Emulsion-Templated Poly(N-Isopropylacrylamide) Shells Formed by Thermo-Enhanced Interfacial Complexation

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The encapsulation of fragile biomacromolecules is crucial in many biotechnological applications but remains challenging. Interfacial complexation (IC) in water-in-oil emulsions proves to be an efficient process for the formation of protective polymer layers at the surface of capsule-precursor water droplets. In this work, the enhancement of conventional IC by introducing thermoresponsive poly(N-isopropylacrylamide) (PNIPAM) strands in the interfacial polymer layer is described. Surfactant-polymer IC is implemented in water-in-fluorocarbon oil emulsions between a water-soluble poly(L-lysine)-g-poly(N-isopropylacrylamide) cationic copolymer (PLL-g-PNIPAM) and an oil-soluble anionic surfactant. Fluorescence imaging demonstrates that the thermal collapse transition of PNIPAM strands, triggered by gentle heating, induces an enrichment of the polymer layer initially formed by IC. Spontaneous co-precipitation of nanoparticles initially dispersed in the aqueous cores—with no specific treatment—is also achieved upon PNIPAM transition. This process is leveraged to irreversibly segregate these nanoparticles in the interfacial polymer layer, resulting in gel-like mixed shells. Thermo-enhancement of conventional IC is thus a promising approach for the straightforward formation, strengthening, and functionalization of capsule shells. As implemented in mild conditions, thermo-enhanced IC is additionally compatible with the encapsulation of proteins, opening new opportunities for delivery systems of biomacromolecules.

1. Introduction

The compartmentalization of molecules or nanoparticles is commonly required for various biotechnological applications including drug encapsulation and delivery,[1–3] cell mimicry,[4–6] advanced sensing,[7–9] or molecular screening and analysis.[10,11] The encapsulation of biomacromolecules such as RNA or proteins is particularly demanding because of their fragility and high sensitivity to their local environment.[12] Achieving efficient encapsulation without altering the integrity and activity of proteins requires specifically to preserve a mild aqueous environment in artificial compartments.[13] Capsular structures have been developed to confine biomolecules within protective shells, avoiding contacts with external apolar solvents or low or high pHs that may be denaturing. In such capsular systems, water-insoluble polymers are typically used to form the external shell wall, allowing not only to control the shell structure (thickness, rigidity, permeability, etc.)[14–16] but also to impart functionalities to the capsule (stimuli-responsiveness, chemical activity, specific recognition, etc.).[17,18] In addition to polymer chains, the capsule shells may also contain organic or inorganic particles that may increase mechanical properties and/or introduce new functionalities.[19,20]

The formation of polymer capsules usually requires a template to guide the assembly of macromolecular chains into shells with well-defined sizes and morphologies. The various procedures commonly used for template-guided shell formation—interfacial polymerization,[21,22] internal phase separation,[23,24] or layer-by-layer assembly[25,26]—may however be stressful and of limited interest for the encapsulation of biomacromolecules. For instance, interfacial polymerization or internal phase separation are usually implemented with oil-based cores and with solvents of polymers that may denature proteins.[27] Layer-by-layer assembly most of the time requires sacrificial solid templates that must be removed in conditions that are inappropriate to fragile biomacromolecules.[28]

In contrast, interfacial complexation has proved to be an easy and versatile approach for the formulation of polymer envelopes delimiting aqueous cores in mild conditions. In this process, two oppositely charged polymers are solubilized in each phase of an emulsion, and spontaneously form a complex at the liquid-liquid interface via attractive electrostatic interactions. Interfacial complexation of polyelectrolytes has been reported in water-in-oil,[29–31] water-in-water,[32–34] or water-in-water-in-water[35,36] emulsions. All-aqueous emulsions have the advantage to readily produce water-compatible capsules useful as cargos for biological applications. Proteins or cells have been successfully entrapped in all-aqueous assemblies but the partitioning of water-soluble species between inner and outer phases may limit the encapsulation efficiency.[37] Improving the yield of encapsulation of fragile proteins in mild conditions remains

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challenging, especially for biological applications that require high molecular concentrations and high activity preservation.

The objective of the work presented here was to form microcapsules that present both (i) a mild and biocompatible aqueous core and (ii) a thick and functionalized polymer shell wall. This approach required a good control on the composition of the core-shell system, without having recourse to sophisticated steps of chemical modifications, cross-linking or capsule post-processing that would increase the probability of loading degradation.

In this context, the use of fluorcarbon oils (FCO) as continuous phase of water-in-oil emulsions turned out to be an interesting alternative to hydrocarbon oils since (i) FCO are biocompatible,[38,39] and (ii) solution components (salts, hydrocarbon species, biomolecules) show an extremely poor solubility in FCO,[40] offering an improved control on the composition of the aqueous droplets. To our knowledge, very few works have implemented surfactant-polymer interfacial complexation (SPIC) in water-in-emulsion for the design of capsular systems. DeJournette et al. have evidenced the formation of a complex layer by SPIC between an FCO-soluble macrorosurfactant (a carboxylate-terminated perfluoro-PEG called Krytox) and a water-soluble diamine (Jeffamine) at the edge of water droplets.[41] More recently, Abell and co-workers showed that SPIC can be leveraged to locally increase the concentration of a polycation at the water/FCO interface and implemented supramolecular cross-linking of adjacent chains by cucurbituril units to form thin polymer capsules.[42,43]

Based on SPIC in water-in-FCO (W/FCO) emulsions, we propose here a generic approach to optimize the deposition of polymer chains at the surface of water droplets, enabling (i) the formation of a 1 μm-thick polymer shell, (ii) the straightforward incorporation of nanoparticles in the shell without any chemical modification of the particles, and (iii) the encapsulation of proteins in the droplet cores. The originality of our strategy relies on the introduction of thermoresponsive properties in the polymer used as the water-soluble complexing agent of SPIC (with Krytox as oil-soluble partner). The polymer in question is a comb-like derivative of poly(L-lysine) (PLL) grafted with temperature-responsive strands of poly(N-isopropylacrylamide) (PNIPAM), yielding the PLL-g-PNIPAM copolymer. The SPIC process induced by the interaction between side amino groups of PLL and Krytox molecules leads to the formation at room temperature of a germinal polymer layer across the W/FCO interface, i.e., at the edge of the aqueous droplets.

