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RESEARCH PAPER

Poly(ethylene glycol)-poly(ε-caprolactone) Iodinated Nanocapsules as Contrast Agents for X-ray Imaging

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ABSTRACT

Purpose Synthesis and formulation of iodinated PCL-mPEG nanocapsules as new original blood pool contrast agents for computed tomography.

Methods PCL-mPEG was synthesized and formulated following the *emulsion–solvent diffusion* process, in the form of iodinated nanocapsules. Physico-chemical characterization of such nano-materials was performed by DLS and transmission electron microscopy. A stability study of the nanocapsules suspension was followed-up to 3 month. Blood biocompatibility was performed. Finally, the nanocapsules suspension radiopacity was evaluated *in vitro* then *in vivo* in mice as micro-CT contrast agent.

Results In this study, the iodine concentration in nanocapsules suspension was about 70 mgI/mL. Besides, these nanocarriers appeared non-toxic, and stable in suspension. *In vivo*, i.v. administration of 10 μL/g of mouse body weight of theses nano-particles induced a vascular contrast enhancement of 168 HU and a half-life in blood of 4.2 +/− 0.5 h. Elimination route of these particles appears mainly performed by the liver, without sequestration in spleen and lymph nodes confirming their stealth properties.

Conclusions This study proposes the first example of iodinated biodegradable polymeric blood pool contrast agent, able to induce an exploitable contrast enhancement. The main advantage of polymeric system compared to lipid ones, lies in their stability and handling, *e.g.* towards drying for storage.

KEY WORDS: blood pool contrast agent · computed tomography · iodine · nano-particles · poly(ethylene glycol) - poly(caprolactone) · polymer · x-ray imaging

ABBREVIATIONS

^{1}H NMR	^{1}H nuclear magnetic resonance
BPCA	blood pool contrast agent
$CDCl_3$	deuterated chloroform
CL	ε-caprolactone
DCM	dichloromethane
d_h	hydrodynamic diameter
FBS	fetal bovine serum
HU	Hounsfield unit
M	molecular weight
Micro-CT	micro-computed tomography
M_{NMR}	molecular weight calculated from nuclear magnetic resonance spectrum
M_{nSEC}	molecular weight determined by SEC-MALLS

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OPR	oil/polymer ratio
PBS	phosphate buffered saline
PCL	poly(ε-caprolactone)
PCL-mPEG	poly(ε-caprolactone) – monomethoxy poly(ethylene glycol)
PD	polymerization degree
PEG	poly(ethylene glycol)
rpm	rotations per minute

INTRODUCTION

Micro-computed tomography (micro-CT), is a very powerful and non-invasive tool used to establish high-resolution images with isotropic voxels in relatively short scan times. This imaging modality is particularly suitable for visualizing and differentiating bones and soft tissues, but has limitation for delimiting different soft tissues. However, for the last decade many recent research efforts allowed to extend the scope of micro-CT application to soft tissue through the design of specific contrast agents, in the form of X-ray opaque, exogenous compounds having a selective biodistribution.

Radiopacity is induced by high Z-number atoms, and linearly dependent to the concentration. Most intravenous contrasting agents are made with iodine since it offers a good compromise between contrasting power, safety and cost (1). Iodinated contrast agents used for human applications are hydrosoluble macromolecules and thus undergo a very quick blood clearance by kidney (2). As well, iodinated oils also exist but their i.v. injection induces vascular emboli (3). To prevent these side effects and limitations, and in order to obtain a long circulating time giving a contrast enhancement in blood and potentially a targeted distribution, the general methods worked out consisted of formulating such contrast agents in the form of nanoparticulate systems. Classical examples are liposomes (4–8), chylomicrons (9,10), dendrimers (11), nanoemulsions (12–15), polymeric nano-particles (16,17) or micelles (18,19). Each solution has specific advantages and limitations, like low contrasting properties, toxicity, laborious formulation processes or poor stability, and unfortunately, no ideal micro-CT contrast agent have to date been formulated.

Polymeric nanoparticles are interesting candidates for blood pool contrast agent (BPCA), since their formulation process can be very simple and reproducible (*e.g.* emulsion–solvent diffusion process) and monodisperse suspensions can present a strong stability for several months (20). Likewise, using specific biodegradable polymers allows reducing the suspension toxicity. However, to date, the only polymeric particles are formulated using inorganic compounds as contrasting materials (*e.g.* gold, bismuth or rare earth). The attempts to combine the advantages of polymeric nanocarriers and iodine were always limited by the low reachable iodine concentration. Herein we propose a new technology based on emulsion–solvent diffusion process, giving rise to biodegradable and non toxic polymeric nanocapsules made with PCL-mPEG, and encapsulating a high concentration of iodine.

The emulsion–solvent diffusion process presents clear advantages compared to the other existing methods, such as avoiding chemical reactions by using preformed polymers, or using pharmaceutically acceptable organic solvents (like ethyl acetate). As well, this methods allows reaching high encapsulation yields of hydrophobic compounds, with high reproducibility, control of the particle size, and easy scaling-up (21). The method consists of a two-step process based on a low-energy nano-emulsification along with the polymer nanoprecipitation induced by the solvent migration from the dispersed phase to the bulk one.

Compared to PCL homopolymers, PCL-mPEG copolymers were included in the formulation since they present several advantages, improving biocompatibility of the nanoparticles (22). As well, these amphiphile copolymers allow obtaining a PEG coating of the nanoparticles surfaces, in order to improve their stealth properties and persistence in blood pool (23–25). In physiological conditions, PCL blocks are slowly degraded resulting in a nontoxic and low molecular weight by-products, which do not create acidic environment such as polylactid (PLA) or poly(lactide-co-glycolide) (PLGA) (26). The hydrophobic cores of the nanocapsules were composed of iodinated derivative of poppy seed oil (Lipiodol Ultrafluid®), having an iodine content as high as of 38 wt.%.

This study investigates the potentials of iodinated PCL-mPEG nanocapsules as a new class of BPCA for CT imaging. The first step was the synthesis of PCL-mPEG diblock copolymers, and their formulation with iodinated oil. The resulting nanocapsules were physico-chemically characterized by DLS, and electron microscopy, and the stability of the suspension was followed-up for 100 days. Then, this contrast agent was evaluated *in vitro* to determine particles stability, blood toxicity and radiopacity, and finally, their efficiency *in vivo* as BPCA was studied in mice through micro-CT imaging, giving a quantification of the contrast enhancement in blood, blood half-life, and disclosing the elimination ways.

MATERIALS AND METHODS

Materials

ε-caprolactone (CL) (Sigma, Saint Louis, USA), CaH_2 (93%, Acros Organics, Geel, Belgium), monomethoxypoly(ethylene glycol) (mPEG) (M: 5,000 Da, Fluka, Steinheim, Germany), toluene (99%, Acros Organics, Geel, Belgium), dichloromethane (DCM) (99%, Carlos Erba, Rodano, Italia), diethyl ether (99%, Acros Organics, Geel, Belgium), 2-éthylhexanoate stannous [$Sn(Oct)_2$] (95%, Sigma, Saint Louis, USA), Poly(ε-caprolactone) (PCL) (M: 14,000 Da, Sigma, Saint Louis, USA), Lipiodol Ultrafluid® (lot

09LU601A, Guerbet, Roissy Charles de Gaulle, France), iobitridol (Xenetix 300®, Guerbet, Roissy Charles de Gaulle, France), Fenestra VC® (Art, Saint-Laurent, Canada), poloxamer 188 (Lutrol F68®, lot WPND546E, BASF, Ludwigshafen, Germany) kindly free purchased by Laserson (Etampes, France), ethyl acetate (99,98%, Fischer Scientific, Leicestershire, UK), $Na_2HPO_4;12H_2O$ (99%, Fluka, Steinheim, Germany), HCl (37%, Acros Organics, Geel, Belgium), colloidal silica particles Percoll® (GE, Waukesha, USA). Distilled water was purified by using Milli-Q® system (Millipore, Bedford, USA).

