A Top-Down Synthesis Route to Ultrasmall Multifunctional Gd-Based Silica Nanoparticles for Theranostic Applications


Abstract: New, ultrasmall nanoparticles with sizes below 5 nm have been obtained. These small rigid platforms (SRP) are composed of a polysiloxane matrix with DOTAGA ligands (1,4,7,10-tetraazacyclododecane-1-glutaric anhydride-4,7,10-triacetic acid)–Gd^{3+} chelates on their surface. They have been synthesised on their own original top-down process: 1) formation of a gadolinium oxide Gd_2O_3 core, 2) encapsulation in a polysiloxane shell grafted with DOTAGA ligands, 3) dissolution of the gadolinium oxide core due to chelation of Gd^{3+} by DOTAGA ligands and 4) polysiloxane fragmentation. These nanoparticles have been fully characterised using photon correlation spectroscopy (PCS), transmission electron microscopy (TEM), a superconducting quantum interference device (SQUID) and electron paramagnetic resonance (EPR) to demonstrate the dissolution of the oxide core and by inductively coupled plasma mass spectrometry (ICP-MS), mass spectrometry, fluorescence spectroscopy, 29Si solid-state NMR, 1H NMR and diffusion ordered spectroscopy (DOSY) to determine the nanoparticle composition. Relaxivity measurements gave a longitudinal relaxivity r_1 of 11.9 s^{-1} mM^{-1} per Gd at 60 MHz. Finally, potentiometric titrations showed that Gd^{3+} is strongly chelated to DOTAGA (complexation constant log b_{10} = 24.78) and cellular tests confirmed the that nanoconstructs had a very low toxicity. Moreover, SRPs are excreted from the body by renal clearance. Their efficiency as contrast agents for MRI has been proved and they are promising candidates as sensitising agents for image-guided radiotherapy.

Keywords: cancer · gadolinium · nanoparticles · silica · theranostic

Introduction

During the past decade, intense research has been carried out in the domain of multifunctional nanoparticles for theranostic applications. These nanoparticles present the opportunity to combine several different features in a single item. They can improve disease diagnosis, enable new treatments, and monitor therapeutic responses. They offer a large number of possible combinations between 1) imaging techniques such as optical imaging, scintigraphy, magnetic resonance imaging (MRI), ultrasound-based imaging and X-ray computed tomography and 2) therapeutic techniques, such as drug and gene delivery, hyperthermia, photodynamic and neutron therapy or sensitising for radiotherapy.[1,2]

Nanoparticles for theranostic applications need to be biocompatible, nontoxic, tuneable in size and suitable for efficient clearance from the body. Silica nanoparticles are one of the most popular probes described in the literature.[3] Multifunctional silica nanoparticles have been reported as contrast agents for several combinations of imaging modalities: optical imaging and positron emission tomography (PET),[7] optical imaging and MRI,[8–10] and optical imaging with both MRI and computed tomography.[11] Some studies combine imaging and therapy: MRI and gene delivery,[12] optical imaging and drug delivery,[13] and optical imaging and photodynamic therapy.[14,15]

The two most common approaches described in literature for silica particle synthesis are the Stöber method and the reverse microemulsion process. The reverse microemulsion process provides particles with sizes ranging from 10 nm to 1.5 µm with good monodispersity.[16,17] However, only low yields are obtained, and the nanoparticles prepared by this method require long purification steps to remove all the surfactants before any biological application. On the other hand, the Stöber method yields particles with sizes ranging from 10 nm to 2 µm with rather good monodispersity.[18–20] With modified Stöber-type syntheses, sizes down to 3–7 nm can be reached.[21]

Grafting functional groups on the particle’s surface always leads to a size increase. The more functionalities that are added, the bigger the particles will be. Nanoparticle size is a major parameter in toxicity concerns. First, particle size
needs to be kept below 50 nm to avoid undesirable macrophage uptake. Second, particle size also plays a key role in the elimination route from the body. Effective renal clearance, which seems to be the most reproducible and most efficient route of excretion, is achieved for very small sizes only. Silica particles with hydrodynamic diameters between 3 and 7 nm combine reasonable circulation lifetimes and efficient renal clearance from the body.[22] Similar studies with quantum dots set the upper limit for renal clearance at a hydrodynamic diameter of 5.5 nm.[23] On the other hand, larger particles accumulate in the liver and intestines, with prolonged retention in the body, and may cause toxicity concerns due to longer exposures.

To reach such small sizes while conserving multimodality, we attempted to reduce the size of nanoparticles previously described elsewhere.[24,25] These nanoparticles are made up of a gadolinium oxide core with a polysiloxane shell and are grafted with poly(ethylene glycol) (PEG) ligands. They present an average initial size ranging from 11 to 14 nm. Here, our approach was to reduce the polysiloxane shell thickness, in order to decrease the size and to increase the ratio between “active” species for imaging and therapeutic purposes (Gd) and inert matter (polysiloxane). To prevent the leakage of any Gd\(^{3+}\) ions from the oxide core, DOTAGA (1,4,7,10-tetraazacyclododecane-1-glutaric anhydride-4,7,10-triacetic acid) was grafted onto the particle surface to collect any dissolved Gd\(^{3+}\).