The PNIPAM is a thermoresponsive polymer exhibiting a hydrophilic-to-hydrophobic transition – switching from swollen coils to collapsed globules – at a Lower Critical Solution Temperature of ≈32 °C in water.[44] Accordingly, the PNIPAM strands grafted onto the PLL backbone in PLL-g-PNIPAM introduced temperature-sensitivity in the polymer layer formed at the water/FCO interface. We show that the thermal collapse transition of PNIPAM significantly increases the amount of polymer chains trapped in the interfacial layer initially self-organized by SPIC. Interestingly we also show that this polymer enrichment persists after cooling back to room temperature. This process called “thermo-enhanced interfacial complexation” being achieved upon gentle heating and in mild pH conditions, it turns out to be compatible with the encapsulation of a model protein.

We also demonstrate that the thermal transition of the PNIPAM strands can be leveraged to irreversibly capture, in the interfacial polymer layer, nanoparticles that were initially dispersed in the droplet cores. This procedure is likely general as long as PLL can spontaneously bind onto the particles of interest, what has been reported for a variety of (anionic) surfaces including silica, ceramics, plastics such as polystyrene, protein particles, etc.[45–48] As compared to the literature on hybrid or nanoparticle-functionalized capsules,[49,50] our approach for the formation of polymer-particles mixed shells only relies on gentle heating of the original batch of droplets, what does not require any specific steps of nanoparticle functionalization or capsule post-processing.

2. Results and Discussion

2.1. Interfacial Complexation of PLL Comb-Like Derivatives in Water-in-Fluorocarbon Oil Emulsions

Poly(L-lysine)-g-poly(N-isopropylacrylamide) copolymer (PLL-g-PNIPAM) was synthesized by grafting reactive PNIPAM strands (NH ester-terminated, Mw = 2 kDa) onto the amine side groups of the PLL backbone (Mw = 15–30 kDa) with a grafting molar ratio of ≈0.20 (see Experimental Section).[48] PLL-g-PNIPAM was labeled with a fluorophore (coumarin or rhodamine, see Experimental Section) to evaluate by fluorescence microscopy the efficiency of surfactant-polymer interfacial complexation (SPIC) between PLL-g-PNIPAM (polymer) and Krytox (surfactant) in water-in-fluorocarbon oil (W/FCO) emulsion. Emulsions containing free PNIPAM chains (labeled with Cy3) were used as a control as PNIPAM was not expected to accumulate at the interface if not associated to cationic PLL (see Figure 1A).

W/FCO emulsions were prepared as follows: the continuous oil phase was made of FC-70 fluorocarbon oil (FCO) with varying concentrations of Krytox. An aqueous solution of PLL-g-PNIPAM (in the wt% range in a 5:95 volume ratio, yielding nano- to micrometer-size water droplets dispersed in the continuous oil phase. This W/FCO emulsion was injected in a glass capillary for microscopy imaging by confocal laser scanning microscopy (CLSM).

At 3 wt% of rhodamine-labeled PLL-g-PNIPAM and 0.05 wt% Krytox, the fluorescence of rhodamine was essentially localized at the edge of the water droplets, i.e., at the water/FCO interface, as shown in Figure 1B. In contrast, the fluorescence of Cy3-labeled PNIPAM free chains (in the absence of PLL) was homogenously distributed in the core of the water droplets (see Figure 1B). The difference of distribution between free PNIPAM and PLL-g-PNIPAM is highlighted in the radial fluorescence profiles (see Figure 1C), with a sharp peak localized at the droplet edge for PLL-g-PNIPAM and a uniform level of fluorescence within the droplet core for free PNIPAM. The higher density of PLL-g-PNIPAM at the interfaces (as compared to both the droplet cores and the continuous oil phase) combined with a high emulsion stability (absence of droplet coalescence for 5 h, see Figure S1, Supporting Information) are indicative of the formation of an interfacial polymer layer by SPIC when PNIPAM is grafted onto the cationic PLL backbone.
For each batch of PLL derivatives, the Krytox/PLL-g-PNIPAM concentration ratio was adjusted to achieve an optimal deposition of PLL-g-PNIPAM at the interface by SPIC, evidenced by (i) the stability of the emulsion and (ii) the formation of a fluorescent layer of PLL-g-PNIPAM at the droplet edge. This optimization is illustrated in Figure 2 in the case of a coumarin-labeled PLL-g-PNIPAM. At low Krytox concentration (typically ≤ 0.01 wt%) the coalescence of droplets onto the capillary side revealed that the surfactant concentration was insufficient to stabilize the water droplets. At high Krytox concentration (typically ≥ 0.05 wt%), the fluorescence of coumarin-labeled PLL-g-PNIPAM was essentially observed in the continuous phase, indicating a massive extraction of polymer chains in the FCO. As the FCO does not solubilize the hydrophilic hydrocarbon-based PLL derivatives, this observation suggests that excess Krytox can disperse hydrophilic compounds in FCO, likely under the form of inverse micelles. At intermediate Krytox concentration (typically 0.03 wt%), fluorescence micrographs showed a bright corona of PLL-g-PNIPAM localized at the droplet edges and negligible fluorescence in the oil. This observation is consistent with a SPIC process where the PLL-g-PNIPAM copolymer is attracted towards the interface upon the formation of coulombic complexes with Krytox as schematized in Figure 1. At high amount of polymer in the aqueous phase (typically ≥ 5 wt%), the interface was saturated in PLL-g-PNIPAM, and excess polymer chains remained soluble within the droplet core, leading to a low fluorescence contrast between the edge and the core of the droplets. Because of the excessive amount of internal polymer content, this condition was not suitable for the formation of hollow polymer capsules.