Pre-emulsions were formulated with a rotor-stator apparatus (Ultra-Turrax T25®, IKA labotechnik, Staufen, Germany). Other devices used were a rotary evaporator associated with a vacuum pump (Rotavapor R144®, Buchi, Flavil, Switzerland), a MicroKros® tangential filter (Spectrum Labotories, Rancho Dominguez, USA), a size exclusion chromatography (Waters, MA, Milford, USA), a refractive index detector Waters 410® (Waters, MA, Milford, USA), a triple-angle light scattering detector MiniDAWN Tristar® (Wyatt Technology, Santa Barbara, CA, USA). Size distributions of the nanoparticle suspensions were accessed by dynamic light scattering (DLS), with a Zetasizer NanoZS® (Malvern, Brookhaven, UK). Transmission electronic microscopy was performed with a Philips CM12® (Philips, Verdun, France). Nuclear magnetic resonance spectrometry was performed at 300 MHz (Brucker DRX 300®, Brucker, Fremont, CA, USA). Finally, specific separation by molecular weight was performed by ultra-centrifugation with Optima MAX-XP® (Beckman Coulter, Brea, CA, USA).

Synthesis of PCL-mPEG Diblock Copolymer

CL was purified by vacuum distillation over CaH_2 and mPEG was dried by an azeotropic distillation with anhydrous toluene under dry nitrogen atmosphere. All the other reagents used in this work were of analytic reagent grade and used as received.

The PCL-mPEG diblock copolymer was synthesized by ring-opening polymerization of CL with mPEG as described by Diab *et al.* (27). Briefly, CL, mPEG, and $Sn(Oct)_2$ (0.1% of CL in molar amount) dissolved in toluene, were stirred under dry nitrogen at 130°C for 12 h. The resulting copolymer was cooled to room temperature, and then precipitated in excess of cold diethyl ether. The obtained copolymer was purified 3 times by dissolving them in DCM and then precipitated in excess of cold diethyl ether. Finally the mixture was filtered and dried at room temperature under vacuum for 24 h.

The degree of polymerization of PCL block was calculated from nuclear magnetic resonance (^{1}H NMR) spectrum.

Characterization of PCL-mPEG Diblock Copolymer

Nuclear Magnetic Resonance Analysis

^{1}H NMR spectrum was recorded by using a NMR spectrometer operating at 300 MHz using deuterated chloroform ($CDCl_3$) as solvent. Chemical shifts (d) were measured in ppm taking tetramethylsilane as internal reference standard.

Absolute Molar Mass Determination by SEC Multiangle Laser Light Scattering / Refractive Index

Absolute molecular weights of copolymer were determined on a Waters Size Exclusion Chromatography (SEC) system coupled with a triple-angle light scattering detector and an refractive index detector with integrated temperature controller maintained at 30°C. The two signals were measured simultaneously by online multiangle laser light scattering / refractive index (SEC–MALLS), giving rise to the copolymers absolute molecular weight. Data collection and processing were performed using two softwares (ASTRA®, Wyatt Technology, and the Empower Pro®, Waters Corporation).

Light scattering dn/dC value of PCL-mPEG diblock copolymer was determined by using 2 ranges of concentrations with polystyrene having both knowledge dn/dC. The range of concentration with the synthesized PCL-mPEG diblock copolymer was the same as ranges of concentrations with known polystyrenes. These ranges were going from 3.3 to 50 mg/mL.

Formulation of Iodinated Nanocapsules

The oily core polymeric nanocapsules were prepared by the emulsion–solvent diffusion process, illustrated in Fig. 1. The first step is the preparation the pre-emulsion, with organic phase containing solvent + polymer + iodinated oil, dispersed in water + surfactant. The dispersed and bulk phases were mutually saturated (respectively with water and solvent), in order to prevent the premature solvent transfer during the pre-emulsification. Precisely, the organic phase contained 1 wt.% of PCL-mPEG diblock copolymer and 3 wt.% of iodinated oil (Lipiodol Ultrafluid®) dissolved in saturated ethyl acetate. The aqueous phase contained surfactant (poloxamer 188) at given concentrations. Pre-emulsion was prepared by pouring organic phase (5 or 20 ml) into the aqueous phase (25 ml), and emulsifiying during 10 min with a rotor-stator device at 24 000 rotations per minutes (rpm). This resulted in an oil-in-water (O/W) macro-emulsion. Next, the nanocapsules were generated by extracting the solvent from the dispersed droplets, with a drastic dilution of the aqueous bulk phase with a large

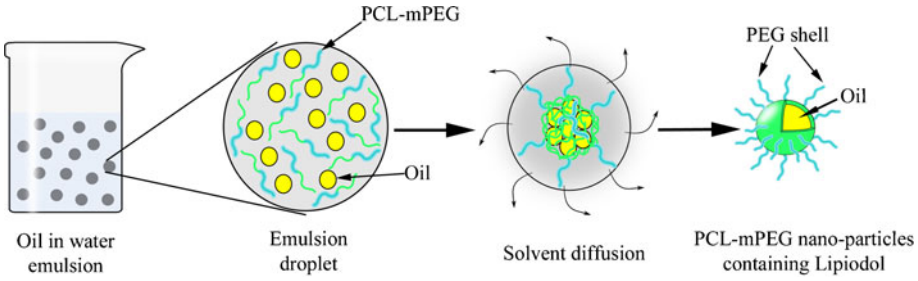

Fig. 1 Schematic description of the proposed formation mechanism of nano-particles containing Lipiodol® by emulsion–solvent diffusion process. In the organic phase droplets, the yellow dots and the blue-green lines are the iodinated oil and polymer respectively.

additional pure water volume (200 ml), under gentle stirring (600 rpm) for 10 min. As a result, the nano-precipitation of PCL-mPEG occurred. The presence of the oily phase likely induced the polymer nano-precipitation located at the periphery of the nanoparticles, creating a core-shell nanocapsule structure. The organic solvent and a part of the water (175 mL) were there after removed by evaporation under reduced pressure. Then, to afford a purified and concentrated suspension, the formulation was filtered by tangential filtration and 3 times washed with phosphate buffer (containing 0.75 wt.% of poloxamer 188 to avoid the particles aggregation during this purification process (28)). Finally, the nanocapsules suspension was filtered with a membrane filter (pore diameter of 0.8 μm).

Nanocapsule Characterization

Hydrodynamic Size

Hydrodynamic diameters and PDI were obtained by dynamic light scattering using a DLS instrument. The helium/neon laser, 4 mW, operated at 633 nm, with the scatter angle fixed at 173° and the temperature was maintained at 25°C. PDI is a measure of the broadness of a size distribution derived from the cumulants analysis of DLS data according to ISO 13321:1996; for a single Gaussian population with standard deviation, σ, and mean size, x_{PCS}, thus $PDI=\sigma^2/x^2_{PCS}$ is the relative variance of the distribution. In other words, it shows the quality of the dispersion. Values≤0.1 reflect a very good monodispersity and quality of the nanoparticulate suspensions. Measurements were performed three times for each point.

Transmission Electronic Microscopy

Nano-particles were visualized using a Philips CM120. PCL-mPEG iodinated nano-particles diluted 1000 times were deposed on a copper grid covered with a formal-carbon membrane. The magnification was 85000.

Density of Nano-Particles

Nanocapsules density was determined by differential ultra-centrifugation, within a density gradient of colloidal silica particles (Percoll®). 1 g of preparations was diluted in 4.5 g of Percoll®. Then, the mix was centrifuged during 30 min at 40,000 rpm, and at controlled temperature of (25±1)°C. Heights of the bands locating the different compounds were measured from the dispersion meniscus to the central point of the band.