Indeed, free Gd\(^{3+}\) is toxic and can be responsible for nephrogenic systemic fibrosis (NSF), a systemic fibrosing disorder that principally affects the skin, but can also involve any tissue in the human body and even result in death. Cases of NSF have been reported after injection of Gd-containing contrast agents into patients, namely gadodiamide, gadopentetate or gadoversetamide, which are linear DTPA derivatives.[26] No case of NSF from macrocyclic DOTA-derivatives (DOTA = 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) has been reported to our knowledge, thanks to their improved complexation constants and slower dissociation kinetics.[27] In the present study, DOTAGA was used instead of the classical DOTA because it has five carboxylic acid groups instead of only four. Thanks to this extra group, eight coordination sites will still remain available for complexing Gd\(^{3+}\) ions after grafting onto the nanoparticle surface.

An unexpected top-down phenomenon occurred after grafting the DOTAGA; nanoparticles with a hydrodynamic...
diameter of 3 nm were obtained. This phenomenon corresponds to Gd₂O₃ core dissolution followed by chelation of Gd³⁺ ions by DOTAGA ligands and simultaneous fragmentation of the polysiloxane shell. These small rigid platforms (SRP) showed no toxicity after injection in mice and efficient renal clearance. They have proven their efficiency as contrast agents for in vivo X-ray and fluorescence imaging, scintigraphy and magnetic resonance imaging. Here, we specifically investigate the top-down process that occurs during the particle synthesis. Therefore, we characterised the SRPs using several complementary techniques. Electron spectroscopy and microscopy, photon correlation spectroscopy, electron paramagnetic resonance and magnetic susceptibility measurements were used to prove the oxide core dissolution. Mass spectrometry, ²⁹Si solid-state NMR and ¹H NMR spectroscopy and inductively coupled plasma mass spectrometry were performed to determine the nanoparticle composition. Finally, potentiometric titrations showed that no Gd³⁺ leaching occurs after chelation by DOTAGA, and cellular tests confirmed a very low toxicity of the SRPs obtained. Such promising results lead us to test our systems in in vivo therapeutic applications. After injection in gliosarcoma-bearing rats and treatment with radiotherapy, the survival of the rats was monitored in comparison with, on one hand, untreated rats and, on the other hand, rats treated with radiotherapy only.

**Results and Discussion**

**Nanoparticle synthesis**

*General description:* Gadolinium oxide cores (Figure 1a), formed according to the equation: $2\text{GdCl}_3 + 6\text{NaOH} \rightarrow \text{Gd}_2\text{O}_3 + 3\text{H}_2\text{O} + 6\text{NaCl}$, were synthesised in diethylene glycol (DEG). DEG was chosen for its high viscosity, which prevents the agglomeration of the growing nanoparticles. These cores present an average diameter of 3.5 nm, as confirmed by photon correlation spectroscopy (PCS) measurements.

Then, a polysiloxane shell was grown from these cores (Figure 1b) using hydrolysis–condensation of aminopropyl triethoxysilane (APTES) and tetraethyl orthosilicate (TEOS), leading to a global diameter of 4.5 nm, as determined by PCS measurements (Figure 2a).

The expected composition of the polysiloxane shell was $\text{Si}_1\text{O}_{1.7}\text{C}_{1.8}\text{N}_{0.6}\text{H}_{4.8}$ per Si atom, with regard to the quantities introduced. A ratio of only 2Si per Gd was chosen here to reduce the thickness of the polysiloxane shell and further improve renal elimination in medical applications (using a ratio of 6Si per Gd leads to a total size between 11 and 14 nm). The construct possesses a diameter of 4.5 nm (mean standard deviation (MSD) of 1.8 nm), corresponding to a 50% yield in the polysiloxane synthesis, if we consider a polysiloxane density of 2. The Gd₂O₃ cores encapsulated in the polysiloxane shell remain well crystallised with a sub-5 nm diameter, as revealed by the high-resolution transmis-
sion electron microscopy (HRTEM) image in Figure 2d. For instance, lattice fringes can be clearly assigned to the (222) and (400) planes with inter-reticular distances of approximately 3.1 and 2.8 Å, respectively. The fast Fourier transform of the imaged area (see inset Figure 2d) confirms the crystalline nature of the Gd$_2$O$_3$ cores, corresponding to the expected cubic crystal structure (space group $Ia3$). Finally, the polysiloxane shell displayed amine groups on its surface for further functionalisation.