Scanning electron microscopy (SEM) imaging was performed to visualize the interface at a higher resolution and to determine the influence of the formulation on the capsules. W/FCO emulsions were prepared as previously described, then deposited on a glass coverslip, dried for three days at ambient air, and metalized by sputtering of a 30 nm-thin layer of Ag prior to SEM.
imaging (see Experimental Section). SEM images of microcapsules prepared from an emulsion containing 1 wt% of PLL-g-PNIPAM in the aqueous phase are shown in Figure 3A. Hollow microcapsules were observed, suggesting that drying-resistant polymer layers were formed at the periphery of the droplets. Their flattened aspect—presumably coming from membrane collapsing during the drying step—allows for an estimate of shell wall thicknesses, which is typically of 0.6–0.8 µm (the apparent thickness of a collapsed membrane being assumed to correspond to two stacked membrane layers, see Figure 3B).

The influence of the polymer (PLL-g-PNIPAM) and Krytox concentrations on the SEM images is detailed in Figure S2 (Supporting Information). Briefly, the SEM observations showed (i) no or unstable capsules at the lowest Krytox concentrations, (ii) the formation of hollow polymer capsules at intermediate concentrations of Krytox, and (iii) the presence of small aggregates or filaments in addition to capsules at high Krytox concentration. This evolution of morphologies is consistent with observation by fluorescence (see Figure 2) of (i) unstable emulsions at low Krytox concentrations, (ii) well-defined droplets with a sharp polymer corona at intermediate compositions, and (iii) partition of the polymer in the oil at the highest Krytox concentrations.

2.2. Influence of the Ionic Strength and the pH on SPIC – Quantification of the Polymer Surface Excess

As interfacial complexation involves coulombic interactions between carboxylate-terminated Krytox and ammonium groups of PLL, the pH as well as the ionic strength of the aqueous phase should have an important impact on the SPIC equilibrium and may enable to optimize the properties of the interfacial assemblies. Beyond qualitative comparisons, a quantitative approach was required to conclude on the SPIC efficiency for the different compositions of the aqueous phase.

To that aim, a robust and reliable quantification of the amount of polymer chains involved in the interfacial layer was implemented. We measured the concentration of polymer chains that remained soluble in the droplet cores (i.e., the residual core concentration of the chains that are not involved in SPIC) and deduced, by difference to the initial composition, the quantity of polymer adsorbed at the interface. Raw values of fluorescence were however biased by optical effects of droplet curvature since the water/FCO interface can deviate the light beam of the microscope. To account for optical biases on fluorescence within a droplet, the core fluorescence of dye-labeled PLL-g-PNIPAM was normalized following a suitable calibration process (described in detail in Figure S2, Supporting Information). Briefly, this calibration includes a correction factor determined by measuring the fluorescence intensity of a soluble Cy3-labeled PNIPAM that was uniformly distributed within droplets (as previously shown in Figure 1) as a function of the droplet diameter from 10 to 50 µm (see Figure S2, Supporting Information). Once the residual PLL-g-PNIPAM concentration in core was obtained, the fraction of PLL-g-PNIPAM recruited in the interfacial layer was calculated as being equal to the loss of the soluble polymer amount. Finally, this fraction of interfacial PLL-g-PNIPAM
was converted in surface excess through normalization by the droplet interfacial area.

To assess the effect of the ionic strength, we compared droplets containing either a $7.7 \times 10^{-3}$ m phosphate buffer (ionic strength $= 17 \times 10^{-3}$ m, pH $= 7.3$), or this buffer supplemented with 100 $\times 10^{-3}$ m or 500 $\times 10^{-3}$ m NaCl. To assess the effect of the pH, we used a 30 $\times 10^{-3}$ m Britton-Robinson (BR) buffer – made of 10 $\times 10^{-3}$ m NaH$_2$PO$_4$/10 $\times 10^{-3}$ m NaOAc/10 $\times 10^{-3}$ m CHES – allowing for a buffering effect on a large range of pH (see Experimental Section). Aqueous phases were prepared in BR buffer at either acidic or alkaline pH, namely at pH $= 4.3$ or pH $= 9.4$.

Irrespective of the ionic strength condition, a bright corona was visible at the edge of the droplets with a low residual fluorescence in the core (see Figure S4A, Supporting Information) and a non-zero surface excess was measured, suggesting that the SPIC was effective even at high NaCl concentration. However, uneven distributions of fluorescence around the droplet edges were noticed in NaCl-containing emulsions, and particularly at 500 $\times 10^{-3}$ m NaCl where “crescent moon-like” shapes of fluorescence became obvious specifically in the smallest droplets ($< 20$ µm). This observation suggests that the presence of salts at high concentration in the aqueous phase led to a patchy segregation of the polymer chains that significantly differs from the uniform coverage of the interface at low ionic strength. The corresponding polymer surface excesses are shown as a function of the residual core concentration and droplet diameter in Figure S4B,C (Supporting Information). The presence of NaCl slightly diminished the surface excess, and markedly broadened the distribution of residual core concentrations. Some droplets containing 100 $\times 10^{-3}$ m or 500 $\times 10^{-3}$ m NaCl had up to 80% of residual polymer chains in their core, suggesting a weakening of the interfacial binding with increasing salt. To prepare capsules, a robust solid-like membrane would be thus preferably obtained at low ionic strength as this offers more homogeneous interfaces and ideally a solid-like interfacial complex phase (comments on the uneven distribution of the polymer at the interface and on a possible fluidification of the complex phase at high ionic strength are given below Figure S4, Supporting Information).