Stability Study

In vitro stability of suspensions of iodinated nanocapsules were checked during 6 months (*i.e.* at 0, 7, 14, 28, 60, 90 and 180 days). The suspensions were stored at (25± 2)°C in dark. In these storage conditions, we performed the following-up of pH, osmolarity, size distribution and PDI.

Biocompatibility Study

The biocompatibility experiments consisted in testing the stability of iodinated nano-particles in fetal bovine serum (FBS) and in evaluating nano-particles blood toxicity. The amount of iodinated nano-particles suspension incubated with the erythrocytes or FBS corresponds to the maximum dosage administrated to mice (10% of the blood pool volume). Regarding the stability study, nano-particules (0.1 mL) were mixed with FBS (1 mL), homogenized and incubated at (37±1)°C under gentle agitation. The blood toxicity was assessed through photometric measurements of hemolysis. Erythrocytes were separated from plasma and leukocytes by centrifugation (10 min, 1,200 g, room temperature). Peripheral blood mononuclear cells and plasma band were drawn with a Pasteur pipette. Pellet (erythrocytes) was washed three times with PBS. Incubation of erythrocytes with nano-particles was performed in PBS at 2%. Erythrocytes suspension was mixed with nano-particles at 10% (vol./vol.), and incubated at (37±1)°C for 2, 3 and 4 h under a gentle magnetic stirring (150 rpm). Next, the samples were spun down for 5 min at 1,500 g and the supernatants were used for measurement. Finally, the photometric measurements of the free hemoglobin in the supernatant, at 414 nm, were expressed as percentage of destroyed erythrocytes. Solution of triton X-100 (1 wt.%) was used as positive control.

Micro-CT Imaging

The experiments were performed in accordance with the Committee on Animal Research and Ethics of the University of Lyon-1.

In the one hand, *in vitro* measurements of radiopacity were performed with a micro-CT scanner (1076 Skyscan®, Kartuizersweg, Belgium). Experimental parameters were: X-ray: 49 keV, 129 μA; resolution: 35 μm; pitch: 0.4°; aluminum filter: 0:5 and 632 ms. These *in vitro* experiments were carried out to determine the radiopacity and iodine concentration of the nano-particles. In this respect, a quantification curve correlating the iodine concentration and radiopacity was beforehand established with a commercial hydrophilic contrast agent (Xenetix 300®, namely iobitridol).

In the other hand, *in vivo* experiments were performed with a micro-CT scanner (INVEON®, Siemens, Munich, Germany). Experimental parameters: X-ray were: 50 keV, 500 μA; resolution: 111.25 μm; pitch: 2°; aluminum filter: 0:5 and 900 ms. The quantification of X-ray attenuation in Hounsfield units (HU) was performed by image-processing software (INVEON® Siemens, Munich, Germany). The open-source software Osirix® was used to perform the 3D volume rendering. 3 Swiss mice were anesthetized with isoflurane during the nano-particles suspension administration as well as during the CT scans. 10 μL of nano-particles/g of mouse weight were intravenously injected (using a catheter) in the tail vein. Scans were performed before administration: immediately after injection, and then 30 min, 1 h, 2 h, 3 h, 4 h and 6 h post-injection.

RESULTS AND DISCUSSION

To be an efficient blood pool contrast agent, the iodinated PCL-mPEG nanocapsules suspensions have to meet some criteria (1). The first one is their X-ray attenuation properties that should be high enough to induce a visible contrast enhancement. For example, Fenestra VC® (as commercialized BPCA) presents an iodine content of 55 mg I/mL (9). Secondly, iodinated nano-particles must have a long circulation time in blood pool. This is performed with a suitable surface modification, generally using hydrophilic polymer like PEG (29,30), however, the size of the droplets is also an important criteria which can strongly impact the blood residence time. Thus, the iodinated nano-particles size must be larger than 10 nm, corresponding to the width of fenestrated capillaries, and also must be small enough (below 200 nm) as higher sizes increase the RES uptake rate (1). Finally, the dispersion has to be monodisperse, decreasing the probability of a heterogeneous *in vivo* behavior of the nano-particles suspension.

PCL-mPEG Diblock Copolymer

The main interest in synthesizing and using diblock PCL-mPEG copolymer, lied in the resulting decoration of the nanocapsules surface by the PEG moiety of the polymer (see Fig. 1). However, it is also to be noted that the presence the PEG block improves the copolymer degradation rate, the higher the molecular weight of PEG block, the higher the degradation rate (31). Amphiphilic properties come from the poor aqueous solubility of the PCL part of the polymer (31). The choice of molecular masses of PCL and mPEG blocks was a compromise between different factors. The ability of PEG to increase the circulation lifetime of nano-particles has been found to depend on both the amount and length of PEG incorporated (23–25). In addition, the molecular weight of the PCL part was long enough to insure a strong anchoring of the PEG moiety (31). On the other hand, the longer the PEG, the bigger the resulting particle, this can increase the risk to obtain particles size over 200 nm (20) and so the RES uptake. Finally, the reproducibility of the polymerization reaction becomes less controllable with the polymerization degrees of PCL block. Our choice for an optimized formulation was finally focused on the synthesis of diblock PCL-mPEG copolymer having molecular masses of 11 400 for the PCL block and 5,000 Da for the mPEG one.

Figure 2 reports the ^{1}H NMR spectrum showing the characteristic resonance peaks of the synthesized PCL-mPEG block copolymer. The singlet (a) for monomethoxy in mPEG end groups ($-OCH_3$) appears at 3.38 ppm. The sharp peak at 3.65 ppm (multiplet, b) is attributed to methylene protons of $–CH_2CH_2O–$ of mPEG units in the block copolymer. Peaks at chemical shifts of 1.40 (multiplet, f), 1.65 (multiplet, e), 2.30 (multiplet, d), and 4.05 ppm (multiplet, c) are assigned to the methylene protons of $–(CH_2)_3–$, $–OCCH_2–$, and $–CH_2OOC–$, respectively, in PCL units. The very weak peaks at 4.22 and 3.87 ppm are, respectively, attributed to the methylene protons of $–O–CH_2–CH_2–$ of mPEG end unit that links with PCL blocks.

The molecular weight (M_{NMR}) of PCL-mPEG copolymers was calculated from ^{1}H NMR spectrum according to the following equations (27):

$$\frac{4m+2}{3} = \int \frac{OCH_2CH_2}{\int OCH_3}$$

$$\frac{2(n+1)}{3} = \frac{\int CH_2OCO}{\int OCH_3}$$

$$M_{NMR} = M_w(PCLblock) + M_w(mPEGblock = 44m + 114n)$$

where $\int -OCH_2CH_2-$ is the integral of the intensity of the methylene proton peak of $–OCH_2CH_2–$ belonging to mPEG blocks at 3.65 ppm; $\int -CH_2OCO-$ is the integral of the intensity of the methylene proton peak of $–CH_2OCO–$

