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Short Communication

A focus of the nanoprecipitation by solvent displacement: example of poly(MAOTIB) intended to *in vivo* applications

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Abstract

Through this study, we propose to specifically focus on a particular stage of the fabrication of polymeric nanoparticles intended to be used as contrast agent for biomedical X-ray imaging. These nanoparticles, made from nanoprecipitation of preformed polymer, poly(MAOTIB) (poly(2-methacryloyloxyethyl(2,3,5-triiodobenzoate))) follow a solvent displacement process. This method, widely used in literature, is sensitive to the formulation and process parameters such as nature and concentrations of surfactant and polymer, solvent / non-solvent ratio, rate of addition of one phase in the other one, respective volumes of the phase, and homogenization shearing rate. On the other hand, in function of the aimed administration route, the final suspension should obey to specific constraints on final product, *e.g.* size range and polydispersity, final particle concentration (*i.e.* iodine concentration) and surfactant concentration. In the present work, we report a specific investigation on the nanoprecipiation of poly(MAOTIB) in tetrahydrofuran, dropped in water or ethanol (as non-solvent) and stabilized by nonionic surfactant. The objective is to show and explain the potentials and limitations of such the process, but also to provide a guidance on the way to optimize it.

Keywords:

Nanoprecipitation; polymeric nanoparticles; poly(MAOTIB); solvent displacement method; biomedical imaging.

Nanoprecipitation or solvent displacement method has been described as an efficient, cost-effective and simple post-polymerization technique to yield monodisperse colloidal polymeric nanoparticles (PNPs), mostly nanospheres and nanocapsules. The typical protocols involve the precipitation of a preformed polymer in presence of surfactant to generate polymeric colloids, as a consequence of its solvent displacement or evaporation. In the case of solvent displacement, the polymer phase is mixed with another phase in which the polymer is not soluble (so-called “non-solvent”), more precisely the polymer is not soluble in the final “solvent”/”non-solvent” mixture. As a results polymer undergoes a controlled nanoprecipitation in the form of nanoparticles. Such process may occur using dialysis or dropping technique, which both require low-cost equipment as well as low energy yields. An important advantage of this method compared to *in situ* polymerization owes to the fact that the preformed polymer have been beforehand purified, preventing the remaining presence of reactive precursors or unreacted monomers in the final nanoprecipitates. As a result manufacturing PNPs does not requires using additives, rendering the process quite relevant for pharmaceutics formulations, reducing the number of potential exogenous components (Fessi, Devissaguet, Puisieux, & Thies, 1992; Hitanga, Sharma, Chopra, &

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Kumar, 2015; Hornig, Heinze, Becer, Ulrich, & Jena, 2009; Lepeltier, Bourgaux, & Couvreur, 2014; Mahapatro & Singh, 2011; Pinto Reis, Neufeld, Ribeiro, & Veiga, 2006; Schubert, Delaney, Jr, & Schubert, 2011).

The interest for PNPs has emerged over the past few decades owing to their promising potential applications in nanomedicine as controlled delivery systems of drugs or active principle ingredients (API) (such as genes, drugs, vaccines, biomolecules, probes for imaging…). PNPs have indeed been pointed out as excellent multifunctional vehicles due to their tunable architecture (size, shape, surface, composition…). Conditioned by the chemical nature of the matrix polymer, PNPs can exhibit a good biocompatibility. PNPs *in vivo* fate can also be monitored and controlled through physiological mechanisms such as biodistribution, targeted accumulation, biodegradation profiles. PNPs may also present a particular ability to interact with selected biological entities like viruses, cell membrane receptors and proteins *via* a specific (small) size coupled with surface functionalization. To summarize, PNPs can be considered a versatile nanocarrier with high potential as drug delivery system, along with industrial scaling-up and to application in Human. The critical point in close relationship with the compatibility with the desired biomedical applications (that PNPs will be dedicated to), remains the method of preparation, formulation and functionalization. Indeed it could be divided-up into two main methodologies: *in situ* polymerization processes and nano-precipitation of preformed polymer (Hans & Lowman, 2002; Mahapatro & Singh, 2011; Pinto Reis et al., 2006; Xu, Zhang, Nichols, Shi, & Wen, 2007).