As for the pH, the SPIC process was more efficient in acidic conditions than in alkaline conditions since significantly higher surface excesses and lower residual core concentrations were observed at pH $= 4.3$ compared to pH $= 9.4$ (see Figure S5, Supporting Information). This may be attributed to the fact that the degree of ionization of PLL is decreasing as the pH is getting closer to the pKa of the PLL side amino groups ($\approx 10.5$). In other words, the fraction of protonated amino groups – likely to be involved in coulombic interactions with Krytox – decreases with the pH, and consequently the amount of PLL-g-PNIPAM that can be captured at the interface by SPIC decreases as well.

2.3. Thermoresponsive Behavior of the Interfacial PLL-g-PNIPAM Layer

The thermoresponsive behavior of PLL-g-PNIPAM was characterized in solution by turbidimetry and compared to free PNIPAM (Figure 4A). The transmittance profiles of either PLL-g-PNIPAM or free PNIPAM solutions similarly decreased between 33 °C and 40 °C, indicating that PNIPAM strands – grafted or not – undergo a coil-to-globule transition in the same temperature window. A linear extrapolation of the curve around the inflexion point indicates the same transition temperature of $T_c$ for both PNIPAM forms (coinciding with the beginning of the decrease in transmittance). In the following, this temperature of collapse transition of PNIPAM strands will be referred to as $T_c$. 

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**Figure 3.** A) SEM micrographs of dried W/FCO emulsions with 1 wt% of PLL-g-PNIPAM or PLL-g-PEG in the aqueous phase and 0.05 wt% of Krytox in the FCO phase (scale bars = 20 µm). B) Thickness profiles of the shell folds indicated by colored arrows in the micrographs and giving an estimation of twice the apparent thickness of the capsule shells (as the folds are presumably formed by the collapse of two shell layers).
To assess the thermal transition of PLL-g-PNIPAM in conditions of SPIC with Krytox (in W/FCO droplets), we used 8-anilinonaphthalene-1-sulfonic acid (ANS) as a fluorescence probe exhibiting enhanced fluorescence signal when confined in hydrophobic regions.\[52,53\] ANS fluorescence was used as a marker of (i) the localization of PLL-g-PNIPAM since polymer-enriched regions are more hydrophobic than the aqueous background of water droplets, and (ii) the thermoresponsive properties of PNIPAM since its collapse transition to a more hydrophobic state would be evidenced by an increase in ANS fluorescence intensity.\[54\] Figure 4B shows confocal micrographs of a W/FCO emulsion with 2 wt% of PLL-g-PNIPAM and 500 × 10⁻⁶ M of ANS in the aqueous phase and 0.05 wt% of Krytox in the FCO phase (scale bars = 50 µm). Bottom: Zoom on the droplet framed in white in the top micrographs, at intermediary temperatures (scale bars = 10 µm). C) Fluorescence radial profiles of the droplet shown in B at increasing temperature. D) Plot of the droplet edge/core intensity ratio – extracted from fluorescence radial profiles – as a function of the temperature (error bars = standard error on 8 droplets).

Figure 4. Evidence for thermal collapse transition of PLL-g-PNIPAM in SPIC conditions. A) Turbidimetry measurement of free PNIPAM (dashed line) and PLL-g-PNIPAM (solid line) solutions at 1 wt% in phosphate buffer. B) Top: Confocal micrographs at T = 20 °C and T = 45 °C of a W/FCO emulsion with 2 wt% of PLL-g-PNIPAM and 500 × 10⁻⁶ M of ANS in the aqueous phase and 0.05 wt% of Krytox in the FCO phase (scale bars = 50 µm). Bottom: Zoom on the droplet framed in white in the top micrographs, at intermediary temperatures (scale bars = 10 µm). C) Fluorescence radial profiles of the droplet shown in B at increasing temperature. D) Plot of the droplet edge/core intensity ratio – extracted from fluorescence radial profiles – as a function of the temperature (error bars = standard error on 8 droplets).

At T = 20 °C the fluorescence signal of ANS in polymer-loaded droplets was non uniform: the intensity was higher at the droplet edge, as highlighted by the sharp peak of fluorescence (three times higher than the core level) observed in the radial profile (see Figure 4C). This observation, suggesting that the droplet edges are more hydrophobic than the droplet cores, is consistent with an initial deposition of PLL-g-PNIPAM at the water/FCO interface as shown in Figure 1. As temperature was increased from 20 °C to 45 °C, we observed an increase in fluorescence contrast between the edge and the core of the droplets (see Figure 4B), due to a concomitant decrease of intensity in the core and increase of intensity of the peak at the edge (see Figure 4C). The ratio between edge and core fluorescence intensities plotted in Figure 4D shows a continuous increase with temperature, with a significant change of slope at T = 34 °C corresponding to Tc. The significant values of the edge/core ratio at high temperature (T > Tc) confirm the occurrence of the collapse thermal transition of PNIPAM at the water/FCO interface at T = Tc. Moreover, the fluorescence level of ANS fell down to almost zero in droplet cores at T > Tc, suggesting that PLL-g-PNIPAM chains that were initially soluble in the droplet cores have enriched the interface upon heating.

The reversibility of this process was assessed by submitting an emulsion to temperature cycles while recording the edge/core ratio of ANS fluorescence (see Figure S6, Supporting Information): initially set at T = 18 °C, the emulsion was heated up to 45 °C, then cooled back to 18 °C and heated again to 45 °C. In samples brought back to T = 18 °C, the fluorescence level in the droplet cores slightly increased compared to T = 45 °C but did not come back to the initial value measured before heating. After the second heating step the fluorescence profile was identical to the one after the first heating step. These observations point out that after a first collapse transition in the SPIC layer, PLL-g-PNIPAM that had accumulated at the droplet edge remained trapped in the interfacial layer and did not significantly redissolve when the sample was
brought back below the transition temperature (at least on the experimental time scale of ∼1 h).