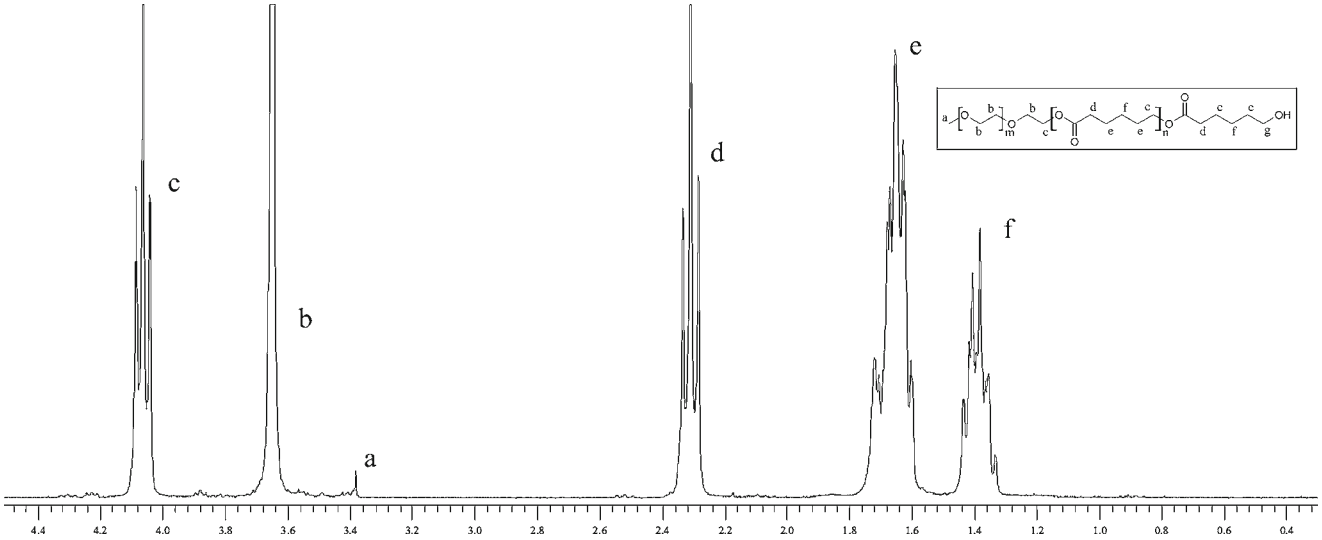

Fig. 2 ^{1}H NMR spectrum of the synthetized PCL-mPEG diblock copolymer in $DCCl_3$.

belonging to PCL blocks at 4.07 ppm; m and n are the corresponding polymerization degrees of mPEG and PCL blocks, respectively. The values 44 and 114 correspond to the molar masses of the repeating units of the mPEG and PCL blocks, respectively. The M_{NMR} obtained values were the same to the theoretical values M (Table I) showing a complete reaction of the synthesis.

The absolute molecular weight of copolymer was also determined by SEC-MALLS, without column calibration of the respective polymer. d*n*/d*C* of PCL-mPEG diblock copolymer was determined at 0.072 mL/g measuring the molecular weight of this copolymer at 15,880 g/mol by SEC-MALLS. As shown in Table I, M_n of the copolymer was determined by two absolute methods (SEC–MALLS and ^{1}H NMR): their very close results confirmed the values of the PD obtained for the PCL block, around 100.

Formulation of Iodinated PCL-mPEG Nanocapsules

The first formulation challenge was to obtain nano-particles exhibiting a monodisperses size distribution between 10 and 200 nm, an important criterion for the formulation of BPCA. The process optimization is illustrated in Fig. 3, that shows the influence of the surfactant amount on the size distribution and polydispersity of iodinated PCL-mPEG nanocapsules. The choice of the surfactant (Poloxamer 188) was done according to a previous study following a similar process (32). Increasing the surfactant concentration in the aqueous phase gave rise to a decrease of hydrodynamic size and polydispersity of iodinated nano-particles. This could be simply explained by the influence of the poloxamer 188 concentration, on the size decrease of the pre-emulsion droplets (*i.e.* before solvent diffusion). As Moinard-Chécot *et al.* demonstrated most of the properties of the nano-particles formed by solvent–diffusion are closely related to the pre-emulsification step (21), correlating the size of organic phase droplets with the one of the resulting nano-particles.

Then above a surfactant concentration of 3.75% (w/v), the size of the nanocapsule undergoes a rise along with a polydispersity is kept constant. This can be explained as the consequence of the accumulation of surfactant and co-polymer at the interface of the pre-emulsion, resulting in a reduction in the solvent diffusion speed. In view of the results, the optimal formulation giving the smaller size and polydispersity appears

Table I Comparison of SEC-MALLS and ^{1}H NMR Results in Terms of Absolute Molecular Weight and Polymerization Degree

Copolymer	M^a	$M_{NMR}{}^b$	$M_{nSEC}{}^c$	$PD_{NMR}{}^d$	$PD_{nSEC}{}^e$	$IP_{nSEC}{}^f$
PCL 11.4 K – mPEG 5 K	16 400	16 400	15 880	100	96	1.18

[a] Theoretical molecular weight as calculated according to the feed ratio

[b] The molecular weight as calculated according to the integrated area ratio of the resonance peaks because of the PCL block at 4.07 ppm and because of the mPEG block at 3.65 ppm

[c] The absolute molecular weight as determined by SEC-MALLS method

[d] Polymerization degree as calculated from ^{1}H NMR data

[e] Polymerization degree as calculated from SEC-MALLS data

[f] Polydispersity index determined by SEC-MALLS method

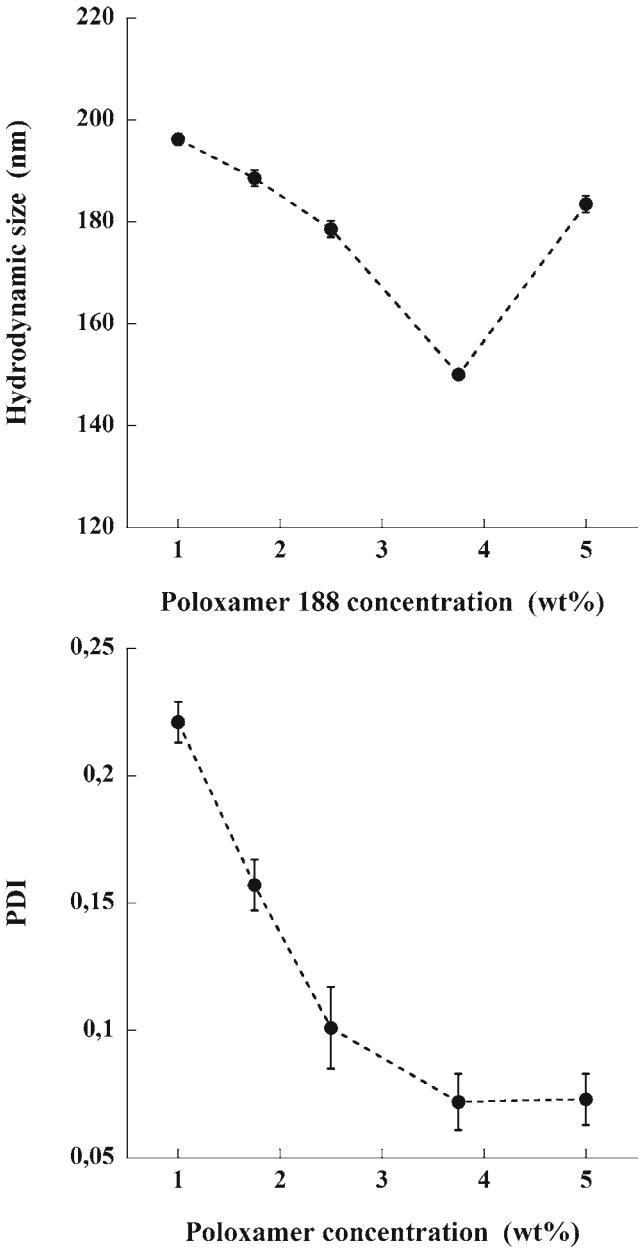

Fig. 3 Nanocapsules formulated by emulsion–solvent diffusion process with PCL-mPEG (M: 11,400 / 5,000), Lipiodol® (iodinated oil) and poloxamer 188 (surfactant). The organic phase (5 ml) containing 1% (w./v.) of polymer and 3%(w./v.) of oil, was emulsified with the aqueous phase (25 mL) containing poloxamer 188 during 10 min at 24,000 rpm. Hydrodynamic diameter and PDI are plotted against the surfactant concentration in the aqueous phase (each essay was repeated 3 times).

at a surfactant concentration of 3.75 wt.%, with a size at 150.1 nm and a PDI around 0.07.