A well-documented application of PNPs regards their use as contrast agent for biomedical imaging, particularly emerging as regards the X-ray imaging modality. In fact, several complementary imaging techniques were approved by the International guidelines for the clinical surveillance and diagnosis of tumor (*e.g.* for hepatocellular carcinoma detection), namely ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI). US technique limits itself to surveillance tests while four-phase CT and dynamic contrast MRI are commonly admitted as first-line diagnosis (Bruix & Sherman, 2011; Cancer, 2012). An excellent example demonstrating the potential of PNPs as nanocarriers for biomedical applications, are Lipiodol®-loaded PNPs –core-shell oil polymer nanoparticles– which were FDA approved as radiopaque contrast agent (CA) for X-ray imaging (Elsabahy, Heo, Lim, Sun, & Wooley, 2015; Idée & Guiu, 2013). X-ray imaging is indeed one of the most used imaging modality for its ability to reveal soft tissues, however only when using specific CAs administrated during X-ray scan is processed (and able to target the imaging region of interest (ROI)). However, while most of clinical radiopaque CAs show a fast blood clearance (due to rapid kidneys excretion of these small hydrophilic molecules), they require the administration of high dose or multiple injections in order to maintain a significant signal over time (Hallouard et al., 2011; Hallouard, Anton, Choquet, Constantinesco, & Vandamme, 2010). This indeed causes adverse effects on kidneys and respiratory functions. Increasing the size of contrast agents higher than 10 nm, with PNPs, prevents the renal elimination and, in turn, make the nanoparticles circulate in blood stream up to reticuloendothelial system (RES) elimination and accumulation in liver and / or spleen (Anton et al., 2017; Attia et al., 2014; Li et al., 2013). Thus PNPs have the ability to carry a high concentration of API and to target the radiopaque cargo towards the ROI (Elsabahy et al., 2015; He, Ai, & Lu, 2015; Key & Leary, 2014; Lee, Choi, & Hyeon, 2013).

One the first promising heavy element-based monomer resistant to polymerization process and with a low molecular weight is an iodinated vinyl monomer, the 2-methacryloyloxyethyl(2,3,5-triiodobenzoate) or MAOTIB monomer. It has been described several times in literature in the fabrication of radiopaque PNPs CAs owing to its suitability *in vivo*, but also for its high iodine content of 62 wt.% (Galperin & Margel, 2006a; Jayakrishnan & Chithambara Thanoo, 1992; Moszner, Salz, Klester, & Rheinberger, 1995). However, the typical preparations methods described, involved working out an *in situ* polymerization process in emulsion, and in general giving rise to microsphere (Galperin & Margel, 2006b; Hagit, Soenke, Johannes, & Shlomo, 2010) or copolymer-based PNPs (Aviv, Bartling, Kieslling, & Margel, 2009) with a limited resulting iodine content (Galperin & Margel, 2006a, 2007). Galperin *et al.*(Galperin et al., 2007) succeeded to produce very small (30 nm) poly(MAOTIB) PNPs by polymerization in emulsion but *in vivo* imaging results remained limited, through a biodistribution highly dispatched between lymph node, spleen, liver and kidneys, *i.e.* a poor specificity and “diluted” X-ray contrast *in vivo*.