As a control experiment, the same imaging process was performed with a non-thermoresponsive poly(L-lysine)-g-poly(ethylene glycol) (PLL-g-PEG) copolymer with the same 0.20 grafting ratio than PLL-g-PNIPAM. A solution of 2 wt% of PLL-g-PEG and 500 × 10⁻⁶ M of ANS was emulsified in FCO and the fluorescence was observed by CLSM at T = 18 °C and T = 45 °C (see Figure S7, Supporting Information). After formulation at T = 18 °C, the droplet edge/core ratio of ANS fluorescence was greater than 1, indicating that PLL-g-PEG has been adsorbed at the water/oil interface by SPIC with Krytox, but was significantly lower than for PLL-g-PNIPAM, probably because both PLL and PEG moieties are highly hydrophilic. When observed by SEM, the capsules made of PLL-g-PEG at T = 18 °C looked very similar to the PLL-g-PNIPAM ones, both in size and effective membrane thickness (see Figure 3). Moreover, no significant change of the edge/core ratio in droplets containing PLL-g-PEG was observed between T = 18 °C and T = 45 °C, indicating that the temperature had no effect on the hydrophobicity of the interfacial polymer layer. These results confirm that the thermoresponsiveness of PLL-g-PNIPAM highlighted by ANS fluorescence at the droplet edges is intrinsically related to the collapse transition of PNIPAM strands.

The thermoresponsiveness of the PNIPAM strands being preserved once adsorbed at the water/oil interface, we presume that these results illustrate a generic approach for imparting various chemical properties to interfacial polymer layers via the use of the PLL-based comb-like architecture. Other functional polymer strands (such as light- or redox responsive ones) could thus be grafted onto PLL to design new functional polymer shells by interfacial complexation.

2.4. Effect of the Droplet Radius and the Temperature on Characteristic Features of the Polymer Interfacial Layer

To characterize the effect of the temperature on the interfacial polymer layer, we implemented the quantitative fluorescence analysis presented in Section 2.2 to measure the polymer surface excess in the droplets (see Figure S2, Supporting Information). As the fluorescence of carboxy-X-rhodamine (referred as rho) is neither subject to bleaching nor sensitive to temperature (see Figure S8, Supporting Information), it was used to evaluate the concentration of rho-labeled PLL-g-PNIPAM (PLLrho-g-PNIPAM) in emulsion over temperature changes.

Figure 5A shows confocal micrographs of a W/FCO emulsion with water droplets containing 3 wt% of PLLrho-g-PNIPAM at T = 18 °C and T = 45 °C. As previously showed in Figure 1, the fluorescence of rho was brighter at the droplet edge, indicating that PLLrho-g-PNIPAM had accumulated at the water/FCO interface by conventional SPIC upon the emulsification process. At T = 45 °C (above Tc), the interface was still highly fluorescent, but the fluorescence signal had almost disappeared in the droplet core. This sharp decrease in core intensity upon heating is highlighted in the radial profiles drawn in Figure 5A. This observation supports the idea of a thermo-induced transfer of core-soluble PLLrho-g-PNIPAM chains towards the interface, probably related to the progressive immobilization of diffusive chains onto the excess interfacial PLLrho-g-PNIPAM layer via hydrophobic interactions. This hypothesized process is depicted in Figure 5B.

The “apparent” thickness of the interfacial polymer layer – corresponding to the width at half height of the fluorescence peak at the droplet edge – had similar values of ∼1.5 μm for different polymer concentrations (in the 2–5 wt% range) and

Figure 5. Effect of temperature on the amount of interfacial PLLrho-g-PNIPAM. A) Top: Confocal micrographs of a representative water droplet containing 3 wt% of PLLrho-g-PNIPAM dispersed in FCO containing 0.05 wt% of Krytox at T = 18 °C and T = 45 °C (scale bar = 10 μm). Bottom: Fluorescence radial profiles of the top droplets. B) Schematic illustration of the accumulation of core-soluble PLLrho-g-PNIPAM chains at the interface upon collapse transition (occurring at Tc = 34 °C) in a W/FCO emulsion. C) Evolution of the surface excess of PLLrho-g-PNIPAM as a function of the droplet diameter at T = 18 °C and T = 45 °C, calculated from fluorescence measurements. The maximum surface excess (black dashed line) corresponds to the ideal case of interfacial adsorption of all the PLL-g-PNIPAM chains initially present in the droplet.
at low and high temperatures (see Figure S9, Supporting Information). Taking into consideration the lack of resolution of fluorescence microscopy, the width of fluorescence peak measured on the confocal micrographs is rather consistent with the thickness observed by SEM (see Figure 2).

The evolution of the polymer surface excess with the droplet diameter at $T = 18 \degree C$ and $T = 45 \degree C$ is shown in Figure 5C. Experimental data were compared to the maximum reachable surface excess, corresponding to the adsorption at the interface of all the polymer chains contained in the droplets. This maximum excess increases linearly with the droplet diameter as it is proportional to the droplet volume/surface ratio. At $T = 18 \degree C$, the experimental surface excess is significantly below the maximum curve and increases with droplet diameter until reaching a plateau for big droplets (> 30 µm). These observations suggest that a significant fraction of the polymer was not involved in interfacial complexation and that a saturation equilibrium between core-soluble and edge-adsorbed chains was presumably achieved at high volume/surface ratios. Interestingly, when switching temperature from $T = 18 \degree C$ to $T = 45 \degree C$ the surface excess almost doubled at a given droplet diameter. For small droplets (< 30 µm), the surface excess coincides with the maximum excess curve, indicating that all the polymer chains contained in the droplet have been segregated at the W/FCO interface. This observation is in agreement with an enrichment of the polymer interfacial layer above $T_c$, confirming the hypothesis of a core draining and an accumulation of polymer chains at the interface via hydrophobic interactions. For big droplets (> 30 µm), the experimental surface excess deviates from the maximum excess curve, suggesting that a saturation level has been reached in the SPIC process and that a fraction of soluble polymer chains could not be recruited in the interfacial complex layer.