In a third step, DOTAGA was grafted to the amine functional groups of the core–shell particles, while the nanoparticles were still dispersed in DEG (Figure 1c). Instead of PEG or diethylene triamine pentaacetic acid (DTPA), the DOTAGA ligands were specifically selected to permit efficient chelation of Gd$^{3+}$ in case of accidental release from the core. Moreover, thanks to their carboxylic acid groups, they make the core–shell particles hydrophilic, conferring them good colloidal stability in water and biological buffers.

Finally the particles were transferred into water, purified and freeze-dried for storage. They can be dispersed again in water before use; the hydrodynamic diameter obtained after dispersion was equal to the diameter before freeze-drying, with less than 5% difference. Colloidal stability was provided by electrostatic repulsion between negatively charged nanoparticles, as reflected by the negative value of the zeta potential, namely $-17$ mV at pH 7.4 (see Supporting Information).

**Evidence of a top-down process:** In order to optimise the nanoparticles’ colloidal stability, different amounts of DOTAGA were introduced in the particle synthesis route, ranging from 0.5 to 3 DOTAGA ligands per Gd atom. The final sizes of the obtained nanoparticles after precipitation, dispersion in water and two days purification were determined by PCS measurements (Figure 3). Surprisingly, the particle size decreased with increasing amounts of DOTAGA. For the lowest amount of DOTAGA (0.5DOTAGA per Gd), the synthesis was non-reproducible and lead to some agglomeration, most probably because the particles were not hydrophilic enough. With 0.75DOTAGA, an average size of 8 nm was obtained (Figure 2b), which is consistent with the expected core–shell structure. Indeed, similar syntheses with short PEG chains (PEG250-(COOH)$_2$) instead of DOTAGA gave hydrodynamic diameters between 6 and 8 nm. With higher DOTAGA amounts, sizes of around 3 nm (smaller than the initial Gd$_2$O$_3$ cores) were obtained, suggesting a possible new top-down mechanism. Based on HRTEM investigations, the addition of 0.75DOTAGA per Gd still preserved the crystalline nature of the Gd$_2$O$_3$ core, as observed in Figure 2e, in which (400) lattice planes can be distinguished, as well as a size of approximately 3.5 nm in diameter. On the other hand, the hybrid systems obtained with 2DOTAGA per Gd do not present any crystallinity, as shown in Figure 2f, despite high Gd concentrations detected by energy dispersive X-ray spect-

![Figure 2. PCS measurements and high-resolution TEM images of the Gd$_2$O$_3$-polysilodane core–shell particles a), d) before DOTAGA addition, b), e) after addition of 0.75 DOTAGA/Gd$_2$O$_3$, and c) f) after addition of 2DOTAGA/Gd.](image)

![Figure 3. Final particle size variation depending on the number of introduced ligands: DOTAGA, PEG and DTPA.](image)
troscopy (EDX) measurements. Apparently, the size decrease of the particles is directly correlated with the disappearance of the crystalline Gd₂O₃ core.

The same experiments were also realised by replacing the DOTAGA by DTPA, which is also a chelator for Gd³⁺, and a similar decrease in size was observed with increasing amounts of DTPA. On the other hand, when the same experiments were realised with PEG, which is not a chelator for Gd³⁺, no significant evolution in size was observed (Figure 3), and the core crystallinity was not affected (TEM images not shown). Therefore, it seems that specific chelating ligands for Gd³⁺, such as DOTAGA or DTPA, played a key role in this process.

All this experimental evidence leads to the hypothesis that, in aqueous solutions, the DOTAGA ligands strongly accelerated the Gd³⁺ ion dissolution from the Gd₂O₃ core. Since the polysiloxane layer was optimised to be rather thin, the presence of possible defects in this layer might favour the migration of Gd³⁺ out of the core and these ions being chelated by the ligands on the particle surface. The thin polysiloxane shell, bearing no more internal support, might therefore collapse on itself and break up into several fragments. Small rigid platforms (SRP), with average sizes of 3 nm, were hence obtained with no remains of the initial core–shell structure. The bottom-up synthesis (consisting of core formation, encapsulation and functionalisation) turns into a top-down route (Figure 1d–f).

**SRP’s visualisation and localisation by electron spectro-microscopy techniques:** To confirm the SRP’s structure, they were also studied by scanning transmission electron microscopy (STEM) by means of high-angle annular dark-field (HAADF) images combined with electron energy loss spectroscopy (EELS) using a spectrum imaging approach. HAADF imaging is known for its high sensitivity to composition, since the image intensity varies approximately as \( Z^{1.7} \), giving rise to a “Z-contrast” image. Figure 4 presents HAADF images of the SRPs, which can be clearly observed at the present resolution. The agglomerated SRPs form a thin film either on the carbon layer (Figure 4a) or self-supported in the sub-micrometer hole of the holey carbon grid (Figure 4b). High-magnification HAADF imaging makes it possible to distinguish individual bright contrasts in the nano-size range that arise mainly from the Gd signal contribution (Figure 4c).