An alternative solution was previously described by our group (Wallyn et al., 2018), developed in order to face these difficulties, was based on the development of new formulations of poly(MAOTIB). The fabrication of iodinated PNPs were then performed according to the polymer nanoprecipitation process. Poly(MAOTIB) was primarily polymerized by radical polymerization, nanoprecipitated, and the resulting PNPs suspension was compatible with an i.v. administration sizing around 164 nm and having an iodine concentration about 59 %(w/w). *In vivo* evaluation provided clear delineation of spleen and liver about 1h after injection (Wallyn et al., 2018). It is noteworthy that, in order to use PNPs as *in vivo* CAs, few stringent requirements must be fulfill. Poly(MAOTIB) PNPs should possess a stable and biocompatible core-shell morphology based on *(i)* a polymeric core with high payload of opacifying heavy element (*i.e.* iodine grafted on the polymer structure); *(ii)* a hydrophilic shell (made by the nonionic surfactants, Kolliphor ELP® Castor oil PEG-35), decorating the nanoparticle and presenting antibiofouling properties; and *(iii)* showing a narrow size distribution, below 200 nm to both avoid embolization and to postpone opsonization (Elsabahy et al., 2015; He et al., 2015; Key & Leary, 2014; Lee et al., 2013; Torchilin, 2002, 2012).

It is important to note that the optimization of the *in vivo*

imaging properties comes by the optimization of the physico-chemical properties of the particulate CAs PNPs themselves (Attia et al., 2014, 2016). It follows therefrom that a better control of the PNPs properties *in vivo* is closely related to their size, concentration, and surface properties, and thus due to the nano-precipitation formulation process itself. Nanoprecipitation is actually sensitive to concentration, solvent / non-solvent ratio, nature of surfactant and polymer, rate of addition of one phase in the other one, respective volumes of the phase, and homogenization shearing rate. A complete study of the effects of these key parameters is generally necessary to tune and control the resulting PNPs physicochemical properties (Bilati, Allémann, & Doelker, 2005; Chorny, Fishbein, Danenberg, & Golomb, 2002; Schubert et al., 2011). In the present *Short Communication*, we propose to precisely focus on this important experimental stage –nanoprecipitation stage– and investigate the impact of the formulation parameters on the properties of the resulting poly(MAOTIB) nanoparticles. The idea is, not only to understand more deeply the process, but also to provide a guidance on the optimization of nanoprecipitation by the dropping technique aiming the optimal PNPs formulation.

Using poly(MAOTIB) polymer is an interesting example, representative of a wide range of polymers, for which the size and nanoparticle concentration (*i.e.* iodine) in suspension, are both very important –since conditioning their efficiency *in vivo*. On the one hand, the main experimental parameters we selected were, *(i)* the polymer concentration, *(ii)* the non-solvent nature and *(iii)* the amount of surfactants. On the other hand, the key properties of the generated PNPs lie in their size distribution, the iodine concentration and viscosity of the final suspension, parameters directly related to the compatibility of the formulation for the parenteral route, used as CAs for preclinical X-ray imaging.

The preparation of poly(MAOTIB) PNPs was performed using poly(MAOTIB) homopolymer according to the dropping method in presence of hydrophilic nonionic surfactant, illustrated in Fig. 1 (the synthesis and chemical characterization of poly(MAOTIB), described in previous reports (Wallyn et al., 2018), was strictly followed). The dropping organic phase, composed of poly-(MAOTIB) (25, 50 or 100 mg) solubilized in tetrahydrofuran (THF, from Sigma, 10 mL), was added dropwise under magnetic stirring (500 rpm) to the non-solvent phase (water or ethanol (EtOH), from Sigma, 40 mL) containing the nonionic surfactant (Kolliphor ELP®, from BASF, amount ranging from 40 mg to 1 g). After the dropping stage, volatile solvents were removed under reduced pressure (rotary evaporator, Büchi, Switzerland). The excess of surfactant was removed by centrifugation / washing cycles: *i.e.* non-solvent washing, centrifugation, removing and replacing supernatant (process varying in function of the experiments followed). PNPs were finally collected and re-dispersed by sonication in phosphate buffer saline (PBS) as isotonic and vehicle compatible with parenteral administration. PNPs suspension were characterized by dynamic light scattering (Malvern NanoZS®, Malvern Instruments),