The evolution of the surface excess as a function of the residual concentration in droplet cores is shown in Figure S10 (Supporting Information). From $T = 18 \degree C$ to $T = 45 \degree C$, the fraction of core-soluble polymer chains decreased from 0.4–0.6 to less than 0.1 concomitantly to an increase in the mean surface excess, confirming that (i) a high proportion of polymer chains were not involved in interfacial complexation in conditions of conventional SPIC (at $T < T_c$) and (ii) a massive accumulation of polymer chains at the interface was observed beyond the collapse transition of PNIPAM (at $T > T_c$). In addition, this process seems to be mainly irreversible as the surface excess remained at the same high level when a sample was cooled back at $T = 18 \degree C$ after a first heating step at $T = 45 \degree C$ (in Figure S11, Supporting Information). This suggests that the enrichment of the interfacial polymer layer is not subject to significant redispersion as PNIPAM chains are brought back to $T < T_c$.

The same quantitative approach was performed on a non-thermosensitive PLL-g-PEG labeled with carboxy-X-rhodamine (PLLrh0-g-PEG) and the results are shown in Figure S12 (Supporting Information). The curves of surface excess superimpose at $T = 18 \degree C$ and $T = 45 \degree C$ and plateau at a saturation level which is twice lower than PLL-g-PNIPAM at $T = 18 \degree C$ (and 4 times lower at $T = 45 \degree C$). These observations indicate that (i) PLL-g-PNIPAM has a higher affinity with the interface than PLL-g-PEG presumably because PNIPAM strands are shorter than PEG ones (18 versus 44 monomers per chain respectively) and (ii) there is no effect of the temperature on the distribution of PLL-g-PEG in droplets, confirming that the process observed for PLL-g-PNIPAM is intrinsic to the thermoresponsive nature of PNIPAM strands.

2.5. Segregation of Nanoparticles in the Polymer Interfacial Layer

The combination of nanoparticles with PLL-g-PNIPAM was investigated in order to achieve multicomponent interfacial layers. As a proof of concept, 50 nm NeutrAvidin-coated fluorescent nanoparticles (fluoNPs) were added in the aqueous phase containing PLL-g-PNIPAM prior to emulsification. Figure 6A show confocal micrographs of a W/FCO emulsion with 2 wt% of PLL-g-PNIPAM and 0.025 wt% of fluoNPs in the aqueous phase at $T = 18 \degree C$ and $T = 45 \degree C$. As PLL-g-PNIPAM was not dye-labeled, the fluorescence signal was only due to the fluoNPs and allowed to visualize their distribution in the water droplets. At $T = 18 \degree C$, the fluoNPs were homogeneously distributed within the droplets. At $T = 45 \degree C$, the fluoNPs were essentially located at the droplet edge, indicating that they had been segregated at the water/FCO interface concomitantly with the collapse transition of PNIPAM at $T_c$. This process of thermo-induced co-segregation of polymer chains and fluoNPs at the interface is depicted in Figure 6B (the Krytox molecules and the positive charges of PLL have been removed for more readability).

The evolution of the emulsion was recorded over time while temperature was increased (from 27 °C to 45 °C) to assess the dynamics of fluoNPs within the droplets (see Supplementary Movie). For the representative droplet shown in Figure 6C, the evolution of the radial profile and the radial kymograph are represented along the temperature sweep. In the radial kymograph, the projections of the radial fluorescence image at increasing temperatures have been put side by side in a continuous image. It illustrates the progressive disappearance of fluorescence in the droplet core concomitant with an accumulation of the fluorescence at the droplet edge. This process is also highlighted by the evolution of the radial profile as temperature rises: the uniform profile of fluorescence at low temperature ($T < T_c$) was progressively replaced by a sharp peak of fluorescence localized at the droplet edge ($T > T_c$). The consistency of the so-formed polymer-NPs mixed layer was assessed by two qualitative observations: (i) micron-size aggregates of fluoNPs that had reached the interface were totally immobilized at $T = 45 \degree C$ as shown in Figure S13 (Supporting Information), (ii) the corona of fluoNPs formed at $T = 45\degree C$ did not redispense as the emulsion was cooled down below $T_c$ (see Figure 6D), suggesting that the co-segregation of PLL-g-PNIPAM and fluoNPs at the interface was irreversible. These data are suggesting the formation of highly viscous or gel-like shells at the water/FCO interface, probably due to PLL-particles interactions that play the role of physical cross-linking. The irreversible capture of nanoparticles in PLL-g-PNIPAM shells has been demonstrated here in the case of one type of nanoparticles (protein-coated) but this approach is likely to be generic as PLL can interact with a great variety of materials (silica, oxides, plastics).

A control emulsion was made with 2 wt% of non-thermosensitive PLL-g-PEG and 0.025 wt% of fluoNPs in the
aqueous phase. The distribution of fluoNPs was uniform in water droplets at \( T = 18 \, ^\circ C \) and \( T = 45 \, ^\circ C \) (see Figure S14, Supporting Information), suggesting that the interfacial capture of fluoNPs observed with PLL-g-PNIPAM is due to the thermoresponsiveness of PNIPAM strands and is not inherent to the nature of fluoNPs.

This new approach of formation of “composite” shells has the advantage to rely on a spontaneous self-assembly upon gentle heating and does not require any pre-modification of the nanoparticles of interest or post-modification of the polymer capsules.

### 2.6. Encapsulation of a Model Protein

The possibility to encapsulate proteins in PLL-g-PNIPAM core-shell droplets was tested using eGFP (enhanced Green Fluorescent Protein) as a fluorescent model protein. An aqueous solution containing 2 wt\% of coumarin-labeled PLL-g-PNIPAM and \( 10 \times 10^{-6} \) m of eGFP was prepared in phosphate buffer (pH = 7.3) and emulsified in FCO phase. The distribution of both the polymer and the protein within droplets was determined by CLSM (see Figure 7). The bright coumarin fluorescence signal at the droplet edge shows that PLL-g-PNIPAM was concentrated at the water/FCO interface as expected, demonstrating that the presence of the protein in the aqueous phase did not affect the formation of the interfacial polymer layer induced by SPIC. The uniform distribution of eGFP within droplets showed that the protein had been efficiently encapsulated in the aqueous droplet cores without significant loss of fluorescence properties (see Figure S15, Supporting Information). The overlay of confocal images clearly shows the “core-shell” distribution of the species, with the polymer at the droplet edge and the protein entrapped in the droplet core.