However, interpreting such contrasts as individual SRPs only based on HAADF images remains rather speculative. Combining Z-contrast images with an EELS spectrum imaging approach allows us to chemically map the different elements and their relative positions. Here, EELS spectra were collected at every position of the probe while scanning the region of interest, typically \( 20 \times 20 \) nm. Figure 5a presents the HAADF intensity profile map acquired simultaneously during the experiment. Carbon, silicon, and gadolinium intensity maps were obtained by extracting the C-K, Si-L₂,₃, and Gd-N₄,₅ edges, respectively (Figure 5b–d). The Si-L₂,₃ map shows almost spherical contrast areas the diameters of which typically span from 1.5 to 2.5 nm, corresponding to the fragmented polysiloxane. In the upper part of Figure 5, the sample is thin enough so that individual polysiloxane cores can be observed, as confirmed by the Si-L₂,₃ intensity.
Ultrasmall Silica Nanoparticles

probed between two spheres going to zero (see example of EELS spectra in Supporting Information). The Gd–N4,5 map presents very localised peaks, which are correlated with the bright spots of the STEM-HAADF image, as expected, since Gd is the heaviest element present in this system. The positions of individual Gd atoms were statistically estimated and indicated in Figure 5d and the Supporting Information. Based on the EELS experiment, we systemically found that every polysiloxane core is observed with 3Gd atoms (in the upper part of Figure 5b, in which Si particles can be separated unambiguously, around 117 Gd atoms are counted in the vicinity of ca. 40Si particles). This value is lower than the one obtained by other techniques (see section on the determination of the SRP molecular structure), but we presume that some Gd atoms might be missed while scanning the region of interest with EELS. The positions of some polysiloxane cores (blue circles) and of some Gd atoms (red circles) have been indicated over the C-K intensity map in Figure 5b. The C and Si distributions are clearly anti-correlated and some Gd atoms can be observed in the C between the particles, confirming the overall structure of the SRPs as depicted in Figure 11a (see below). The Supporting Information presents an example of the EELS-based elemental localisation of a single SRP by combining the Si-L2,3, Gd-N4,5, and C-K intensity maps displayed in Figure 11b–d, respectively. A fragmented polysiloxane core is surrounded by five individual Gd atoms, while the C is located in the interstitial space. This clearly illustrates the 2D elemental structure of a SRP as proposed in the present study.

To go now further in the characterisation of the mechanisms involved in the top-down synthesis, several characterisation experiments have been performed: magnetic susceptibility measurements (performed with a superconducting quantum interference device, SQUID) and electron paramagnetic resonance (EPR) measurements will be presented below, to investigate the disappearance of the oxide core. Nanoparticles with 2 DOTAGA per Gd were chosen for all the following experiments, because they are right on the plateau in the size measurements. This should provide both good core dissolution and good reproducibility in the syntheses.

Characterisation of the top-down mechanism

Evidence of core disappearance by susceptibility measurements: Magnetic susceptibility measurements were performed using a SQUID to validate the absence of any gadolinium oxide core in the SRPs. Two control samples were used: 3.5 nm sized gadolinium oxide stabilised with PEG and paramagnetic Gd3+–DOTA chelates. All samples were introduced to the SQUID in the solid state (freeze-dried solutions). On one hand, gadolinium oxide showed paramagnetic behaviour at room temperature (RT) and antiferromagnetic behaviour below the Neel temperature (1.72 K). This low-temperature transition, compared with that obtained in bulk oxide (17–18 K), is entirely explained by size effects (mainly reduction of magnetic interactions). On the other hand, both SRPs and Gd3+–DOTA chelates, the Neel temperatures of which were 0.11 and 0.13 K, had a behaviour characteristic of non-interacting Gd3+ ions isolated by diamagnetic matrices. Only negligible amounts of oxide core remain then in the SRPs (less than 5% of the starting amount), confirming TEM observations, in which most of the Gd was located in Gd3+–DOTAGA chelates.