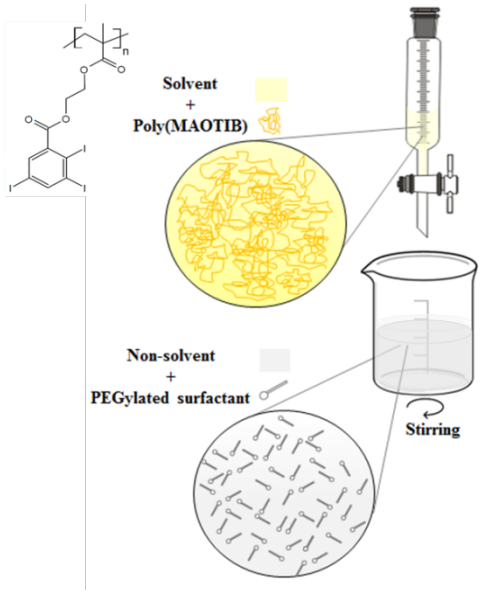

Figure 1: Nanoprecipitation procedure of the poly(MAOTIB) by the dropping technique.

experiments were performed in triplicate.

The first investigation concerned the impact of the nature the non-solvent phase and surfactant concentration, on the PNPs properties and size distribution. To this end, a given concentration of polymer (2.5 mg/mL) in THF (10 mL) was dropped into the non-solvent (water or EtOH, 40 mL). Different quantities of surfactant beforehand solubilized in the non-solvent. The results were reported in Fig. 2 (a), as the mean diameter of the nanoparticles against the surfactant concentration, for the two different non-solvent phases. Surprisingly, the curves are not monotonous, and both, first, slightly decrease and then increase at the higher surfactant concentrations. In addition, the influence of the nature of the non-solvent appear relatively important. As regards the nanoprecipitation in EtOH, the PNPs sizes follow an initial plateau ranging from 151 nm to 142 nm, from 5 mg/mL to 15 mg/mL, respectively, and then above 25 mg/mL undergoes a sudden size increase reaching 230 nm. On the other hand, using water as non-solvent phase allows decreasing the size around 125 nm at higher surfactant concentrations around 17.5 to 20 mg/mL. The values of the polydispersity indexes (PDI) in general followed the similar trend, increasing at the highest concentrations.

This global trend appears relatively surprising, since in general, *e.g.* when compared to spontaneous emulsification processes –that are close to nanoprecipitation– increasing the surfactant concentration actually decreases the droplets size (Anton & Vandamme, 2009; Attia et al., 2014; Li et al., 2013). In that case –nano-emulsification– the surfactants find a thermodynamically stable configuration at the water / oil interface and that therefore

