Phosphatidylcholine-Coated Iron Oxide Nanomicelles for In Vivo Prolonged Circulation Time with an Antibiofouling Protein Corona

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Abstract

We report the synthesis of micellar phosphatidylcholine-coated superparamagnetic iron oxide nanoparticles as a new long circulation contrast agents for magnetic resonance imaging. Oleic acid-coated Fe3O4 nanoparticles were first prepared through thermal degradation and then encapsulated into small clusters with a phosphatidylcholine coating to obtain hydrophilic nanomicelles. A thorough characterization confirmed the chemical nature of the coating and the excellent colloidal stability of these nanomicelles in aqueous media. Magnetization and relaxivity properties proved their suitability as magnetic resonance imaging (MRI) contrast agent and in vitro cell viability data showed low toxicity. Vascular lifetime and elimination kinetics in the liver were assessed by blood relaxometry and by in vivo MRI in rats and compared with “control” particles prepared with a polyethylene glycol derivative. These micellar particles had a lifetime in blood of more than 10 h, much longer than the control nanoparticles (2 h), which is remarkable considering that the coating molecule is a small biocompatible zwitterionic phospholipid. The protein corona was characterized after incubation with rat serum at different times by highthroughput proteomics, showing a higher proportion of bound apolipoproteins and other dysopsonins for the phosphatidylcholine particles. The antibiofouling properties of this corona and its resistance to the adsorption of proteins corroborate the observed enhanced stability and prolonged systemic circulation.

Introduction

The development of nanotechnology has led to major medical advances, particularly in diagnostic-based imaging and drug and gene delivery.[1] Among other nanomaterials, superparamagnetic iron oxide nanoparticles (SPION) are widely used for preclinical and clinical applications, especially as contrast agents in magnetic resonance imaging MRI).[2] One of the key challenges in the development of SPION for biomedical applications is their functionalization to ensure good circulating and targeting properties in vivo.[3, 4] High-temperature decomposition of organic iron precursors in organic solvents yield the best SPION in terms of size, size dispersion, crystallinity, and reproducibility of the synthesis.[3] However, this method affords SPION that are only stable in organic solvents, therefore they require a second step to make them stable in aqueous media suitable for in vivo applications. The main approaches used for this are direct chemical modification of the oleic acid structure, ligand exchange, and stabilization within a hydrophilic

coating matrix.[5–7] These hydrophilic coatings should confer aqueous stability, good biocompatibility and hinder SPION from the reticuloendothelial system (RES).[8] Small zwitterion molecules were proposed as an alternative coating with antibiofouling properties. However, they are generally anchored or covalently conjugated to the surface of other type of inorganic NPs[9–11] and few studies have reported SPION stabilization within micelles made of only small molecules,[12, 13] mainly because encapsulation of inorganic NPs inside such micelles is a chemosynthetic challenge. Indeed, the hydrophilic/hydrophobic balance of the coating is crucial for the formation of hydrophobic SPION-encapsulated micelles, a requirement that often makes small amphiphilic molecules unsuitable, leading to unstable structures that form aggregates, or fail to incorporate the SPION cargo.[14] Consequently micelles formed from amphiphilic polymers whose hydrophilic part often includes polyethylene glycol (PEG) chains, like diblock copolymers or lipid-derived polymers,[14–16] are often preferred for easier synthesis, good drug-delivery properties, and long circulating lifetimes as antibiofouling coatings.[17]

We report here oleic acid (OA)-Fe3O4 NPs encapsulated into nanomicelles of small phosphatidylcholine (PC) molecule by a nanoemulsion method and we investigate whether physicochemical properties, in vivo behavior, and vascular circulating times comparable to micelles of PEG coating can be obtained. We selected zwitterionic PC because it plays important biological roles as the major component of mammalian cell membrane. Also, its phospholipid structure allows easy formation of a micelle over the OA of the hydrophobic Fe3O4 NPs as shown by its usual inclusion in amphiphilic nanostructures like liposomes.[18] For comparison, we selected the PEG derivative polysorbate 80 (P80), a polymeric coating known by its blood pool properties.[17] We estimated the vascular life time of the both probes and studied the rate of PC SPION elimination in the liver by MRI in rats. The biological fate and uptake of NPs by the RES is strongly influenced by the composition of the protein corona, being specific to each nanomaterial.[19, 20] It is now an ongoing issue to understand better how the SPION synthetic identity influence the composition of the corona to improve the biological fate of the probes.[21] To investigate the correlations between the difference in the vascular lifetimes obtained with the PEG and PC SPION with their protein corona, we resolved by proteomic analysis, the composition of the corona of the both nanomicelles incubated in vitro with rat serum.

Results and Discussion

Synthesis and physicochemical characterization of the PC SPION

Oleic acid-coated magnetite OA Fe_3O_4 NPs were synthesized by decomposition of iron(III) acetylacetonate [$Fe(acac)_3$] at high temperature in an oleylamine (OM)–OA mixture.[22] NPs were uniform, with a polydispersity index (PDI) of 0.24, a hydrodynamic size of (103) nm and a core diameter of (72) nm. Transmission electron microscopy (TEM) confirmed the particles to be spherical and well dispersed (the Supporting Information, Figure S1).[23] These hydrophobic SPION were then stabilized within a micelle composed of the amphiphilic molecule PC. We used a nanoemulsion method that involved mixing a small volume of the OA Fe_3O_4 NPs in n-hexane within a larger volume of aqueous phosphate buffer containing PC. Use of hexane encourages the spontaneous formation of a PC monolayer; attempts with other solvents such as $CHCl_3$ were unsuccessful.[24] Under sonication and stirring, an oil-in-water emulsion forms that converts progressively into a single aqueous solution after hexane evaporation.[25] In this process, the hydrophobic fatty acid PC tails surround the OA aliphatic chain of the OA Fe_3O_4 NPs through hydrophobic interactions, whereas the hydrophilic choline heads line up around the outer surface providing water-dispersibility (Scheme 1).

Successful formation of hydrophilic nanomicelles was first indicated by the non-redispersion of PC SPION after mixing with hexane (Figure 1a, inset). The nanomicelles had hydrodynamic size of 74.9 nm with a polydispersity index (PDI) of 0.14, which show that the method yields a very homogeneous dispersion (Figure 1a). The difference in hydrodynamic size between OA Fe_3O_4 NPs precursors and the PC SPION is attributable to the formation of micelles containing several OA Fe_3O_4 NPs packed together, confirmed in TEM images showing OA Fe_3O_4 NPs in small assemblies (Figure 1b). A large excess of PC compared with OA NPs was used to favor the formation of stable micelles of small size, and a lower proportion of PC renders large micelles less stable in water. High-resolution TEM showed the lattice fringes on the Fe_3O_4 cores, demonstrating excellent crystallinity (Figure 1b).[4] To evaluate the colloidal stability of the nanomicelles, we measured zeta potential (z) versus pH. PC is a zwitterionic molecule, with a negative charge due to the glycerophosphate group and a positive charge due to the trimethylethanolammoniun group. Accordingly, the measured value of z (Figure 1c) predicts excellent stability of PC SPION over the whole range of pH below and above pH 5, at which the micelles surface has no net electrical charge.