Evidence of core disappearance by EPR spectroscopy: To confirm the absence of cores, EPR experiments were performed on different Gd species: SRPs, Gd3+–DOTA complexes and, this time, bulk Gd2O3 (Figure 6). Bulk oxide exhibits a negligible signal as compared to Gd3+–DOTA complexes (Figure 6, inset) which should make it possible to distinguish Gd-based cores from SRPs bearing Gd complexes unambiguously. Gd3+–DOTA complexes are characterised by a g-value of 1.99, a field distance between the two spectrum extrema, ∆Hpp, of 62 G (Supporting Information) and a slightly asymmetric signal. These values are similar to those reported in literature, with ∆Hpp depending on the metal-ion concentration and temperature[32,33] The SRPs present a similar EPR spectrum as the Gd3+–DOTA complexes, meaning that similar species are present, but with a larger ∆Hpp of 100 G. This small difference between the two EPR spectra indicates the presence of weak magnetic interactions between the Gd atoms, which is consistent with the SRP structure in which neighbouring chelates lie on the polysiloxane surface. The broadening of the EPR line might be also related to some dispersion in the surface density of the Gd3+–DOTAGA groups, with such a dispersion inducing a distribution of interaction strengths between the neighbouring chelates.
In addition, the evolution of the particles during the purification step (ageing) in water was monitored by EPR spectroscopy in order to better characterise the disappearance of the Gd-based cores. At the beginning of the purification \((t=3 \text{ h})\), the SRP signal increased quickly, indicating a rapid formation of \(\text{Gd}^{3+} \text{-DOTAGA} \) chelates after core dissolution. This evolution slowed down significantly after a few hours, and the EPR signal reached a maximum at \(48 \text{ h} \) (Figure 6). If we consider that the system does not evolve any more after 7 days, we find that 13.4% \(\text{Gd}_2\text{O}_3\) is remaining after 3 h, 5.5% \(\text{Gd}_2\text{O}_3\) after 20 h, and that the core dissolution is complete after 2 days. These results are consistent with both TEM and magnetic susceptibility measurements showing the absence of \(\text{Gd}_2\text{O}_3\) core after this duration. Finally, they helped us to determine and fix a purification time of 2 days for our standard particle synthesis.

At this stage, we have demonstrated that the SRPs discussed are likely composed of a polysiloxane matrix, carrying, a priori, both DOTAGA and DOTAGA–Gd\(^{3+}\) chelates on their surface. Any crystalline \(\text{Gd}_2\text{O}_3\) has disappeared from the nanoconstruct so obtained.

**Determination of the SRP molecular structure**

*Global chemical formula of the SRPs obtained by inductively coupled plasma mass spectrometry (ICP-MS):* To investigate the SRP chemical composition, chemical analysis was performed by ICP-MS. The obtained compositions in Gd, Si, N and C, in atomic and mass ratios, are given in Table 1. Before functionalisation of the core–shell particle, the measured Si/N ratio (after purification in DEG against ethanol) showed that the composition of the polysiloxane was not modified during the hydrolysis-condensation of its precursors (60% APTES, 40% TEOS before reaction, 60% R-APTES, 40% R-TEOS after reaction, the letter R indicating that the precursors have reacted so that their chemical formulas have changed (Supporting Information); to simplify the notations, this letter will be omitted in the following). Assuming that the APTES/TEOS ratio will also remain constant during the further functionalisation and purification steps (which will be verified later by other techniques), one easily finds that the SRPs contain around 1.5 DOTAGA ligands per Gd (Table 2). This implies that two types of DOTAGA are attached on the surface: those that are complexed by \(\text{Gd}^{3+}\) and the others that are not complexed by any ion. Calculations also show that some residual DEG remains in low amounts in the final product (0.65 DEG molecule \((\text{C}_4\text{O}_3\text{H}_{10})\) per Gd). HPLC measurements indicated a high purity of the colloidal solution; the residual DEG is then attached to the SRPs surface. Finally, the analyses performed make it possible to obtain the synthesis yield for each group of species: it varies from 7% for DOTAGA to 19% for APTES and TEOS (Table 2). In conclusion, an SRP average composition can be deduced from all the data (Table 2): \(\text{Gd}_{1.9}\text{APTES}_{2.3}\text{TEOS}_{1.3}\text{DOTAGA}_{1.5}\).

**Chemical formula of a unique SRP obtained by mass spectrometry (MS):** To obtain the chemical formula of a single construct knowing the global formula given by Tables 1 and 2, an exact estimation of its size, or better, its mass is required. Since PCS provides only a hydrodynamic size and TEM observations are not sufficient to delineate ultrasmall hybrid particles with enough precision, we tried to get the molecular weight of a single SRP by electrospray mass spectrometry measurements. Macromolecules that contain multiple groups capable of being ionised (e.g., Gd–DOTAGA complexes) can sustain multiple charges so that distributions of charge states are often observed in the mass-to-charge spectrum. This is observed for SRPs (Figure 7a), for which a clear distribution of charges is observed in the \(m/z\) range between 1000 and 4000 Th. The results obtained can be exploited since the “envelope” of the peaks is stable and the relative abundance of the different peaks is found to be only slightly dependent on the electrospray ionization (ESI) MS experimental conditions. A multiplicative correlation algorithm (MCA)\(^{[34]}\) was then used to estimate the mass of the nanoparticles (see Figure 7b, the mass spectrum generated). This spectrum is characterised by a main population at 8.5 kDa (MSD of 0.5 kDa) associated to a broader one peaking at 9.7 kDa (MSD of 0.5 kDa). This mass is consistent with all the morphology information obtained until now, including the mass deduced from centrifugal filtration using membranes (Vivaspin) with different pore sizes (data not shown).