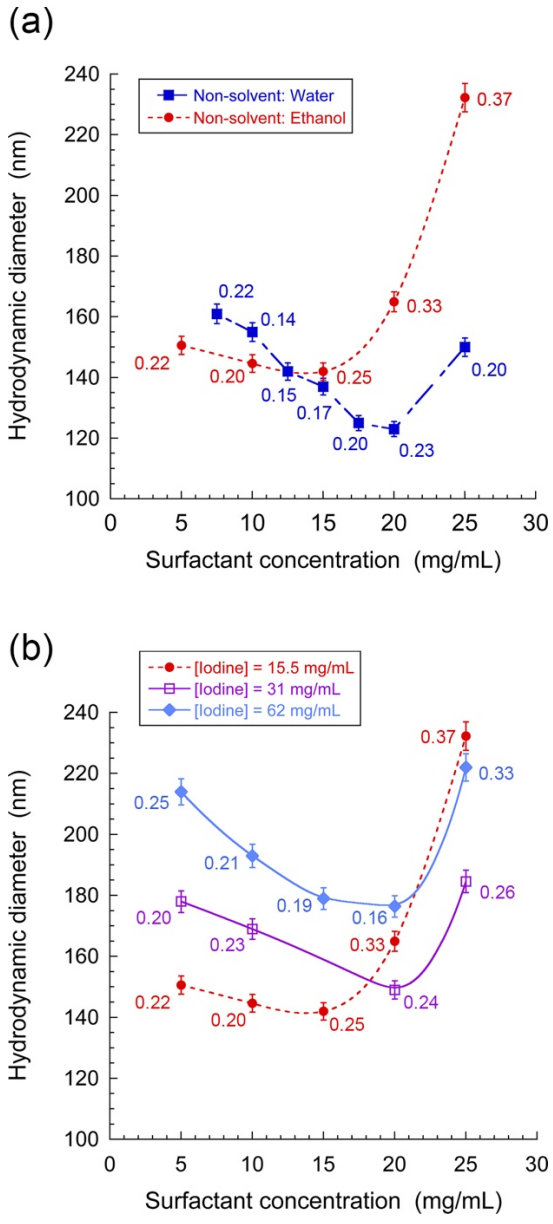

Figure 2: Effect of surfactant concentration in the non-solvent phase, on size distributions of poly(MAOTIB) PNPs suspension, after EtOH evaporation it was redispersed in PBS (n=3). (a) impact of the nature of the non-solvent phase, concentration of polymer in THF = 2.5 mg/mL (*i.e.* 15.5 mg of iodine by mL in the final suspension); (b) impact of the polymer concentration in THF, 2.5, 5.0 and 10.0 mg/mL (*i.e.* 15.5, 30 and 62.2 mg of iodine by mL in the final suspension, respectively), non-solvent being EtOH.

trends to increase the interfacial area. In contrast, in the case of polymeric nanoparticles, the surfactants are "trapped" between external phase (in which it is soluble) and the precipitated polymer shell. It follows therefrom that its amphiphilic role –actively impacting the size– is only played in the very short whiles prior to the polymer precipitation. After that, its role changes, and becomes a coating material. This difference compared to the emulsification could likely explain the size increasing for the higher surfactant concentrations.

On the other hand, the nanoprecipitation process by solvent displacement is driven by the velocity of the displacement of the "solvent" phase towards the "non-solvent", and thus by the affinities between solvent and non-solvent. In addition –and this is important in our case– the addition of molecules solubilized in these phases can modulate their relative affinities. This likely explains the significant difference we observe between water and ethanol (Fig. 2(a)): for instance, for the lowest surfactant concentration (below 10 mg/mL), for the same polymer and surfactant concentrations, using EtOH gives rise to significant lower sizes compared to water. The only explanation comes from the respective affinities between THF and non-solvent, as well as the ones between the polymer and non-solvent. In addition, along with an increase to the surfactant concentration –in the non-solvent– this effect is modulated even giving better results for water around 20 mg/mL (Fig. 2 (a)). At the highest surfactant concentrations, the solvent diffusion process seems disturbed by the number of species –surfactants in non-solvent– already dissolved and the size of the resulting PNPs increases importantly.

In summary, optimal formulations arises as a compromise between the surfactant amount and nanoparticle size: around 10-15 mg/mL of surfactants appears interesting for both non-solvents types, size ranges still remain acceptable respective to the aimed –parenteral– administration route. On the applicative point of view, the solvent displacement method produces quite diluted product, while X-ray imaging requires a high iodine concentration to visualize a contrast enhancement. This is why, strictly speaking, using EtOH could appear more suited since it can be more easily evaporated than water. Even if freeze-drying is a possibility to concentrate aqueous samples, the added value-to-cost ratio will certainly not be enough justified, all the more so since EtOH can provide similar results.