Such good colloidal properties are confirmed in literature concerning the zwitterionic coatings.[11] At physiological pH we obtained a negative potential of 11.5 mV. This moderate value indicates that, in combination with repulsive electrostatic interactions, steric repulsion is also playing a role in the stabilization of the micellar preparation.in combination with repulsive electrostatic interactions, thanks to the bulky "head" and conical geometry of PC. The nanomicelles also indeed showed excellent stability in the commonly used culture media DMEM and RPMI, and even in PBS 10, with no increase in hydrodynamic size over time, even in 10PBS (Figure 1d). The composition of PC SPION was fully characterized by infrared spectroscopy (FTIR), thermogravimetric analysis (TGA) and mass spectrometry (MS) (the Supporting Information, Figure S2). PC SPION showed the characteristic absorption spectrum of PC, with bands at 2920 (n_a CH), 2850 (n_s CH) and 1700 cm^1 (n_s C=O) for the fatty acid chains, and at 1150 (n P=O) and 1000 cm^1 (n NC) for the choline head. TGA displayed that PC coating represented representing less than 10% of the weight of the micelles was a monolayer. The mass spectrum of the PC SPION organic coating (diluted in MeOH/H_2O and analyzed in an acidic mixture to promote ionization, with m/z=760) was representative of the expected PC adducts.

Magnetic properties of the PC SPION as MRI contrast agent

The PC SPION maintained a superparamagnetic behavior with a saturation magnetization value of 60 $emug^1$, in the same order as the hydrophobic OA Fe_3O_4 NPs precursors (70 $emug^1$; Figure 2a), which illustrates that there was low surface oxidation of the iron core during the nanoemulsion procedure. The good superparamagnetic behavior of PC SPION is mostly a consequence of the thermal preparation step, which provides highly crystalline iron oxide cores. To assess the efficacy of the nanomicelles as contrast agents for T_2weigthed MRI, NMR relaxometric properties were investigated. The longitudinal (R_1) and transverse (R_2) relaxation rates were measured as a function of the iron concentration for a set of diluted PC SPION (Figure 2b). Longitudinal (r_1) and transverse (r_2) relaxivities were calculated from the slope of the linear regression, yielding values of 1.3 and 147.4 s^1 mm^1, respectively. The very low r_1 value is due to the reduced accessibility of water and limited influence of the SPION in the middle of the cluster.[26,27] The major factors contributing to the high r_2 value are the size and crystallinity of the iron oxide core. Cluster size has also a strong influence on r_2, with an optimum value of 80 nm, precisely in the range of our nanomicelles.[28]

Cytotoxic effects

The potential toxicity of PC SPION was evaluated by incubation with C57BL/6 mouse embryonic fibroblasts (MEFs) over 72 h. Iron-uptake analysis confirmed that the MEFs effectively internalized the nanomicelles in a time- and concentration-dependent manner (Figure 3a). Cell growth and viability analysis (propidium iodide staining of necrotic cells) showed low toxicity for the 40 $mgmL^{1}$ dose (Figure 3b). Detailed cytometry analysis confirmed that a small proportion of MEFs exposed to PC SPION underwent apoptosis (the Supporting Information, Figure S3) possibly caused by the high internalization of PC SPION inside the cells. These results were confirmed by cell proliferation experiments (Figure 3c.), which showed a slight inhibition of cell population growth in the presence of 40 $mgmL^{1}$ PC SPION at 48 h (at 72 h, stabilization of population growth of control cells reflected cell confluence) These toxicity data suggest that low-doses of PC SPION can be safely used in vivo as a T_2-MRI contrast agent. Low cytotoxicity for PC SPION could be anticipated because PC (a component of lecithin) is nontoxic and extensively used in a wide range of applications.[29] This low toxicity is also indicative of the robustness of the micellar assembly, since liberation of hydrophobic OA Fe_3O_4 NPs would have toxic effects.

Circulation lifetime and in vivo MRI of PC SPION

Vascular lifetime after intravenous administration (i.v.a) of PC SPION was first estimated by T_2 relaxometry of rat blood samples. After i.v.a of the probe, blood aliquots were collected at different times post-injection and their transverse T_2 relaxation times measured (Figure 4a). T_2 shortening below basal levels is an index of the presence of the iron-containing nanomicelles in the blood aliquots. The analysis showed extended circulation of injected PC SPION for about 10 h. This circulation time was complemented by measuring the clearance rate in rat liver from the loss of the MRI signal in this organ. The negative signal enhancement in the liver was measured at different times after i.v.a. by averaging signal intensities from a selected region of interest and normalizing to the basal image (Figure 4b and c). We observed a signal decrease reaching a minimum 19 h after injection. This circulation time is significantly longer than those traditionally obtained by SPION designed with coatings such as PEG that support prolonged circulation in blood; thus even the best pegylated SPION candidates (including polymeric micelles, liposomes, or lipoplexes) have a vascular lifetime in rats of only a few hours.[30–32] Longer circulating times are achieved with ultrasmall superparamagnetic iron oxide NPs (<50 nm), which have reported lifetimes of dozens of hours, within the range detected here.[33] To confirm the performance of PC SPION we therefore

compared them with nanomicelles prepared by the same nanoemulsion method but coated with the PEG-derivative P80, a large nonionic molecule with a lipophilic OA moiety attached to PEG polyether groups.[34] The main physicochemical properties of P80 SPION are compared with PC SPION in Table 1, and detailed characteristics P80 SPION are presented in Figure S4 (the Supporting Information). These control nanomicelles had a hydrodynamic size of 25 nm, an almost neutral charge at physiological pH, and magnetic properties of the same order as the PC SPION. However despite their smaller size, the vascular lifetime of P80 SPION in rats after i.v.a., determined by the relaxometric technique, was around 2 h only. The PC coating thus provides micellar SPION with properties that ensure a signifiand this may contribute to the long circulation time of the PC SPION.[32,36]

To characterize the composition of the hard protein coronas (i.e., strongly adsorbed proteins) of PC and P80 SPION, we incubated in vitro the nanomicelles in rat serum for 15, 90, and 180 min, isolated the NP-protein complexes, and analyzed them by using high-throughput liquid chromatography (LC)MS. The incubation times were chosen in accordance with the estimated vascular lifetime of the probes and based on representative changes found in the literature.[37] The sensitive analysis used allowed us to identify 300 proteins in each corona, with a false discovery rate (FDR) below 1% (results of the proteomics characterization, Appendix A in the Supporting Information). Bioinformatic analysis showed that the identified proteins are representative of pathways related to inflammation and immune system, among others.