### 3. Conclusion

In this work, we demonstrated the possibility to enhance the formation of polymer shells by interfacial complexation in water-in-fluorocarbon oil (W/FCO) emulsions by using the thermoresponsive properties of poly(N-isopropylacrylamide) (PNIPAM) strands grafted on a poly(L-lysine) (PLL) polyelectrolyte backbone. A polymer layer made of PLL-g-PNIPAM comb-like chains was formed at the edge of water droplets upon interfacial complexation between the cationic groups of PLL and an oil-soluble anionic surfactant (Krytox). By controlling the composition of the emulsion, this interfacial complexation resulted in the formation of well-defined hollow capsules with typically 1 \( \mu \)m-thick shells and aqueous cores with controlled pH and ionic strength. In mild conditions of formulation, a model protein was readily encapsulated in the core of the
droplets without interfering with the formation of the polymer shell, demonstrating that our approach is promising for the storage of potentially fragile biomolecules.

We showed that the thermo-responsive behavior of PNIPAM strands – switching from hydrophilic coils to hydrophobic globules at a transition temperature of 34 °C – was preserved in the interfacial complexation state. Our approach demonstrates that comb-like derivatives of PLL can be used as a generic platform to bring functional polymer chains at the surface of aqueous droplets. We believe that this strategy can be readily implemented to impart other properties to the so-formed polymer shell, such as light- or redox responsiveness.

In this study, we used the LCST transition of PNIPAM to trigger the collapse and aggregation of polymer chains upon gentle heating (T > 34 °C). Above the temperature of collapse transition, we observed a massive clustering of PLL-g-PNIPAM at the edge of the droplets, likely due to hydrophobic interactions between PNIPAM strands. This process contributed to enrich the interfacial polymer layer as demonstrated by the increase in polymer surface excess measured from fluorescence images. Interestingly, the persistence of high surface excesses when droplets were cooled back to room temperature suggests that the thermo-induced accumulation of polymer chains in the shell wall was irreversible. This possibility to reinforce polymer shells without having recourse to cross-linking is a major advantage of this approach.

Additionally, the thermal transition of PLL-g-PNIPAM was leveraged to achieve a straightforward capture of nanoparticles in the polymer shells. As a proof of concept, fluorescent nanoparticles that were initially dispersed in the droplet cores were irreversibly segregated in the interfacial layer of PLL-g-PNIPAM after heating above the transition temperature. The capture of nanoparticles turned out to persist even after cooling back to room temperature, suggesting that a gel-like polymer-particles shell had been formed at the droplet edge. This simple procedure of formation of “composite” shells does not require any pre- or post-modifications of the nanoparticles or capsules since it relies on the combination of (i) the propensity of PLL to spontaneously adsorb on various types of nanoparticles (essentially anionic) and (ii) the thermo-induced accumulation of PLL-g-PNIPAM towards the interface upon gentle heating. We thus believe that this general strategy based on “thermo-enhanced” interfacial complexation will deserve future studies on the possible design of a vast variety of functional capsules (for instance magnetic- or light-responsive capsules by interfacial capture of iron oxide or gold nanoparticles).

4. Experimental Section

Materials: Poly(L-lysine) hydrobromide (PLL, M_w = 15–30 kDa), (N-hydroxysuccinimidyl ester)-terminated poly(N-isopropylacrylamide) (PNIPAM-NHS, M_w = 2 kDa), azide-terminated poly(N-isopropylacrylamide) (PNIPAM-N3, M_w = 5 kDa), 5(6)-carboxy-X-rhodamine N-hydroxysuccinimidyl ester (rho-NHS), 7-(diethylamino)coumarin-3-carboxylic acid N-succinimidyl ester (coum-NHS), dibenzocyclooctyne-Cy3 (DBCO-Cy3) and 8-anilino-1-naphthalenesulfonic acid (ANS) were purchased from Sigma-Aldrich. (N-hydroxysuccinimidyl ester)-terminated poly(ethylene glycol) (PEG-NHS, M_w = 2 kDa) was purchased from Rapp Polymere. Fluorinert FC-70 fluorocarbon oil (FCO) was purchased from Sigma-Aldrich. Krytox 157 FS(L) (M_w = 2.5 kDa) was purchased from Samaro. FluoSpheres NeutrAvidin-Labeled Microspheres (40 nm diameter, 1 wt% in pure water) displaying yellow-green fluorescence (505/515 nm) were purchased from ThermoFisher Scientific. Unless otherwise noticed, all chemicals were used without further purification. Enhanced Green Fluorescent Protein (eGFP) had been produced and purified according to a protocol described in the literature.[56]

If not otherwise specified, aqueous solutions were systematically prepared in a home-made 7.7 × 10^{-3} M phosphate buffer, prepared by dissolving 360 mg of NaH_2PO_4 (3 mmol) and 670 mg of Na_2HPO_4.
(4.7 mmol) in 1 L of DI water (phosphate = 7.7 ± 10−3 m, pH = 7.3, ionic strength = 17 ± 10−3 m, Debye length = 2.3 nm). For the specific experiments on the influence of the pH, a modified Britton-Robinson (BR) buffer was prepared by dissolving 480 mg of NaH₂PO₄ (4 mmol), 328 mg of NaOAc (4 mmol) and 828 mg of CHES (4 mmol) in 0.4 L of DI water (pH = 5.7, ionic strength = 30 × 10−3 m, Debye length = 1.7 nm). The pH was adjusted at 4.3 (resp. 9.4) by addition of 150 µL of 1 m H₃PO₄ (resp. 500 µL of 1 m NaOH) in 30 mL of initial BR buffer.