Let us consider the impact of the polymer concentration in solvent phase –and thus of the iodine concentration– on the size and polydispersity of the suspensions. The resulting iodine concentration in the whole suspension is reported in the Fig. 2 (b). It follows that the chosen polymer concentration in THF before nanoprecipitations in ethanol were 2.5, 5.0 and 10.0 mg/mL, resulted in the final suspension of iodine concentrations of 15.5, 30 and 62 mg/mL. As a function of the surfactant concentration in the non-solvent phase, the trends of the curves followed a similar scheme whatever the poly(MAOTIB) concentration: a first size decrease and a sudden increase at the higher surfactant concentration ranges (around 15-20 mg/mL).

Increasing the polymer concentration logically leads to generate bigger PNPs. The question to be raised here is the

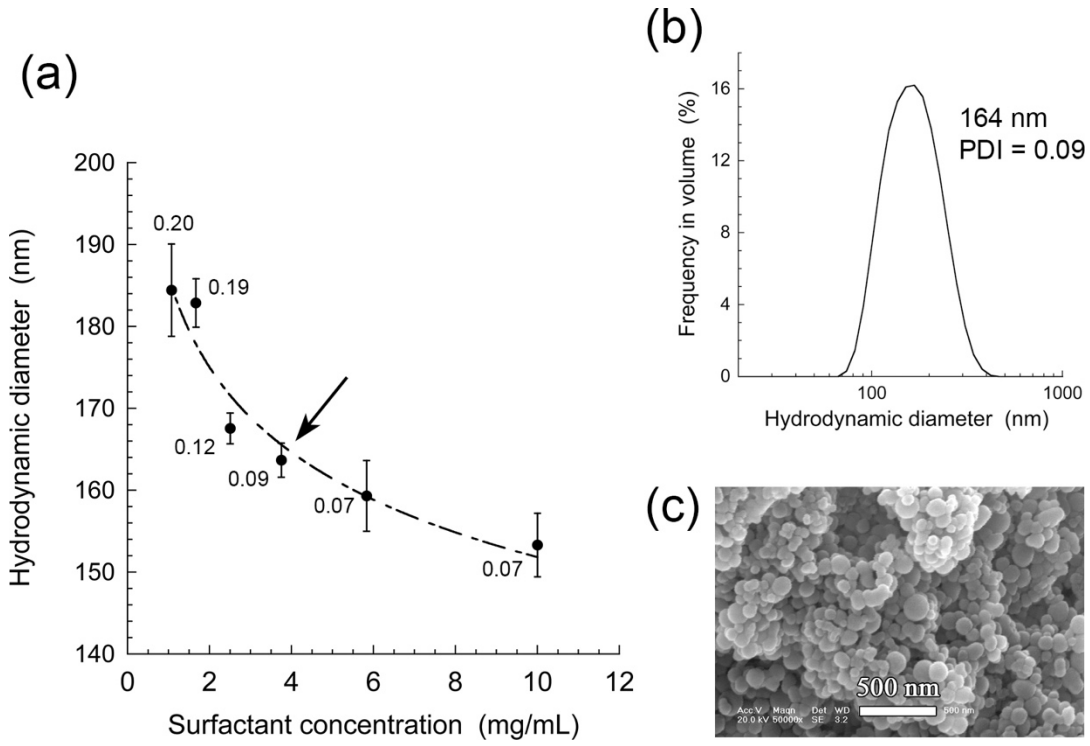

Figure 3: (a) Detailed study of the influence of the surfactant concentration in non-solvent phase, after extensive PNPs washing and redispersion in PBS, arrow indicates the compromise chosen between surfactant amount and size. (b) Size distribution and (c) transmission electron microscopy, of the PNPs corresponding to the optimized formulation shown by an arrow in (a).

balance between *(i)* size PNPs size –related to compatibility with *in vivo* injection and limited RES uptake, *i.e.* assuming the correct below 200 nm– but also *(ii)* the iodine concentration –related to their efficiency as contrast agents, and finally *(iii)* the surfactant concentration the lowest possible to prevent potential toxicity and with low viscosity.