The corona compositions of the two SPION were qualitatively similar at all three incubation times (Figure 5), which is expected since both SPION are micellar structures. Proteins exclusive to the corona of one micellar SPION type were generally detected in lower abundance, with a spectral peptide count (SPC)<4 (Appendix A in the Supporting Information). These lower-abundance proteins are unlikely to have a significant influence for the in vivo behavior of the micelles. For semi-quantitative analysis, the percentage in weight of each coronal protein was estimated on the assumption that the number of peptides identified per protein is roughly proportional to its concentration after normalization.[38] Proteins with reported fouling (opsonins) and antifouling (dysopsonins) properties were grouped and classed according to their biological function in the circulatory system. Both types of micellar SPION showed a decrease over time in the relative amount of dysopsonins in the corona (Figure 6), which is consistent with a progressive opsonization process. However, at each time point, PC SPION bound more dysopsonins than P80 SPION. In

contrast, opsonin levels were lower in the PC corona at the 15 and 90 min incubation times. Thus, the in vivo opsonization shall be slower and less pronounced for a longer circulating time to the PC SPION. Similar studies also concluded that a strong representation of dysopsonins in the corona of NPs favor their longer vascular circulation time.[20,31,32]

For further details, we listed the key proteins that underlie these differences in the biological fates; looking among the abundant coronal proteins (relative proportion in weight >0.6%), the proteins present at an incubation time of 15 min were significantly more abundant (threshold set >1.25 fold) in the PC SPION corona than in the P80 SPION corona. Most of these proteins have antifouling properties, many of them being apolipoproteins or regulators of the complement immune system (the Supporting Information, Table S1a) and are also reported to interact with phospholipids. Many of the proteins of this set specific to PC SPION were also among the ones that underwent significant decrease in their coronal abundance from 15 to 180 min (relative fold threshold set <0.8, the Supporting Information, Table S2), which confirms the important role of these proteins in postponing opsonization. We also observed that the amounts of the dysopsonin apolipoprotein B100 (the most abundant protein in all the coronas) decreased sharply over time in the P80 SPION corona, but remained stable in the PC one, thus ensuring a more sustained antibiofouling behavior for PC SPION (Figure 6). Moreover, at an incubation time of 180 min, the listed key proteins that were bound in a significantly higher proportion to the P80 SPION (threshold >1.25, the Supporting Information, Table S1b) have reported biofouling roles, particularly in relation to vesicular transport and endocytosis promoting a faster and stronger opsonization. In this case, this set was not correlated with the profile of proteins that significantly increased their coronal abundance over time (Appendix A in the Supporting Information, similar for the two SPION, majority of the proteins belongs to the acute phase group: myosin9, clathrin, vault protein.). The proteomics analysis, with caution due to the in vitro aspect of the experiments, thus provides evidence that 1) dysopsonins/lipoproteins have a strong representation in the PC SPION corona, because of a special affinity for the coating and 2) protect these nanomicelles from opsonization, which 3) follow a similar pattern than P80 SPION but delayed and less pronounced. The nature of the coating is the important parameter determining the larger antibiofouling feature of the PC SPION corona. Literature reports that zwiterionic molecule coatings increase surface packing density of non-fouling groups, preventing ion pairings between NP surface and proteins, enhancing hydration or promoting steric repulsion with a monolayer type protein coverage instead of a 3D one in case of flexible polymers.[32,36,39]

Conclusion

A popular method for aqueous stabilization of OA Fe_3O_4 NPs prepared by thermal decomposition is the use of organic micelles formed through intercalation of amphiphilic polymers or loading in polymeric micelles. Nanoemulsion is an easy and convenient synthetic method that allows precise control of the micelles. Here, we developed an alternative probe for T_2 MRI by encapsulating OA Fe_3O_4 NPs into nanomicelles composed of the small zwitterionic molecule PC. The PC SPION are easily and reproducibly prepared, and their final hydrodynamic size of 80 nm (PDI 0.14) is mostly composed of small clusters of the encapsulated OA Fe_3O_4 NPs. The PC SPION have equivalent or superior physicochemical, colloidal and magnetic properties than most of the reported micellar-stabilized SPION micelles with organic polymers. Above all, the PC SPION have a prolonged circulation time in blood (>10 h in rats), which was reflected by the composition of the protein corona with high affinity of a set of dysopsonins for the PC coating and resistance against the adsorption of nonspecific proteins (opsonization). Keeping NPs in the circulation, together with the EPR effect, for example, accumulation in tumors or targeting other diseases in which the endothelium becomes leaky are promoted.

Hence, these nanomicelles are promising contrast agents for preclinical and clinical in vivo MRI. Moreover, small hydrophobic drugs or molecular imaging probes can be easily encapsulated in the nanomicelles together with the OA Fe_3O_4 NPs leading to potential candidates for a multimodal drug-delivery platform.

Experimental Section

Materials

All chemicals for the preparation of the nanomicelles were purchased from Sigma–Aldrich Co. (St. Louis, USA). All reagents were of analytical grade except for the phosphatidylcholine (90%) and were used without any further purification. Distilled water (milliQ) or phosphate buffered saline PBS were used throughout the experiments.

Synthesis

Oleic-acid-coated superparamagnetic iron oxide NPs, OA Fe_3O_4 NPs: The OA Fe_3O_4 NPs were synthesized by using iron acetylacetonate as precursor and phenyl ether as the solvent. A mixture of [$Fe(acac)_3$] (0.71 g, 2 mmol), 1,2-hexadecanediol (2.38 g, 10 mmol), oleic acid (1.69 g, 6 mmol), oleylamine (1.60 g, 6 mmol), and phenyl ether (20 mL) were added to a three-neck flask. The reaction mixture was heated under mechanical stirring and a flow of nitrogen gas up to a temperature of 2008C. This temperature was maintained for 120 min and the solution was then heated under reflux at 2548C for 30 min with a nitrogen balloon on the top of the condenser. Subsequently, the solution was cooled to room temperature. To remove side products, ethanol was added to the reaction mixture and the resulting solution was centrifuged at 8500 rpm for 10 min. The supernatant was decanted, hexane (20 mL) and oleic acid (0.05 mL) were added to the NPs, and the suspension was centrifuged at 8500 rpm to remove aggregates and obtain a stable suspension.

Micellar phosphatidylcholine-coated superparamagnetic iron oxide nanoparticles (PC SPION): Phosphatidylcholine (60 mg, 0.078 mmol) was first dispersed in 15 mL of PBS (pH 7.2, 5 mm). A 1 mL aliquot of OA Fe_3O_4 NPs (10 mg $FemL^{1}$) dispersed in hexane was then added to the solution and the resulting mixture was sonicated (Branson 250, 42 +/6 KHz) under robust stirring for 20 min at 378C. The oil in water (o/w) nanoemulsion was kept under sonication for a further 1 h to evaporate all traces of hexane, resulting in the formation of a homogenous aqueous solution. Aggregates were removed by filtration (0.22 mm, MILIPORE, SterivexGP) and excess phosphatidylcholine was removed by gel filtration in a PD-10 column (GE Healthcare).