The second formulation challenge regarded contrasting or X-ray attenuation properties of the nanocapsules suspension, linearly linked with the concentration of iodine. Actually, the loading rate must be at least higher than 5.5 wt.%, that is the one of the commercial lipid-based reference product, Fenestra VC® (9). In this way, this contrast agent will be efficient as BPCA. To obtain such iodine content in the nanocapsules suspensions, several strategies were evaluated in order to increase the droplet concentration in the suspension (thus improving the iodine concentration), and their impact on the nano-particles size and polydispersity were evaluated (Table II).

The first strategy consisted in studying what is the influence of increasing the proportion of organic phase during the emulsion step. Two formulations were studied, for which the volumes of organic phase were 5 and 20 mL, along with keeping constant the aqueous phase at 25 mL. As a result, no influence on the polydispersity was measured immediately after formulation; the particles size was lowered of about 15 nm (see Table II). Nevertheless, we had intentionally maintained an aqueous phase volume greater than organic phase one to prevent any potential emulsion inversion, no compatible with the whole process.

On the other hand, two others kinds of experiments for improving the nanocapsules concentration were performed once they were formulated. The first one was based on the water evaporation under reduced pressure, allowing simultaneous extraction of ethyl acetate (polymer solvent). A limit appeared below a critical volume around 50 mL, for which the nanocapsules begun to stick and melt on the evaporating flask forming a polymer film. Finally, performing a tangential filtration (also called cross-flow filtration) on the nanocapsule suspension allowed a further increase of their concentration, up to iodine concentration of 5 wt.%. The suspension undergoes a circulation tangentially to the filter, thereby reducing the risk of nano-particles deterioration compared to other methods such as centrifugation or dead-end filtration (33). Likewise, cross-flow filtration is largely used in industrial continuous processes, without risks of blinding the filter (with the formation of "cakes"). This technology allowed decreasing the residual volume from 60 to 3.25 mL along with a conservation of the size and low PDI value, corresponding to a theoretical iodine content increased to 70 mg I/mL (Table II).

Concerning formulation purification (Table II), tangential filtration had also allowed purifying formulation by washing them three times with phosphate buffer. This buffer contained 0.75 wt.% of poloxamer 188 to avoid the particles aggregation during this purification process because the adsorption of this surfactant on particle is reversible (28). To prevent vascular emboli, we filtered formulation with frontal filter having a pore diameter of 0.45 µm. This last purification step had no influence on particle polydispersity and lightly increased particle size.

Nanocapsules suspension and pure Lipiodol Ultrafluid® were compared after ultracentrifugation on a density gradient, in order to evaluate whether non-encapsulated iodinated oil remained in the formulation. The results are reported in Fig. 4, and evidenced different specific locations between

Table II Influence of Organic Phase Proportion and of Tangential and Dead-End Filtration on Particles Size and Polydispersity. Nano-Particles were Formulated by Emulsion–Solvent Diffusion Process. The Organic Phase Composed of 1 wt.% of PCL-mPEG (M 11400/5000 Da) and of 3 wt.% of Lipiodol® (iodinated oil), was Emulsified with 25 mL of a Solution of Satured Water with 3.7 wt.% of Poloxamer 188 (surfactant)

	Organic phase	Residual volume	Hydrodynamic size	PDI
Formulation A	5 mL	60 mL	150.1 +/− 0.808	0.072 +/−0.011
Formulation B	20 mL	60 mL	166.0 +/− 1.508	0.800 +/− 0.019
Before tangential filtration	20 mL	60 mL	166.0 +/− 1.508	0.800 +/− 0.019
After tangential filtration	20 mL	3.25 mL	166.6 +/− 1.473	0.075 +/− 0.019
Before 0.45 μm filtration	20 mL	60 mL	160.9 +/− 0.666	0.111 +/− 0.002
After 0.45 μm filtration	20 mL	60 mL	164.5 +/− 1.136	0.092 +/− 0.010

the iodinated PCL-mPEG nanocapsules and the iodinated oil. This means that all the iodinated oil is well trapped in the core of the nanocapsules with a narrow distribution of the nanocapsule weight. To confirm this hypothesis and to understand how the iodinated oil is encapsulated in PCL-mPEG nano-particles, we observed these particles with a TEM. Actually, thanks to the X-ray contrasting properties of the nanocapsules (*i.e.* iodinated oil) they will be visible by transmission electron microscopy without using staining agent. In the micrographs, presented in Fig. 5, the iodinated oily core appeared the most contrasted. Besides, polymeric shell is also (but less) visible, likely due to a slight entrapment of iodinated molecules within the precipitated shell. These isolated particles exhibit a size consistent with the one given by DLS (d_h = 155 nm in aqueous solution). Likewise, the shell thickness can be evaluated around 36 nm.

In storage conditions, the retention of iodinated oil in nano-particles was investigated during 3 months by following the relative density of PCL-mPEG nanocapsules containing iodinated oil (Fig. 4). To enhance sensibility with this method, 1 g of nanocapsule suspension having a theoretical iodine content of 70 mg I/mL was dispersed within 4.5 g of a density gradient of colloidal silica particles. This explains why the broadness of bands. Figure 4 showed no apparition of compounds at the relative density of free Lipiodol® and no decrease of the relative density of nanocapsules indicating no significantly iodinated oil release during this period. These results also demonstrated that there is no risk of emboli during injection by the presence of free iodinated oil in the formulation.

The last formulation challenge, before we began *in vitro* and *in vivo* evaluations, was to select the most stable suspension of iodinated PCL-mPEG nanocapsules. Stability is of basic importance for aiming the development of pre-clinical or a clinical product. The formulation that showed the best stability

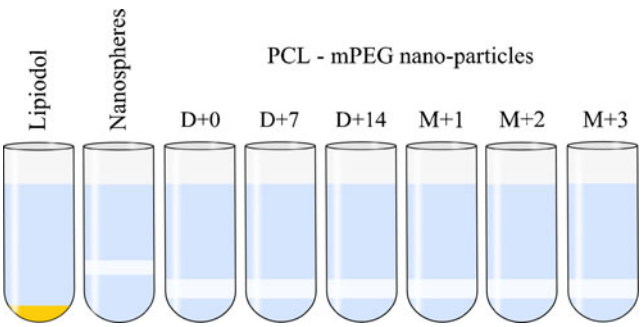

Fig. 4 Density comparison for Lipiodol® (iodinated oil), PCL-mPEG nano-spheres and iodinated PCL-mPEG nano-particles. Likewise *in vitro* density evolution of iodinated PCL-mPEG nano-particles at (25 ± 2)°C was followed during 3 months. Nano-spheres and nano-particles were formulated by emulsion–solvent diffusion process. The organic phase (20 mL) composed of 1%(w./v.) of PCL-mPEG (M 11,400 / 5,000 Da) and of 3%(w./v.) of Lipiodol® (in the case of nano-particles), was emulsified with 25 mL of a solution of satured water with 3.75%(w./v.) of poloxamer 188. Density were determined by centrifuge with Percoll® (silica colloidal solution) at 40,000 rpm during 30 min at (25.0 ± 0.1)°C.