The result can be further refined (in particular to get information about the internal structure of the SRPs) by using MS/MS experiments.\(^{[35]}\) The technique consists in a first step by selecting a species in the first \(m/z\) spectrum before provoking its dissociation by collision with He atoms. The dissociated elements are then studied by a second mass analysis giving a second \(m/z\) spectrum. Here, the species corresponding to the broad peak centred at \(m/z = 1405\) in the spectrum of Figure 7a (MS spectrum) was chosen: as seen above, it is the SRP itself with a 6+ charge due to the electrospray technique in the positive mode. The second \(m/z\) spectrum (MS/MS spectrum) corresponding to the dissociated SRPs is given in Figure 7c. We observed the peak at \(m/z = 1405\) corresponding to the 8.5 kDa SRPs with a charge state of +6.
The peak is not isotopically resolved but presents abundant neutral losses on its left, characteristic of the different groups that have been cleaved from the SRP/C29s surface. This fragmentation of “surface groups” has already been observed in the case of gold nanoclusters.[36–39] The MS/MS spectrum is also characterised by 1) low-mass singly charged DOTAGA ligands, complexed or not to Gd$^{3+}$, and polysiloxane fragments made up of APTES and TEOS, and 2) two intense doubly and triply charged ions between 1500 and 2000 Th (Figure 7c). Interestingly, these peaks correspond to molecular masses of about 3520 and 4910 Da the sum of which is roughly equal to that of the initial SRP, approximately 8500 Da. The two corresponding sub-particles certainly result from the cleavage of the SRP in two parts, as already observed for CdS nanoclusters.[40,41] Moreover, the sum of the charges of the two sub-particles is equal to the 6$^+$ charge hypothesised for the SRP if we add one singly charged molecular degradation product (low-mass singly charged DOTAGA ligand). These two parts cannot be assigned directly by MS/MS experiments due to a lack of mass accuracy and resolution of the instrument. However MS/MS/MS experiments performed by successively choosing these two parts to obtain a third $m/z$ spectrum make it possible to propose a composition of these two parts: Gd$_7$APTES$_{16}$TEOS$_{11}$DOTAGA$_{10}$ and Gd$_7$APTES$_{16}$TEOS$_{10.5}$DOTAGA$_{10.5}$.

Confirmation of two kinds of DOTAGA—complexed or non-complexed to Gd$^{3+}$, measurement by fluorescence titration: The presence of both types of ligands (complexed or not to Gd$^{3+}$) is suggested by the formula of the SRP itself, since, according to it, the nanoconstruct contains more ligands (10) than Gd cations (7). This observation can be also directly verified by the two peaks present in the low $m/z$ range of the MS spectrum (Figure 8a), since they correspond to two singly charged APTES–DOTAGA species: one non-complexed (594.30 Th) and the other bearing a Gd$^{3+}$ ion (749.22 Th).

To obtain the quantitative proportion of the complexed DOTAGA [Eq. (1)] and compare it to the formula deter-
minded by mass spectrometry \((p = 0.66)\), resolved time luminescence experiments were performed after the addition of different amounts of Eu\(^{3+}\) ions to a solution containing SRPs. It is possible to differentiate the non-complexed ligands from the complexed ones, since only the first are able to chelate additional Eu\(^{3+}\) ions. This chelation can be easily followed by the correlative change in the Eu luminescence. Indeed, free Eu ions have a luminescence almost completely quenched by the OH\(^-\) oscillators present in water, whereas the complexed Eu\(^{3+}\) ions are prevented from quenching by the protecting surrounding ligand. Several sets were prepared by the addition of different amounts of Eu\(^{3+}\) ions to the nanoparticles, ranging from 0 to 2 times the Gd content (noted 0 to 200% on Figure 8b). Gadolinium content was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Namely, europium chloride \((\text{EuCl}_3\cdot 6\text{H}_2\text{O})\) was dissolved in water, and added to the solutions containing the nanoparticles. Because of the H\(^+\) release during rare-earth complexation, the pH of the solutions was adjusted to pH 6, which is high enough for fast Eu\(^{3+}\) complexation, but simultaneously low enough to prevent hydroxide formation. The mixtures were stirred for 48 h, and the effective complexation of Eu\(^{3+}\) ions by DOTAGA was controlled by MS (see Supporting Information). Luminescence measurements were made in solutions containing 0.1 mm Gd. Eu\(^{3+}\) ions in water were excited at 395 nm and that resulted in two emission peaks: \(\text{D}_{n=2}\text{F}_1\) at 592 nm and \(3\text{D}_{n=2}\text{F}_3\) at 616 nm.