Micellar polysorbate 80-coated superparamagnetic iron oxide nanoparticles (P80 SPION): Polysorbate 80 (150 mg, 0.11 mmol) was first dispersed in PBS (12 mL, pH 7.2, 5 mm). A 1 mL

aliquot of OA Fe_3O_4 NPs (10 mg $FemL^{1}$) dispersed in hexane was then added to the solution and the resulting mixture was sonicated (Branson 250, 42 +/6 KHz) under robust stirring for 20 min at 378C. The oil in water (o/w) nanoemulsion was kept under sonication for a further 1 h to evaporate all traces of hexane, resulting in the formation of a homogenous aqueous solution. Aggregates were removed by filtration (0.22 mm, MILIPORE, Sterivex-GP) and excess polysorbate 80 was removed by gel filtration in a PD-10 column (GE Healthcare).[34]

Physicochemical characterization of the NPs

The hydrodynamic size, polydispersity index and zeta potential of the nanomicelles were measured with a Zetasizer Nano ZS90 (Malvern Instruments, UK) using folded capillary cells. Morphology and core size were determined using a 200 keV JEOL-2000 FXII transmission microscope (Jeol Ltd. Japan) at the National Center of Electron Microscopy of the University Complutense of Madrid. For the preparation of the sample, a drop of a dilute magnetic nanoparticle suspension was placed on a carbon-coated copper grid and the solvent allowed to evaporate at room temperature (RT) for 24 h. Fourier transform infrared spectroscopy (FTIR) spectra were obtained on a Perkin–Elmer Spectrum 400 Series spectrometer (Perkin–Elmer, USA); each spectrum was obtained by averaging 32 interferograms with a resolution of 1 cm^{1}. Thermogravimetric analysis (TGA) spectra were obtained with a Seiko TG/ATD 320 U, SSC 5200 (Seiko Instruments, Japan) at the Institute of Materials Science of the University Autnoma of Madrid. The dried PC SPION were heated from 208C to 10008C at 108Cmin^{1} under an air flow of 100 mLmin^{1}. Mass spectrometry was performed with in a Bruker Esquire 3000 apparatus (Bruker Daltonik, Germany) equipped with an ESI source and an ion trap analyzer, coupled to an Agilent 1100 capillary LC system (Agilent Technologies, USA). The sample was diluted 1:10 in water/methanol (1:1) before the LC/MS analysis. The analysis were carried out by FIA (flow injection analysis), working in both polarities, using a 0.1% formic acid/ methanol (50:50) mix as the mobile phase to promote ionization, at 0.1mLmin^{1}.

Magnetic characterization

Magnetic characterization of the samples was carried out in a vibrating sample magnetometer using 100 mL of solution in a special sample holder. Magnetization curves were recorded at room temperature by first saturating the sample in a field of 1 T. The magnetization values were normalized to the amount of iron to yield the specific magnetization (emug^{1} Fe). The initial susceptibility of the suspensions was measured in the field range 100 Oe, and the saturation

magnetization values (Ms) were evaluated by extrapolating to infinite field the experimental results obtained in the high field range in which the magnetization linearly increases with 1/H. For determination of the NMR relaxometric values, the T2 and T1 relaxation times were measured in a Bruker MQ60 (Bruker Biospin, Germany) with a T2 cp and T1 ir mb sequences. The relaxation rate R_i values ($1/T_i$, s^1, i=1, 2), obtained from the measured relaxation times (T_i, s) were corrected by subtracting the water relaxation rate in the absence of the contrast agent. Linear fitting of the data gives straight lines whose slopes are the relaxivities (r_i, $s^1\,mm^1$) related to the iron concentration (mm): $R_i = R_{bi} + r_i\,[Fe]$.

Cell toxicology

Cell lines and media: C57BL/6 mouse embryonic fibroblasts (MEFs) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillinstreptomycin and 1 mm sodium pyruvate. Cytotoxicity and iron uptake were assessed in MEFs exposed to PC SPION at different Fe concentrations (10 or 40 $mgmL^1$) and times of incubation (24, 48, and 72 h). Control cells were treated with vehicle (water).

Cytotoxicity assays: In the presence of Ca^{2+}, annexin V binds to phosphatidylserine residues exposed on the outer surface of the plasma membrane of apoptotic cells. We collected 106 cells in PBS (500 mL) and washed them. Cells were pelleted and resuspended in 195 mL binding buffer (10 mm HEPES/NaOH, pH 7.4; 140 mm NaCl;

2.5 mm $CaCl_2$). APC-Annexin V (BD PharmingenTM) was added (5 mL) and cells were incubated for 15 min at 258C in the dark. The viability marker propidium iodide was then added to a final concentration of 0.001% (w/v), and cells were analyzed by flow-cytometry using the BD FACSCanto II system. All experiments were performed in triplicate. As a result, viable cells are negative for both APC Annexin V and PI; early apoptotic cells are APC Annexin V positive and PI negative; and late apoptotic and dead cells are both APC Annexin V and PI positive. Cytotoxicity was estimated by comparing the proportion of viable cells in populations exposed to the PC SPION with that in control cells.

Iron-uptake quantification

Approximately 1106 treated cells were lysed with 300 mL lysis buffer (50 mm NaCl, 50 mm TrisCl pH 8, 0.2% SDS) for 3 h at 558C and mixed with the same volume of 10 mm HCl. Then, 150 mL

1.4m HCl and 150 mL 4.5% KMnO4 were added. After 2 h at 608C, 90 mL of a detection solution containing 6.5 mm ferrozine, 6.5 mm neocuproine, 2.5m ammonium acetate and 1m ascorbic acid was added. The absorbance at 550 nm was measured after a further 30 min. The concentration of internalized Fe was calculated from a standard curve of $FeCl_3$ (0 to 300 mm).

Vascular circulating times and clearance

All animal experiments conducted in this work were approved by the ethics and animal welfare committee at CNIC and were developed according to the Spanish and UE legislation.

Blood relaxometry

Rats (n=2, Wistar male, 6 weeks old) were anesthetized with 2% isoflurane in a mixture of N_2/O_2 (80:20). The baseline blood sample (200 mL) was collected through the tail vein before administration of PC SPION (0.15 $mgkg^1$). Blood samples (200 mL) were collected at intervals from 5 min to 24 h. T2 relaxation times of the samples were measured in a Bruker Biospec spectrometer with a T2 Carr– Purcell–Meiboom–Gill sequence (Bruker Biospec 47/40, 1.5 T, Bruker Biospin, Germany) and plotted against the T2 of the baseline blood sample.

Evaluation of nanomicelles uptake in liver by MRI

Rats (n=2, Wistar male, 6 weeks old) were anesthetized with 2% isoflurane in a mixture of N_2/O_2 (80:20). Baseline images were acquired before intravenous administration of PC SPION (0.15 $mgkg^1$). MR images of rat liver were acquired at intervals from 5 min up to a few hours after injection. The images were acquired with a 7 T Agilent/Varian 7T DD1 spectrometer (Agilent 2) with a 31 cm horizontal bore, using a 72 mm inner diameter quadrature birdcage volume coil (Rapid Biomedical GmBH, Germany). The rats were kept anaesthetized with the isoflurane-gas mix through a facial mask and placed prone in a customized plastic holder. Body temperature was kept constant by delivering warm air to the magnet bore, and the respiratory cycle was monitored constantly. For each animal, 8 axial 1 mm thick slices were acquired to image the liver. Images were acquired in free-breathing animals, using a gradient echo sequence with 4 ms/40 ms echo/repetition times, BW of 100 kHz, FOV of 66 cm, for a total acquisition time of about 80 seconds; the flip angle (FA) was fixed at 20 degrees.

Characterization of the protein "hard corona" of PC SPION and P80 SPION

PC SPION or P80 SPION (200 mL in PBS, 0.1 mgmL^{1} iron concentrations) were incubated in 80% rat serum (800 mL) at 378C with gently stirring at 100 rpm. After incubation for 15, 90, or 180 min, the micelle-protein corona complexes were immediately separated from the serum by centrifugation (2 h, 16000 g, 108C). Pellets were collected and redispersed in PBS before another centrifugation cycle. After three similar washing steps the micelle-protein corona complexes were processed for proteomics.