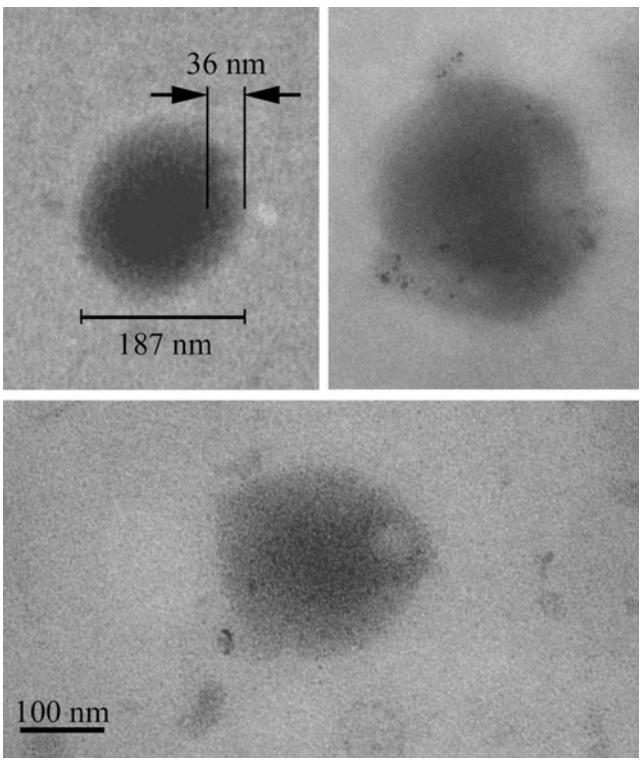

Fig. 5 TEM micrograph of PCL-mPEG nano-particles containing Lipiodol® (an iodinated oil) produced by emulsion–solvent diffusion. The magnification was the same for each three particles.

results for a period of 3 months (reported in Fig. 6) was produced by emulsifying, at 24 000 rpm during 10 min, the organic phase (20 mL) composed of 1% (w/v) of PCL-mPEG (M 11,400/5,000 Da) and 3% (w./v.) of Lipiodol Ultrafluid®, with 25 mL of aqueous phase containing 3.75% (w./v.) of poloxamer 188. The obtained suspension was concentrated until a theoretical iodine concentration of 70 mgI/mL. The parameters followed during this stability study at room temperature were hydrodynamic particles size, particle polydispersity, pH, osmolarity (reported in Fig. 6), and particle relative density (reported in Fig. 4). Osmolarity increased and pH decreased, reflecting the hydrolysis of copolymer PCL-mPEG. Specifically, the hydrolysis of PCL block produces water and CO_2, a slight acid, which nevertheless can impact on the acidity of the nanocapsules suspension. Variation rates of size and PDI seem linked to this phenomenon. Moreover, since the polymer framework of the nanocapsules prevents classical destabilization processes of nano-emulsion (like Ostwald ripening) to occur, the destabilization of the suspension may be the result of the particle aggregation. A change in the bulk composition can likely induce change in the stabilization mechanisms, *i.e.* decrease of pH gives rise to screening surface potentials and inter-particle repulsion forces. On the other hand, it is to be noted that the polymer degradation kinetic depends on the particle surface in contact with water (*i.e.* the smaller the nanocapsules, the higher the degradation rates). Nevertheless, the density of the capsules even after 3 months remains constant (see Fig. 4), eventually signifying that the relative proportion of hydrolyzed polymer remains quite weak. We can conclude that, in aqueous suspension, the iodinated PCL-mPEG nanocapsules could be used up to 3 months after their preparation.

In Vitro Evaluation of Iodinated PCL-mPEG Nanocapsules

In vitro evaluation concerns evaluation of the radiopacity as well as the blood toxicity of the suspension of iodinated nanocapsules.

The Hounsfield unit (HU) scale is a linear transformation of the raw linear X-Ray attenuation data, normalized for water and air (at standard pressure and temperature), with respective values of 0 and −1,000 HU. Therefore, using as reference a commercial product having known iodine content (like Xenetix 300®) allowed the quantification of iodine concentration in our nanocapsules suspension. The selected nanocapsule suspension described above as *most stable* (*i.e.* composed of 1 wt.% of PCL-mPEG (M_w 11,400/5,000 Da) and of 3 wt.% of Lipiodol Ultrafluid® (iodinated oil), and emulsified with 25 mL of a solution of saturated water with

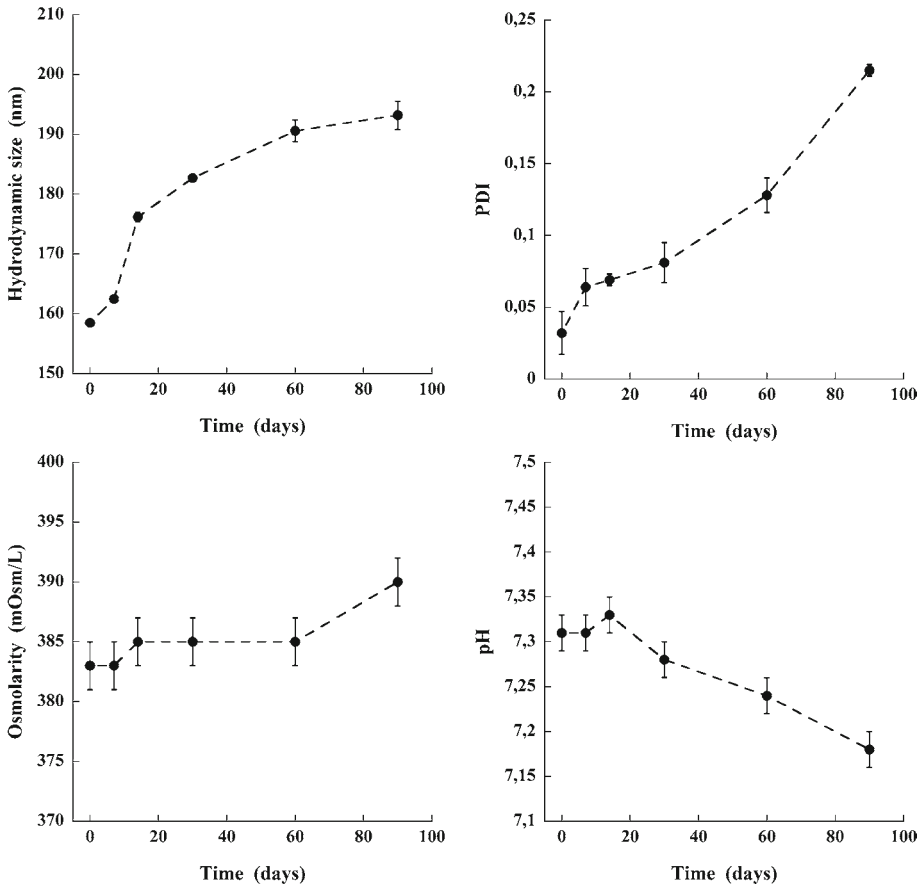

Fig. 6 *In vitro* stability of selected iodinated nano-particles suspension at (25 ± 2)°C during 3 months, $n = 3$. Nano-particles were formulated by emulsion–solvent diffusion process with PCL-mPEG (M: 11,400 / 5,000), Lipiodol® (iodinated oil) and poloxamer 188 (surfactant). The organic phase (20 mL) containing 1%(w./v.) of polymer and 3%(w./v.) of oil, was emulsified with the aqueous phase (25 mL) containing poloxamer 188 during 10 min at 24,000 rpm. This suspension was concentrated until a theoretical iodine concentration of 7 wt.%.

3.75 wt.% of poloxamer 188) corresponded to a calculated iodine concentration after tangential filtration around 70 mgI/mL. On the other hand, the experimental iodine quantification, reported in Fig. 7, was determined at 68.2 mgI/mL. Actually, the difference between calculated and experimental could come from several origins, like inaccuracy of the final volume measurement (±0.1 mL) due to the dead volume of the filters, as well as overestimation of the chemical iodine content in the oil (that was calculated from its chemical structure). Furthermore, the radiopacity of commercial contrast agent for micro-CT (Fenestra VC®) was measured by this way, giving an iodine concentration at 53 mg I/mL, corresponding to the one expected (9), thus validating the methodology. The literature gives that iodinated BPCA are usable with concentration from 22 mg I/mL, but can also reach 130 mg/mL (4–14,16–19). The present nanocapsules suspension, with 68.2 mg I/mL, exceeds the commercial reference and is totally compatible with such applications. Nevertheless, besides this interesting iodine concentration, our system is the first example of iodinated polymeric nanoparticles reaching such high concentration (16,17).