\[
p = \frac{[\text{complexed DOTAGA}]}{[\text{complexed DOTAGA}] + [\text{non-complexed DOTAGA}]}\tag{1}
\]

For small amounts of added Eu\(^{3+}\) (<60% per Gd), the intensities of both emission peaks increase linearly with the amount of Eu\(^{3+}\), demonstrating the formation of increasing quantities of luminescent DOTAGA–Eu\(^{3+}\) chelates. As expected, for large amounts of added Eu\(^{3+}\) (>80%), the luminescence intensity reached a plateau (Figure 8b); the additional Eu\(^{3+}\) remained free (and non-luminescent) in solution. Therefore, we can determine the ratio of free DOTAGA ligands to be \((70 \pm 10)\%\) of the amount of Gd present in the particles. In other words, the proportion of complexed DOTAGA is found by this method to be equal to \((59 \pm 5)\%\), in rather good agreement with the value deduced from ICP-MS \((66 \pm 10)\%\).

Quantitative evaluation of two kinds of DOTAGA—complexed or non-complexed to Gd\(^{3+}\) by potentiometric titration: Another way to determine the proportion of complexed ligands anchored at the periphery of the nanoparticles consists of performing potentiometric titrations of these acid–base entities. Direct pH titrations of the nanoparticles, followed by data modelling with PROTAF software, can lead to the number of protons released during the course of the titration and then to the number of free DOTAGA ligands on the surface. Indeed, from pH 3 to 12, the complexed ligands do not release any H\(^+\), whereas the non-complexed ones release 3H\(^+\). The model was based on the knowledge of the acidity constants of the non-complexed ligands, which were assumed to be similar to the DOTA acidity constants.[49] The presence of supplementary ionisable protons at the surface of the nanoparticle (Si–OH and Si–NH\(_2\) protons) has to be taken into account in the calculation. Consequently, at the end of the optimisation process, a free DOTAGA concentration of 0.519 mm was calculated for a global Gd\(^{3+}\) concentration of 0.8 mm. These values correspond to 65% of non-complexed DOTAGA per Gd and then to a proportion \(p = 61\%\) of complexed DOTAGA. This result is in a good agreement with both the values determined from luminescence experiments \((p = 66\%)\) and ICP-AES \((p = 59\%)\).

Characterisation of the polysiloxane structure by means of nuclear magnetic resonance (NMR) spectroscopy: In order to better characterise the polysiloxane structure, solid state \(^{29}\text{Si}\) NMR experiments were performed on the SRPs (Figure 9). To avoid the perturbing contribution of Gd\(^{3+}\), Gd\(^{3+}\) was replaced by Lu\(^{3+}\), which is diamagnetic instead of paramagnetic. This substitution should not modify the nature of the nanoconstruct obtained (Lu-SRP), since all the lanthanides present similar properties and reactivity. Interestingly, Lu\(^{3+}\) and Gd\(^{3+}\) have also similar complexation constants with DOTA. This explains why all the characterisations performed on Lu-SRPs give the same results as those obtained with (Gd) SRPs (size, zeta potential, etc.). The \(^{29}\text{Si}\) NMR signal can be de-convoluted into six contributions that correspond to six different Si environments. They are of two main types: \(\text{Cs}[(\text{OSi})_n\text{O}_{1-n}\text{Si}((\text{Si})_{-m}\text{O}_{-m}\text{Si}]\) commonly labelled \(T_m\) and \(Q_m\), respectively. \(T_m\) species are formed from APTES and \(Q_m\) from TEOS. The de-convolution gives a \(T/Q\) ratio of 62:38, in perfect agreement with the initial APTES/TEOS ratio of 60/40. Moreover, the con-
and 4 ppm, correspond to all the CH₃ of the tetraazaacyclocdecane unit, all the CH₂ of the acetates, the γ-CH₂ of the APTES (functionalised or not), and the remaining protons of the glutaric spacer. The signal at δ = 8 ppm might be associated to the amide proton (NHCO) formed during reaction between aminopropyl and DOTAGA. Therefore, ¹H NMR spectra show that a large part of the APTES has been functionalised by DOTAGA, which is in good agreement with the chemical formula of the SRP: 10.5DOTAGA for 16APTES.

