxylic acid screened by the PEO brush.

As a conclusion, fluorescent PEO-*b*-PAA or P(AA-*co*-PEOA) core-shell nanoparticles can easily be functionalized, using the same grafting efficiency, with different organic amines such as fluoresceinamine.

## Conclusions

Several core-shell nanoparticles with a hydrophobic core based on polystyrene and hydrophilic shells made of acrylic acid and/or PEOA have been synthesized *via* a RAFT miniemulsion process in water using neither free surfactants nor hydrophobic additives. It was shown that the chemical composition and molar mass of the hydrophilic shell had a major impact on the control of the polymerization, as well as on the size and morphology of the nanoparticles. Using a PPEOA homopolymer macro-RAFT agent in the polymerization process, particles with a void in their center, *i.e.* nanocapsules, were obtained, while pure PAA led to full nanoparticles. With P(AA-*co*-PEOA) random copolymers, either full particles or nanocapsules were formed. After having established robust polymerization conditions for three types of core-shell nanoparticles with shells made of PPEOA<sub>11</sub>, P(AA<sub>6</sub>-*co*-PEOA<sub>6</sub>) or P(AA<sub>11</sub>-*co*-PEOA<sub>11</sub>), the corresponding fluorescent nanoparticles with the same particle morphology could be synthesized. It appeared that the incorporation of 1 mol % of fluorophore (about 5 wt%) did not change the size of the particles and did not disturb the control of the polymerization. The obtained fluorescent nanoparticles of a mean diameter of  $\approx 80$  nm were approximately 1500 times brighter than a single fluorophore, which makes them 100 to 1000 times brighter than CdSe/CdS Quantum dots (based on the estimated brightness) and ranks them among the brightest polymer-based fluorescent nanoparticles. In addition, it appeared that these nanoparticles, with a bulky *comb-like* shell of P(AA-*co*-PEOA), were more fluorescent than particles stabilized by *linear* PEO-*b*-PAA polymers analyzed in a previous study, while using less fluorescent monomer during the synthesis. This was mainly attributed to the fact that FNPs with *comb-like* shells exhibit much less carboxyl groups close to the BODIPY monomers at the periphery of the particles, and that, in this region, the (hydrophobic) chains segments are less densely packed (thanks to the bulkiness of the stabilizing shell) limiting thus BODIPY (interchain) aggregation. We believe that this result is really promising in terms of chemical economy and time saving. Moreover, those nanoparticles that have a void in their middle (nanocapsules) could be of interest for the encapsulation of hydrophilic drugs or active molecules. At last, the possibility to graft pH-sensitive amines, such as fluoresceinamine, on the nanoparticles was demonstrated. Both *comb-like* P(AA-*co*-PEOA) and *linear* PEO-*b*-PAA copolymers could be successfully grafted by fluorescein. For P(AA-*co*-PEOA) core-shell nanoparticles, it was possible to covalently link 520 FA and 1960 BODIPY on the same nanoparticle. Similar nanoparticles exhibiting only FA in their shell and no  $\pi$  in the core have already successfully been used for early monitoring the growth of *Escherichia Coli* bacteria in the presence or absence of antibiotics.<sup>59</sup> In this context, we believe that the combination of two fluorophores in the same nanoparticle will be a powerful mean for designing ratiometric sensors for cell detection and quantification.

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## Notes and references

‡ The diameter of the core of the FNPs was estimated by TEM (DTEM, Table 2). Indeed, in contrast to DLS, this technique analyses dried collapsed samples. In addition, electron dense structures, such as polystyrene, can easily be observed, whereas less dense structures, such as the PEO/PAA-based shell, are generally hard to be observed without using contrast agents. Therefore the TEM diameters were associated to the diameter of the particles core.

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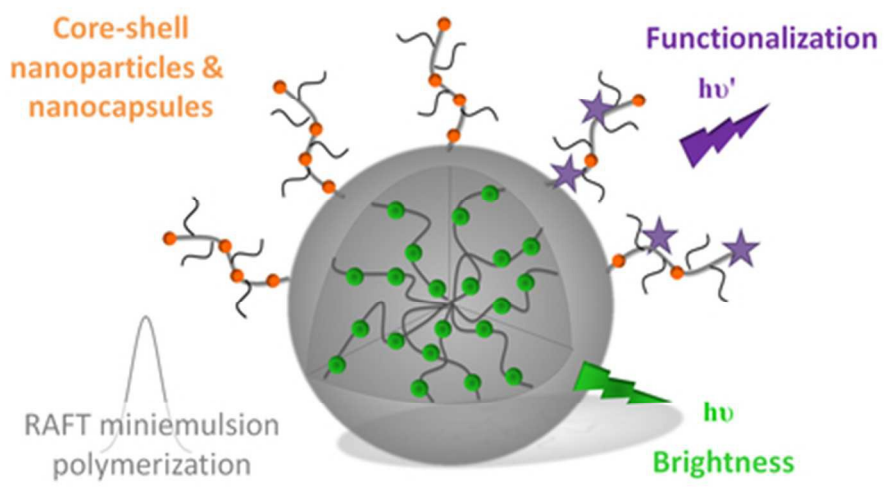


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Ultra-bright fluorescent nanoparticles with comb-like shells based on PEG and acrylic acid were obtained in water using an additive-free miniemulsion polymerization process



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