The *in vitro* toxicity studies on serum and erythrocytes were performed on the above-selected nanocapsules suspension. The visual observation of the fetal bovine serum samples mixed with the contrast agents suspension appeared very stable and homogeneous up to 4 h, without any signs of precipitation or coagulation. Results regarding the erythrocyte hemolysis experiments, at 2, 3 and 4 h of contact with the contrast agent are reported in Table III. Overall, the values stayed under 2%, confirming the compatibility of the contrast agent with blood, a critical feature in the development of new products intended to parenteral administration route.

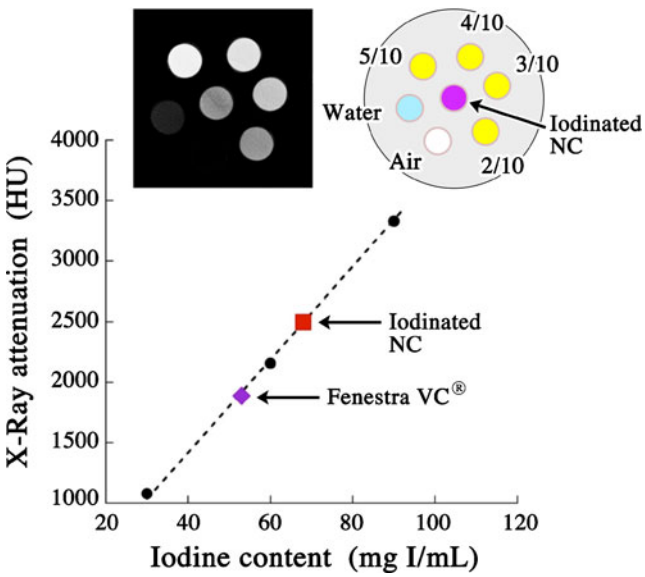

Fig. 7 *In vitro* radiopacity of iodinated nano-particles, water, air and various dilutions of iobitridol (Xenetix®, a commercial hydrophilic contrast agent at 300 mg I/mL). Nano-particles were produce by "emulsion – solvent diffusion" process. The organic phase (20 mL) composed of 1% (w./v.) of PCL-mPEG (M 11,400/5,000 Da) and of 3% (w./v.) of Lipiodol® (iodinated oil), was emulsified with 25 mL of a solution of satured water with 3.75% (w./v.) of poloxamer 188. (*top-left*) Raw data from micro-CT apparatus, (*top-right*) the corresponding locations of the samples. (*bottom*) The X-ray attenuation quantification curve established with iobitridol (*filled circles*). The values of Fenestra VC® and of iodinated nano-particles were represented respectively by a filled lozenge and a filled square.

In Vivo Evaluation of Iodinated PCL-mPEG Nanocapsules

The selected iodinated nanocapsules suspension was injected in caudal vein of 3 SWISS mice at a dosage of 10 µL of nanoparticles suspension/g of mouse weight. No clinical troubles were detected in all mice, up to 3 months.

Immediately after injection, a vascular contrast enhancement arised as shown in Fig. 8 (top). These results validate the efficiency of the contrast agent, as well as the high radiopacity of the suspension disclosed *in vitro*. Then, the *in vivo* becoming of this contrast medium in blood was followed by micro-CT in different key organs: heart, liver, kidney and bladder. The mean half-life of enhanced blood radiopacity was around 4.2+/− 0.5 h, in the same range that the reference contrast agents reported in literature, whatever the chemical nature (*e.g.* chylomicrons, nano-emulsions or micelles) (9,14,19).

By micro-CT, the *in vivo* pharmacokinetic visualized corresponds to the contrasting element of the contrast medium, that is to say iodine, and not the iodinated nanocapsules. After injection, iodine could be in three different forms: nanocapsules containing iodinated oil (Lipiodol®), released iodinated oil from nanocapsule; and free iodide from iodinated oil. However, pharmacokinetics of free iodide and Lipiodol® is well characterized (3,34). It is also possible to deduce the stability of these nanocapsules in blood by comparing the visualized pharmacokinetic with those of free iodide and Lipiodol®.

Table III *In vitro* Toxicity of Iodinated Nano-Particles by Emulsion–Solvent Diffusion Process, Evaluation of Erythrocyte Hemolysis. The Organic Phase (20 mL) Composed of 1 wt.% of PCL-mPEG (M 11,400/5,000 Da) and of 3 wt.% of Lipiodol® (iodinated oil), Was Emulsified with 25 mL of a Solution of Saturated Water with 3.75 wt.% of Poloxamer 188. Nanoparticles Suspension Represented 10% (v./v.) of the Mix Nano-Particles/Erythrocytes. Positive Control was Performed with Triton X-100 (1 wt.%); Negative Control with PBS

	Erythrocyte hemolysis (%)		
	2 h	3 h	4 h
Iodinated nano-particles	0.1 +/− 0.008	0.0 +/− 0.004	1.5 +/− 0.013
Positive control	100 +/− 1	100 +/− 2	100 +/− 0.3
Negative control	0.0 +/− 0.1	0.0 +/− 0.1	0.0 +/− 0.4