Protein digestion

Control of the sample quality was previously performed with state of art SDS polyacrylamide gel electrophoresis. The proteins of the corona bound to a fixed amount of NPs were eluted by boiling in Tris-SDS gel loading buffer containing 50 mm DTT, and loaded onto 10% SDS-polyacrylamide gels to concentrate the proteins in a single band at the stacking/separating gel interface. Briefly, after band visualization with colloidal Coomassie Brilliant blue staining, the acrylamide band was cut into 1 mm^{3} plugs for protein digestion. Gel pieces were incubated with 10 mm DTT (Sigma Aldrich) in 50 mm ammonium bicarbonate (99% purity; Sigma) for 30 min at 568C. After reduction, samples were alkylated with 55 mm iodoacetamide (Sigma Aldrich) in 50 mm ammonium bicarbonate for 20 min at RT. Gel plugs were washed with 50 mm ammonium bicarbonate in 50% acetonitrile (gradient, HPLC grade, Sigma), and dried in a Speedvac. Dry gel pieces were then embedded in sequencing grade modified porcine trypsin (Promega, Madison, WI, USA) at a final concentration of 20 ngmL^{1} (at 40:1 protein/trypsin (w/w) ratio) in 50 mm ammonium bicarbonate and 5% acetonitrile. After digestion at 378C overnight, peptides were re-extracted with 30% acetonitrile in 0.5% trifluoroacetic acid (99.5% purity; Sigma Aldrich), dried in a Speedvac, and finally desalted onto C18 OasisHLB cartridges (Waters) and dried-down.[40] The digested samples were resuspended in 10 mL Buffer A (0.1% (v/v) formic acid) for LCMS/MS separation and analysis.

Mass spectrometry data collection and analysis

Samples were analyzed by LC-MS/MS using a nano-HPLC system (EASY-nLC 1000, Thermo-Proxeon) coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Peptides were separated using an Acclaim PepMap 100 C18 nano-column (75 mm

I.D.25 cm, 2 mm particle size; Thermo Fisher Scientific) with Buffer A at a flow rate of 200 nLmin^{1}, and eluted with a linear gradient from 0–40% Buffer B (90% acetonitrile, 0.1% formic acid

(vol/vol)) for 120 min. A survey scan was performed in the Orbitrap analyzer using a mass range of m/z 390–1500, followed by data-dependent MS/MS scans of the twenty most-intense ions in profile mode. The survey scan was done at 35,000 resolution using a target value of 1,000,000 ions, 60 ms of injection time, and 1 microscan. Fragmentation was performed by CID with a 1.5 Da isolation mass width, 17,500 resolution, a target value of 50,000 ions, and 80 ms of injection time. Proteins were identified using the SEQUEST algorithm (Proteome Discoverer 1.4, Thermo Fisher Scientific). The raw MS/ MS files were searched against the rat Complete Proteome database (Uniprot at July 23th, 2013; 49,050 sequences) and a pseudoinverted version of the same database. SEQUEST searches were performed allowing optional modifications (methionine oxidation) and fixed modifications (cysteine carboxamidomethylation), 2 missed cleavages, and 600 ppm and 1.2 Dappm of mass tolerance for precursor and fragment ions, respectively. False discovery rate (FDR) was determined by the probability ratio method,[41] followed by a post-search 12 ppm precursor mass filtering and the refined FDR calculation method.[42] Only peptides with FDR below 1% and identified with at least two peptides were selected for further analysis. Statistical analysis of data were performed using QuiXoT.[43,44]

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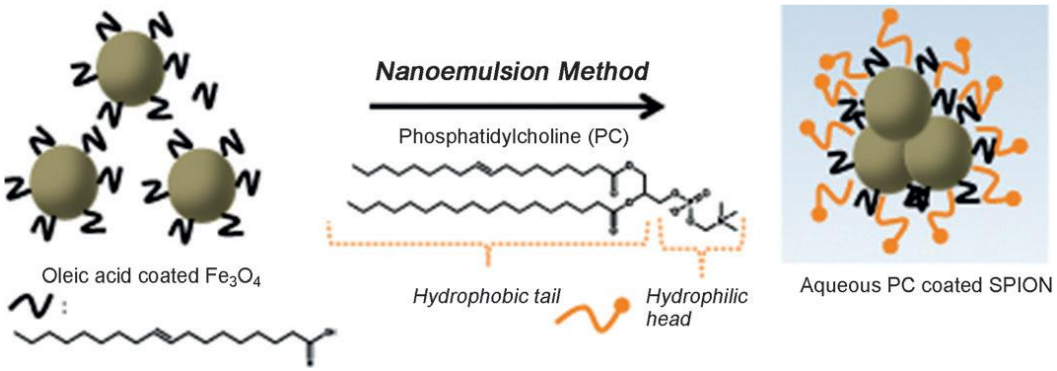
Table 1. Comparison of the physicochemical characterization of micellar PC and P80 SPION.

	PC SPION	P80 SPION
hydrodynamic size [nm]	74.9	25
PDI	0.14	0.19
zeta potential (pH 7.1) [mV]	−11.5	−4
saturation magnetization	70	65
relaxivity [s^{-1} мм$^{-1}$]	r_1: 1.3 r_2: 147.4	r_1: 2.3 r_2: 127.2
estimated vascular lifetime (relaxometric method)	10 h	2 h



Scheme 1. Formation of the PC SPION nanomicelles. Particles dissolved in hexane were added to a large amount of PC dissolved in phosphate buffer. After sonication, the organic phase is evaporated and the micelle is formed.