For comparison, the common lipid / cholesterol-based market solutions (*e.g.* Fenestra®) propose an iodine concentration around 50 mg of iodine / mL (Hallouard et al., 2011, 2010; Li et al., 2013) giving a X-ray contrast enhancement significant enough for this modality. Accordingly, the best suspension of poly(MAOTIB) nanoparticles, corresponding to the requirement listed appear to be the one made with [poly(MAOTIB)] = 10 mg/mL (see Fig. 2 (b)) and surfactant concentration of 10 mg/mL, giving rise to PNPs sizing around 195 nm. Next step of the process is the centrifugation / PBS washing, that allows removing the excess of surfactants and ultimately replacing their vehicle. As a last remark, it is important to note that each individual samples exhibited excellent colloidal stability, confirming the effective PEG coating of the PNPs surface. The polydispersity of the formulations reported in Fig. 2, showing PDI values that remain relatively high, around 0.2, whatever the nature of the non-solvent, the surfactant and the polymer concentrations.

After the first screening of the impact of the formulation parameters on the properties of the nanoparticle dispersion, let us investigate the fine optimization of the formulation prepared and purified up to the conditions required for the *in vivo* administration. It regards the process with [poly(MAOTIB)] = 10 mg/mL in THF, investigating the compromise between the smallest surfactant concentration to be used, and the highest acceptable size (results reported in Fig. 3 (a)). It is noteworthy that these results have been performed after two-consecutive washing cycles with PBS, and finally re-suspension in PBS. The whole PNPs sizes appear globally reduced, which is possibly explained by the desorption of free surfactants. Basically, mean hydrodynamic diameter distribution showed a first drastic decrease from ~184 nm to ~167 nm (surfactant concentrations from 4 to 10 mg/mL, respectively), followed by a slower decrease of only ~10 nm (from 10 to 40 mg/mL). Again, the similar trend is followed by the values of the PDI, showing a distribution relatively large below 10 mg/mL and extremely narrow as the surfactant amount is increased. This is slightly contrasting compared to the PDI values reported in Fig. 2, and explained by the only difference –between these formulations– that is the treatment post-formulation, namely the extensive washing of the free surfactants. Indeed, free surfactant at these concentrations form micelles that can interfere in the DLS measurement as a second population.

An interesting compromise arose for the surfactant concentration equal to 15 mg/mL, even if the surfactant excess can be easily removed during the washing stage. Redispersion in 1 mL of PBS was made possible by a slight sonication, giving rise to a stable and non-aggregating PNPs suspension. The size distribution is reported in Fig. 3 (b). On this sample, iodine titration revealed a concentration at 59 mg I/mL (Wallyn et al., 2018), which is quite enough for providing an important contrast enhancement. As an additional characterization of the PNPs morphology, by scanning electron microscopy (performed on samples spread on a glass slide, died at room temperature, metallized by a thin palladium layer under vacuum, and observed using SEM Philips SIRION FEI apparatus operating at 20 kV), confirmed the size and fine distribution given by DLS (arrow in Fig. 3 (a) and Fig. 3 (b)).

To sum up, herein we investigated the impact of the formulation parameters applied to the nanoprecipitation of iodinated PNPs. Since the ultimate objective is an *in vivo* application, the formulation have to obey to particular constraints regarding: the size polydispersity, the iodine concentration and the sample viscosity. In contrast with the widely spread spontaneous emulsification followed with liquid lipid systems, this study shows that nanoprecipitation have to be considered differently. Affinity of the displacing solvents towards the different compounds and their concentrations, was indeed identified to be the important parameters impacting on the resulting PNPs properties. In this way, by applying a step-by-step approach, the nanoprecipitation of poly(MAOTIB) was fully understood and mastered, elucidating how to find good balance between crucial key parameters of the nanoprecipitation process.

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