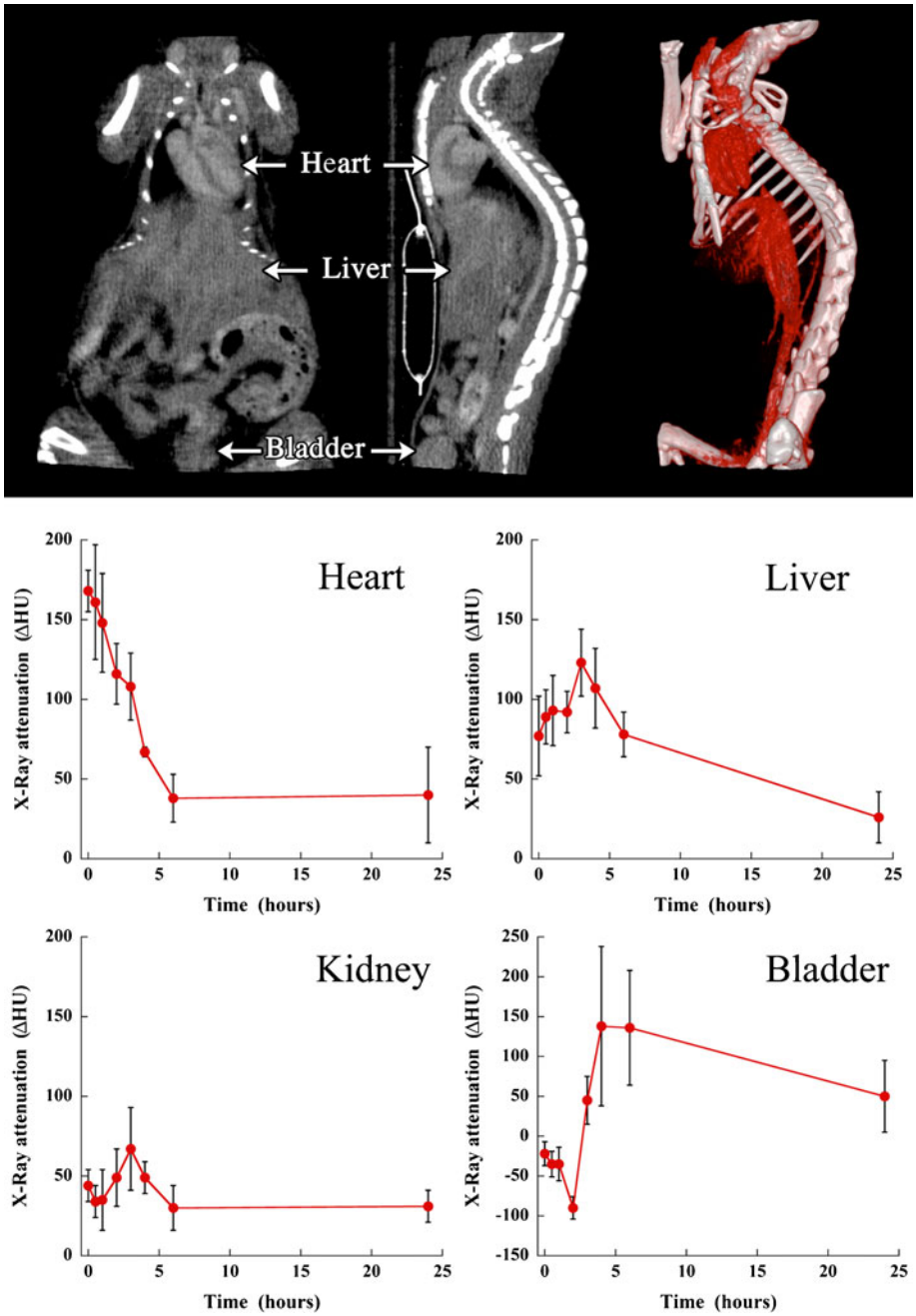

Fig. 8 Iodinated nano-particles injected in mice. The micro-CT scans (*top left*) show sections of the heart, liver, kidneys and bladder, 30 min after i.v. administration. A whole body 3D reconstruction (*top right*) shows mouse skeleton (in *white*) and the vascular bed (in *red*). A movie is available as Supplementary data. Regions of interests (ROIs) in the form of cylindrical volumes are placed in the left ventricle (Heart), the hepatic parenchyma (Liver), the left kidney (Kidney), and the bladder (Bladder), and the evolution of X-ray attenuation was followed-up during 24 h after injection. Nano-particles were produce by emulsion–solvent diffusion process. The organic phase (20 mL) composed of 1% (w./v.) of PCL-mPEG (M 11,400/5,000 Da) and of 3% (w./v.) of Lipiodol® (iodinated oil), was emulsified with 25 mL of a solution of saturated water with 3.75% (w./v.) of poloxamer 188; $n = 3$.

The elimination of free Lipiodol® in blood, the manufacturer indicated a rapid excretion by kidney and predominant by liver (3). Lipiodol® emulsions with poloxamer being not stable (17), it is impossible to compare the pharmacokinetic of such contrasting formulations with the pharmacokinetic observed in Fig. 8. Nevertheless, nano-emulsions of Lipiodol® with PEG-35 castor oil having a size about 160 nm are stable and their distribution in SWISS mice were described (14). These emulsions showed like free Lipiodol®, a progressive elimination by liver and immediate excretion by kidneys decreasing progressively. Both elimination ways seems to have the same importance. In additions, an half-life in blood of 2.5 +/− 0.77 h was observed with emulsions having a residual concentration of 1 wt% of surfactant. At 10 wt% of PEG-35 castor oil, this half-life rise to 4.1 +/− 1.1 h due to renal inhibition by this surfactant.

Figure 8 illustrate the evolution of X-ray attenuation in heart, liver, kidneys and bladder during 24 h after injection of iodinated nanocapsules. In bladder and kidneys, we observed firstly a strong decrease of the radiopacity during 3 h followed by an increase of the X-ray attenuation. This may be explained by a the first elimination of the exceeding blood volume due to the injection and an absence of renal

excretion of iodinated compounds. Indeed, water have a lower radiopacity (0 HU by definition) than bladder and kidney tissues (124.5 +/− 24 HU and 92 +/− 7 HU respectively before injection). Then, increased contrast in bladder and kidneys was induced by the gradual renal elimination of iodinated compounds; enhanced radiopacity in kidneys and in bladder reflecting respectively the excretion rate of iodinated compounds and the total amount of iodinated compounds excreted by kidney since injection. As regards the hepatic elimination route, the accumulation rate appears overall twice more important than renal extraction. The maximum intensity in liver was reached for 4 h post-administration, and then undergoes a continuous slight decrease. In additions, there was an acceleration of liver extraction between 3 and 4 h after contrast medium injection.

The difference of the observed pharmacokinetic and those with emulsified Lipiodol® with PEG-35 castor oil, indicated that Liopiodol contained in PCL-mPEG nanocapsules did not release during at least 3 h. Then, it seems to be a progressive release of iodinated oil from nanocapsules 3 h after injection by the progressive increase of renal excretion and of the acceleration of hepatic excretion (summing the elimination rate of released oil and nanoparticles by liver). This is the consequence of an accelerated hydrolysis of PCL-mPEG in blood which contains several enzymes like esterase increasing degradations of such polymers. Thus, the pharmacokinetic of these nanocapsules can be followed by micro-CT during the first 3 h post-administration. These particles seems to be eliminated only by the liver where they are in few hours metaboliszed and eliminated. This predominant elimination way by the liver is in accordance of nanoparticulate systems having a diameter between 100 and 200 nm (1). Nevertheless, it is important to note that the chemical nature of the oil plays a decisive role in the retention times in liver (13–15). For three similar formulations (nano-emulsions) only varying in the nature of the iodinated oily core, the retention time in liver can pass from 24 h with Lipiodol® to 4 months with iodinated alpha-tocopherol.

The difference of enhanced contrast half-life in blood between emulsified and encapsulate Lipiodol® showed the utility to formulate PCL-mPEG nanocapsules to improve the blood persistence of this oil. In additions, there was also no retention observed in spleen or lymph nodes was observed, indicating that any activation of the RES occurred, and confirming as well the stealth properties due to the presence of PEG layer at the particles surface. Besides, no sequestration was observed in thyroid, a very eager organ of free iodide (34). This demonstrated the absence of free iodide in blood during the 24 h following contrast medium injection.

Finally, the developed PCL-mPEG nanocapsules not only appears very interesting for structural imaging of blood pool and liver, but also the presence of lipophilic inner core can open the doors of the additional encapsulation of active substances (21), and thus to the fabrication of new tools for the personalized therapy also called theragnostics. Actually, most of the time, the efficacy of a therapy depends on the amount of drug available brought on its action site. In so pathologies such as pulmonary embolism or solid tumors, the distribution of the drug can significantly vary from one patient to another, or even in oncology, from one tumor to another in the same patient. This high variability associated with the urgency of the situation (pulmonary embolism) or severity of side effects of chemotherapy (solid tumors), is still a critical challenge and should be each time adapted to each patient. The association of contrast agent and drug in the same career can allow the caregivers a quick evaluation of the drug distribution in the body, and therefore of the efficacy of the treatment adapted to each patient, before the first clinical signs.

CONCLUSION

This study presents for the first time the formulation of iodinated polymeric nanocapsules as non-toxic and efficient contrast agent for preclinical micro-CT imaging. This contrast agent is a suspension of radiopaque nanocapsules composed of commercial iodinated oil (Lipiodol Ultrafluid®) encapsulated in a polymeric shell of PCL-mPEG diblock co-polymer (M 11,400 / 5,000 Da), formulated by emulsion–solvent diffusion method. The strength of this new contrast medium lies on its biocompatibility, its low blood toxicity, and its high iodine encapsulation rate giving concentration (of iodine) in the suspension about 70 mgI/mL, that is to say 1.3 times more loaded than commercial reference of preclinical CT imaging (Fenestra®). *In vivo*, theses polymeric nanocapsules did not show any sequestration in spleen and lymph nodes confirming the efficient stealth properties of theme nano-objects, conferred by the PEG decoration at their surface. Half-life of enhanced contrast in blood of was measured at 4.2+/−0.5 h after injection of PCL-mPEG nanocapsules containing Lipiodol® . By comparing the observed pharmacokinetics post-injection with those of free iodide and Lipiodol® emulsions, we show that there was no release of iodide and that iodinated oil release progressively from 3 h after nanocapsules administration. These capsules were excreted only in liver and release Lipiodol was eliminated by kidneys and liver. Next steps will be the evaluation of the possible co-encapsulation of lipophilic drugs for the development of new tools for personalized predictive therapy.

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