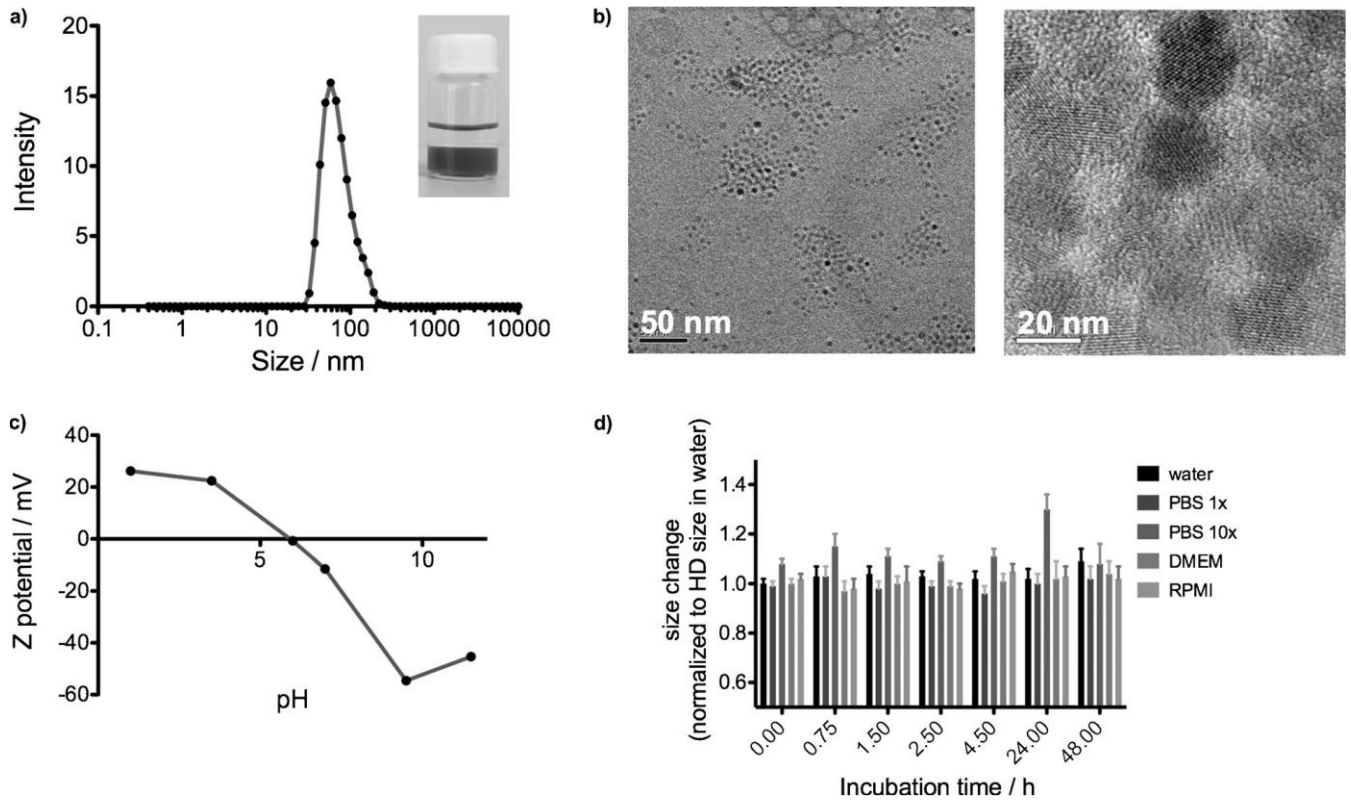


Figure 1. a) Hydrodynamic size of PC SPION. b) TEM images of these nanomicelles at two magnifications. c) Zeta potential of PC SPION as a function of pH. d) Change of hydrodynamic size of these nanomicelles with time in high ionic-strength solutions.

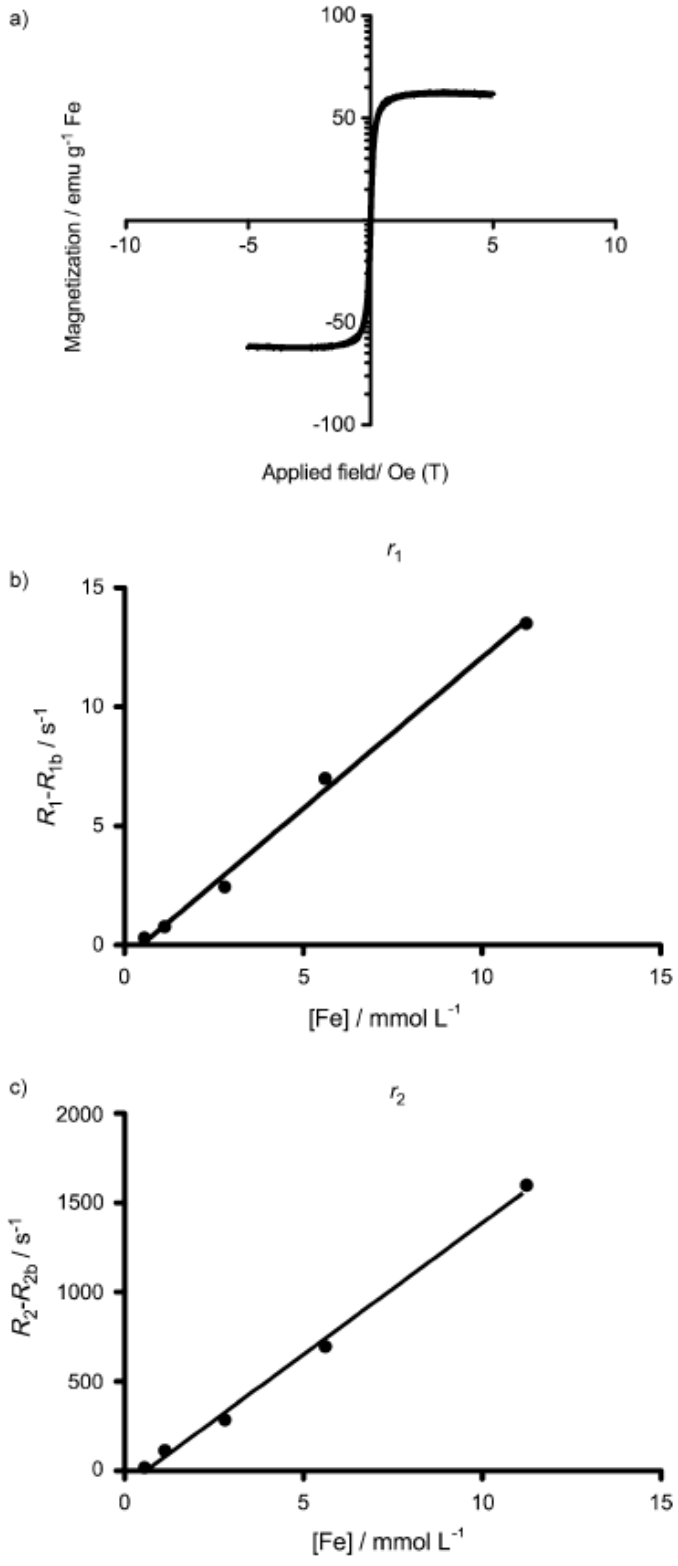


Figure 2. a) Magnetization curve at 298 K (1 Oe=10000 T) and plot of b) longitudinal (T1) and c) transverse (T2) relaxation rates against iron concentration of PC SPION.