**Polymer Synthesis and Functionalization**

PLL was grafted with NHS-terminated strands (PNIPAM-NHS or PEG-NHS) and labeled with NHS-terminated fluorophores (rho-NHS or coum-NHS). The number of NHS-terminated strands was adjusted as a molar fraction of total NHS-terminated fluorophores (rho-NHS or coum-NHS). The number of functionalized lysine units/total theoretical grafting ratio τ follows: PNIPAM-N3 (MW = 15–30 kDa, 15 mg) was dissolved in sodium tetraborate buffer (5 mL, pH = 8.5). PNIPAM-NHS (MW = 2 kDa, 48 mg for τ = 0.33) and rho-NHS (430 µL of rho-NHS at 1 g L⁻¹ solution in DMSO for DMSO as τ = 0.01) were then added and the solution was stirred at 4 °C until complete dissolution of the polymer. Then, the solution was stirred for an additional 4 h at room temperature. The resulting solution was dialyzed against DI water for 3 days in a Labconco Freezone Plus 2.5 apparatus, yielding a colored fluffy powder (3.3 mg, yield = 83%). ¹H NMR (400 MHz, D₂O, δ: 1.0–1.3 (lysine [CH₂] + PNIPAM [CH₂–CH–CO–NH–CH(CH₃)₂], 46H), 1.3–2.2 (lysine [δ–δ–CH₂] + PNIPAM [CH₂–CH–CO–NH–CH(CH₃)₂], 31H), 2.8–2.9 (lysine [non-grafted ε–CH₂], 1.7H), 3.20 (lysine [grafted ε–CH₂], 0.41H), 3.90 (PNIPAM [CH₂–CH–CO–NH–CH(CH₃)₂], 7.6H), 4.30 (lysine backbone, 1H). The experimental grafting ratio τ exp of PLL by polymer strands was determined by comparing ¹H NMR integration values of protons of PLL backbone as reference protons (1H): τ exp = 0.41/2.0 = 0.20. The functionalization of PNIPAM-N₃ by DBCO-Cy₃ was performed as follows: PNIPAM-N₃ (MW = 5 kDa, 100 mg) was dissolved in DI water (5 mL). DBCO-Cy₃ (23 µL at 1 g L⁻¹ in PBS buffer) was added and the solution was stirred for 5 h at room temperature. The resulting solution was dialyzed against DI water for 3 days (Slide-A-Lyzer cassette, MW cutoff = 3.5 kDa) and finally freeze-dried for 2 days, yielding a pink fluffy powder.

**Turbidimetry**

Turbidimetry by UV–Visible Spectrophotometry: UV–Visible spectra were recorded on a single cell Thermo Scientific Evolution Array UV–vis spectrophotometer equipped with a Peltier temperature-controlled cell holder (+0.1 °C). Sample solutions were injected in a 60 µL quartz micro-cuvette (OPL = 3 mm) and submitted to an increase of temperature from 20 °C to 60 °C by steps of 2 °C. A spectrum was recorded at each step temperature after 3 min thermalization and the mean transmittance between 650 and 750 nm was used to plot the turbidimetry curve.

**Emulsion Formulation and Observation**

Emulsion Formulation and Observation: 5 vol% water-in-FCO emulsions were prepared as follows: 5 µL of aqueous phase (containing the polymer at 1–5 wt% in the appropriate buffer, see above and 95 µL of FCO phase (containing 0.01–0.05 wt% Kryoxy) were mixed in a 0.5 mL Eppendorf tube and manually emulsified until the mixture became turbid. For microscopy experiments, a few µL of emulsion were injected in a hollow rectangle capillary (H × W = 0.1 × 1 mm, CMScientific) previously coated with PLL-PED to prevent the breakage of water droplets at the contact with bare glass side. The internal sides of capillaries were coated as follows: capillaries were filled with an aqueous solution of PLL-PED (MW = 2 kDa, 1 g L⁻¹ in phosphate buffer) and incubated for 30 min before evaporation on a heating plate at 60 °C. Then DI water was introduced by capillarity and evaporated at 60 °C.

Phase contrast imaging was performed with a LEICA DM IRE2 microscope equipped with a long-focal × 63 air objective. Images were acquired with a Retina 6000 Q-imaging camera and processed with Micro-Manager 1.4 software (ImageJ).

**Confocal Laser Scanning Microscopy**

Confocal images were acquired on a Zeiss LSM 710 META Laser Scanning Microscope equipped with a Plan Apochromat ×20 (0.8 NA) air objective and a temperature-controlled heating stage (25–50 °C). To ensure emulsion thermalization, capillaries filled with the emulsion were placed on a pierced supporting copper slide that was fixed on the heating stage of the confocal microscope. Low temperatures (16–20 °C) were achieved by placing a glass petri dish full of ice on top of the supporting copper slide close to the capillary. The actual temperature of the supporting copper slide was measured with a REED ST-640B thermocouple. The 405 nm excitation of ANS or coumarin was performed using a 30 mW CW/pulsed diode laser. The 458 nm excitation of eGFP or FluoroSpheres NeutrAvidin-Labeled Microspheres, and the 514 nm excitation of rhodamine or Cy3 were performed using a 25 mW argon laser. Images were acquired using LSM ZEN 2009 software and processed with Fiji software (ImageJ).

**Scanning Electron Microscopy**

SEM imaging was performed on 5 µL emulsion dried on a glass coverslip for three days at ambient air. Prior to imaging, the dried capsules were metalized by deposition of a 30 nm-thin layer of Ag using an Emitech K675X Sputter Coater (Ag sputtering for 60 s at 120 mA). SEM images were recorded with a Hitachi TM3030 Tabletop Microscope at 15 kV accelerating voltage.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords**

interfacial complexation, PNIPAM, polymer capsules, thermoresponsive polymers, water-in-oil emulsions

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