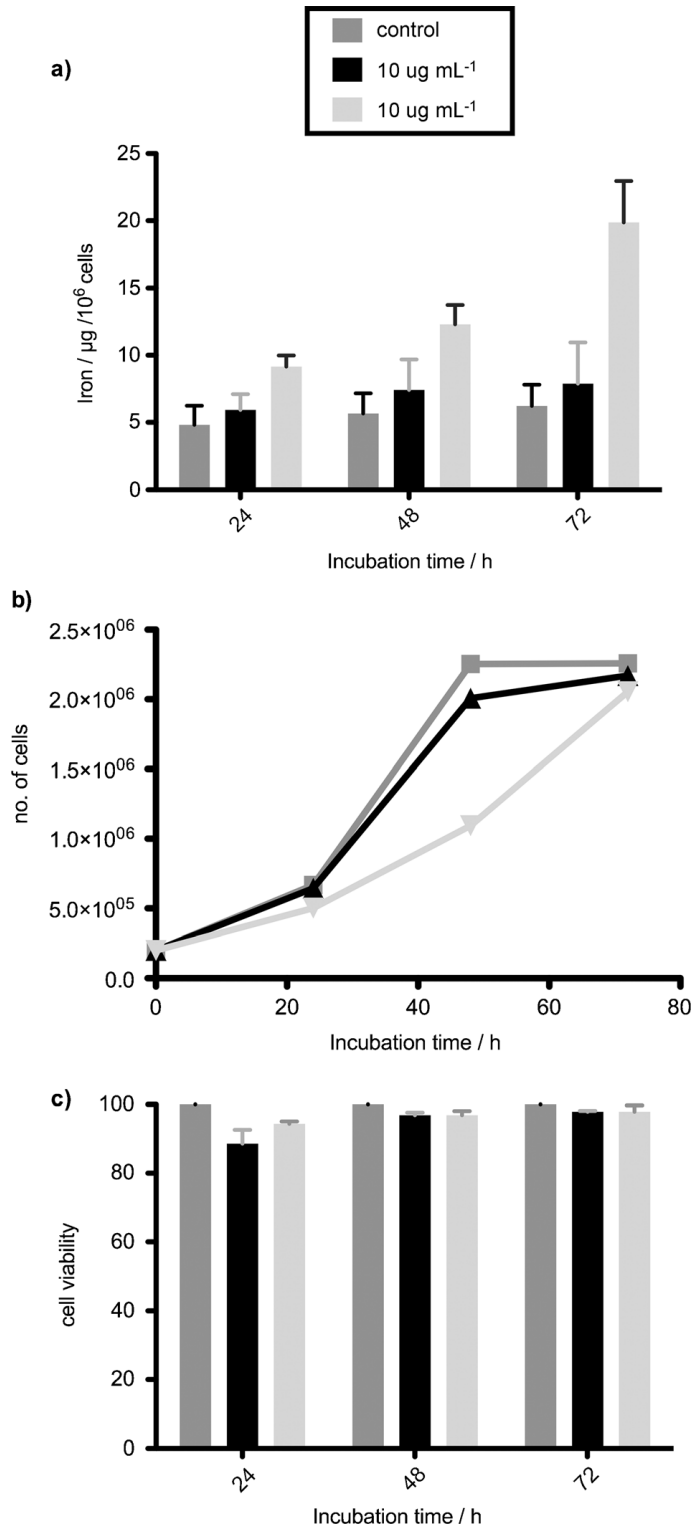


Figure 3. a) Iron uptake by MEFs incubated with PC SPION (at 10 mgmLu1 and 40 mgmL 1 iron concentrations; 24, 48, 72 h). b) Cell viability of MEFs incubated with PC SPION (at 10 and 40 mgmL 1 iron concentrations; 24, 48, 72 h). c) Number of MEFs cells after incubation with these nanomicelles (at 10 mgmL 1 and 40 mgmL 1 iron concentrations; 24, 48, 72 h).

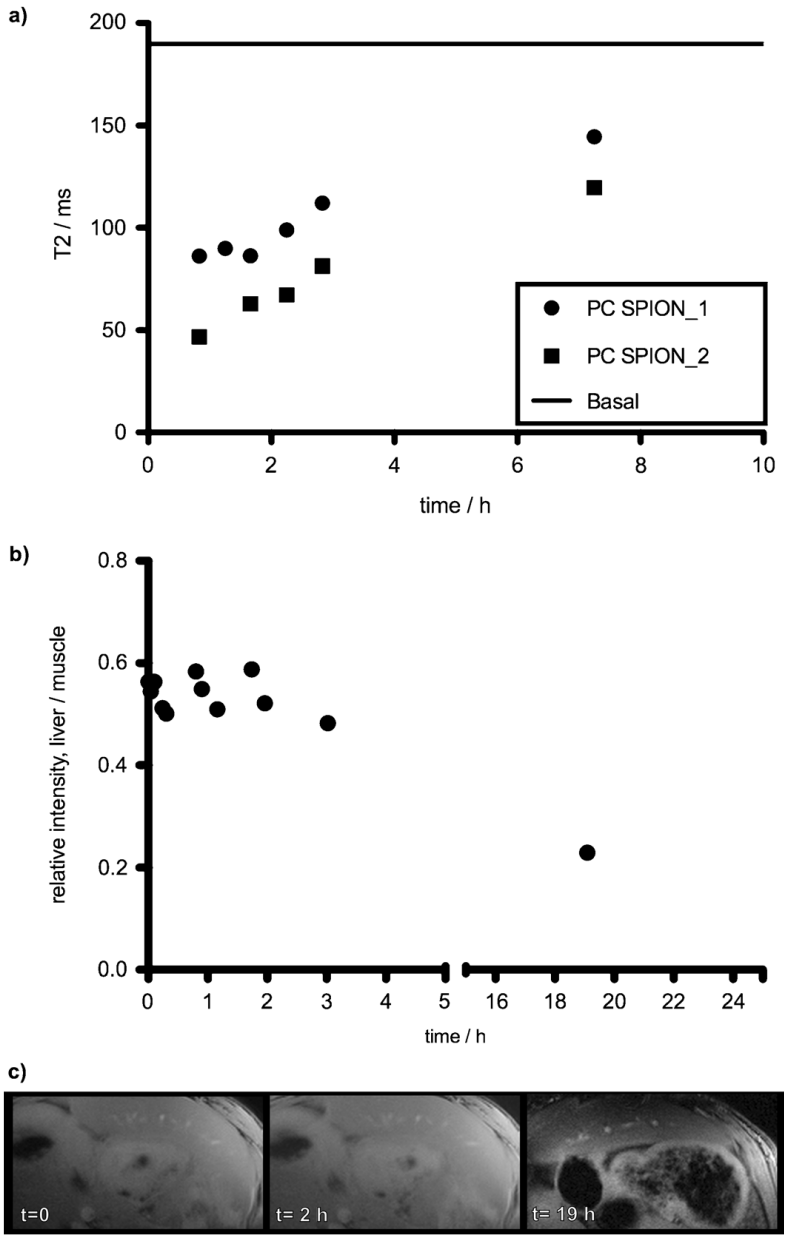


Figure 4. a) T2 relaxation time of rat blood samples after i.v.a. of PC SPION plotted over time relative to the T2 blood baseline value (black line). b) Negative signal enhancement for a set of region of interest (ROI) in rat liver. c) Liver MR images after i.v.a. of PC SPION.

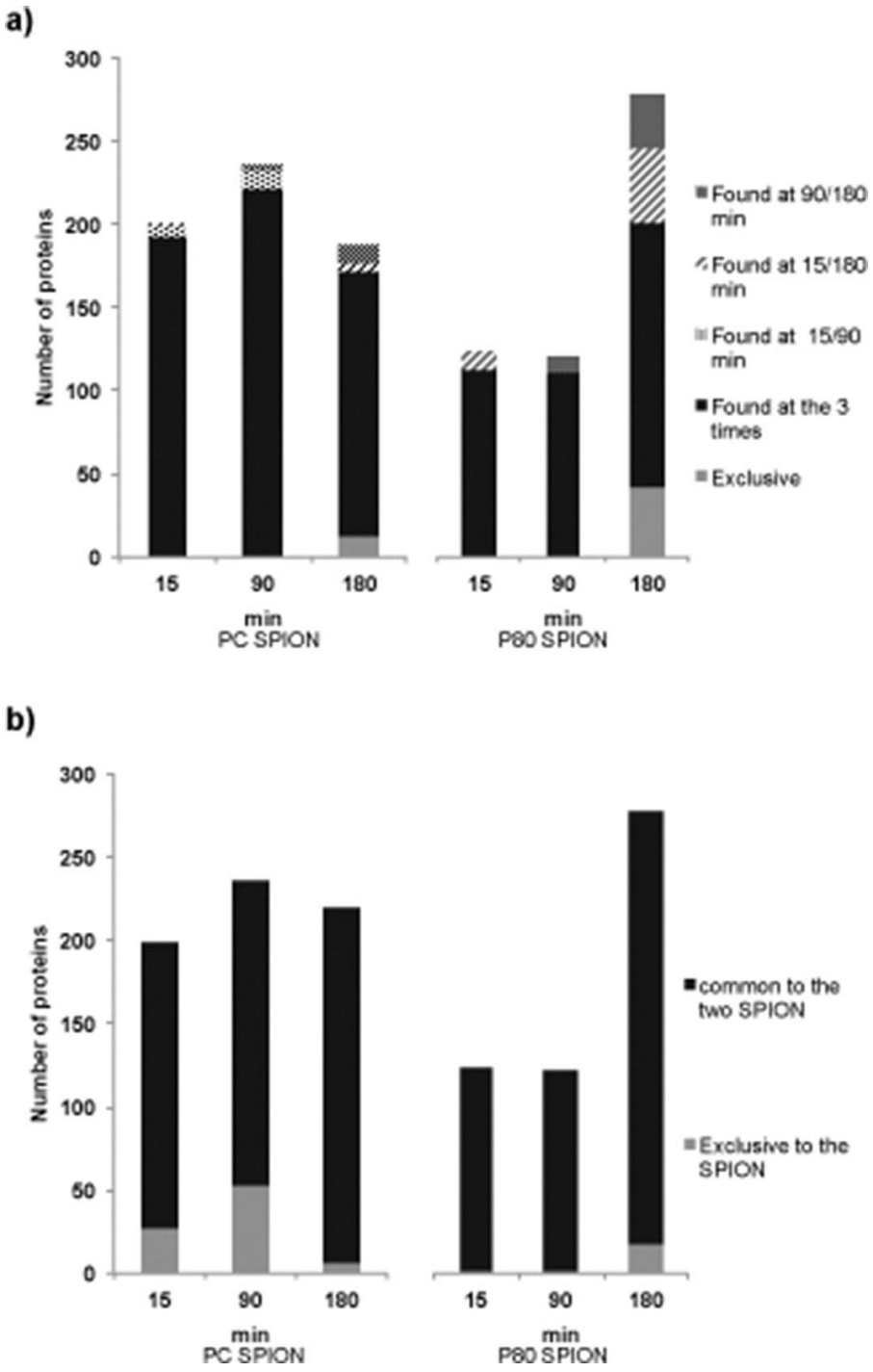


Figure 5. a) Qualitative comparison of protein coronas over time (15, 90, and 180 min) for the PC and P80 SPION. The charts show the numbers of same proteins (SPC>2, FDR>1%) present in the coronas at the three times of incubation, at two times of incubation, and exclusive to one time of incubation. b) Qualitative comparison of PC and P80 SPION coronas at each time. The charts show the numbers of same proteins common to both nanomicelles types or exclusive to one (SPC>2, FDR>1%).

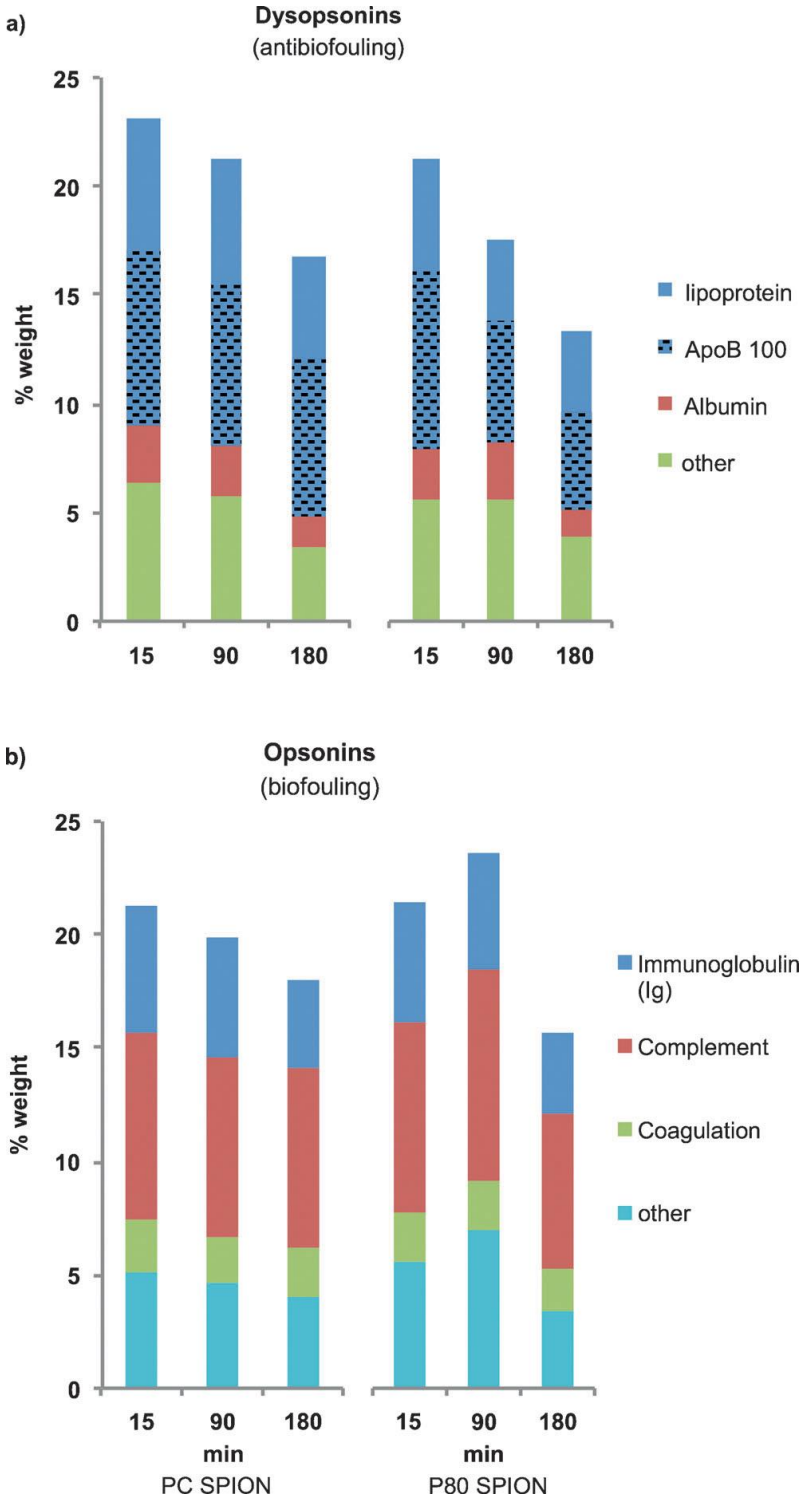


Figure 6. Relative% weight of the proteins with known a) antibiofouling properties (dysopsonins) and b) biofouling properties (opsonins) classified by their biological function in the coronas of micellar PC and P80 SPION at 15, 90, and 180 min in vitro incubation in rat serum. Data label represent the % weight of the single Apo B 100.