To go deeper into the characterisation of the SRPs, Lu-SRPs were also characterised by ¹H diffusion ordered spectroscopy (DOSY) NMR spectroscopy. In order to amplify the signal of free species and appreciate better the propensity of the species to be released, a purification of only one day (instead of two) was voluntarily performed. The pulsed-field gradient echo experiments gave access to the diffusion coefficients for the different species, which makes it possible to obtain the sizes of the objects attached to them.[43,44] The 2D ¹HDOSY NMR spectrum of Lu-SRPs (Figure 10b) showed that, except for DEG, all the resonances were associated to the same diffusion coefficient of around 1.10⁻¹⁰ m²s⁻¹. On the other hand, DEG is associated to two diffusion coefficients: one equal to that of the other species, namely 1.10⁻¹⁰ m²s⁻¹, and another one of 7.10⁻¹⁰ m²s⁻¹.[45] This means that all the species (except a portion of the DEG) are attached to the SRPs. Indeed, 1.10⁻¹⁰ m²s⁻¹ corresponds to a hydrodynamic diameter of 3.8 nm, very close to the SRP size obtained by PCS. Also, the observed spread of the hydrodynamic size covers values ranging from 2.4 to 6.4 nm, again in very good agreement with the size distribution estimated from PCS (Figure 2c). Concerning DEG, a portion attaches to SRPs later, whereas an approximately equivalent portion remains free in solution, indicating that DEG is the only species needing more than one day of purification. The other species belonging to the SRPs seem, in contrast, to be very strongly attached to the nanoconstruct. This indicates that the SRPs keep their integrity in solution, which is of great interest for biomedical applications.

Finally, taking into account all the results obtained until now (and particularly the proportions of each Tₙ and Qₘ species) a global molecular scheme can be proposed for a single SRP (Figure 11a). It appears that the average size of 3 nm is in very good agreement with the average mass of 8500 kDa found by MS. Moreover, the scheme corresponds almost perfectly to an SRP imaged by EELS cartography (Figure 11b–d), the SRP observed presenting five ligands bearing Gd.

Biomedical applications

Gd enrichment of SRPs: In vivo radiotherapy and magnetic resonance imaging (MRI) applications require that the agent used (here the SRP) possesses the highest possible content of Gd. To achieve this, the 40% non-complexed ligands were chelated with additional Gd³⁺ ions by adding an excess of Gd³⁺ and stirring for 24 h at room temperature at
pH 6 just before purification. The verification that all the non-complexed ligands were effectively chelated was made by relaxometry and fluorescence titration experiments. Moreover, ICP-MS analyses showed that, during the stirring step under an excess of Gd$^{3+}$ ions, the quantity of ligands complexed by Gd$^{3+}$ varies effectively from 60 (Gd/Si = 25%) to 100% (Gd/Si = 39%). In all the following sections, enriched SRPs will be used except for potentiometric titration, for which the presence of non-complexed ligands is required.

Preliminary toxicity studies: To demonstrate the absence of toxicity from such SRPs, several types of experiments were performed. With the aim to prevent any Gd$^{3+}$ release in vivo applications, we verified first that the DOTAGA grafted on SRPs has approximately the same complexation ability as free DOTAs, which are considered the most complexing ligands for Gd$^{3+}$.

The complexation constants of different complexes [log$\beta_{110}$, Eq. (2) in the experimental section] were obtained by means of potentiometric measurements. To take into account that the complexation process of macrocyclic ligands at low pH is slow, the kinetic process was accelerated by incubating at 37°C for six weeks. Data modelling using a series of 96 points between pH 2.4 and 4.5 (Figure 12a) led to log$\beta_{110} = 24.78$ for the SRPs. This value indicated that the complexation of Gd$^{3+}$ by the SRP ligands is, for pH > 3, almost as efficient as by DOTA (log$\beta_{110} = 25.58$).[17] For comparison, $\beta_{10}$ is several orders of magnitude higher than for acyclic commercial contrast agents such as Magnevist (Gd–DTPA, log$\beta_{110} = 22.1$) or Omniscan (Gd–DTPA–BMA, log$\beta_{110} = 16.9$). To give a more complete picture of the complexation efficiency of the DOTAGA grafted to the SRP surface, the free-Gd/coordinated-Gd ratio is given over the whole pH range, for grafted DOTAGA and DOTA (Figure 12b). As expected, this ratio is very low and less than one order of magnitude lower for SRPs than for DOTA. This means that the structure of the SRPs ensures strong complexation of Gd$^{3+}$ and should then prevent any release of Gd$^{3+}$ in the body after in vivo injection.

Immunotoxicity was also evaluated using dendritic cells that are responsible for the immune response of the body and, for this reason, need to survive with all their functionalities after SRPs internalisation. Dendritic cells were incubated with nanoparticles for 24 h and then labelled using 7-AAD for necrosis detection. Figure 13a shows that incubation with SRPs up to a Gd concentration of 1 mM has strictly no effect on the viability of the cells and that necrosis becomes significant only for the highest concentrations, with a LD50 (quantity required to kill 50% of cells) close to 5 mM Gd. Moreover, to determine if nanoparticles perturb the

Figure 11. a) Molecular structure of the Gd-based SRP. On average, 27 Si atoms (in yellow) build up the polysiloxane structure. This structure holds 10 DOTAGA ligands, and 7 Gd$^{3+}$ ions (in green). Some DOTAGA ligands remain free from any ion. Carbon atoms are represented in cyan, N in blue, and O in red. b), c) and d) Intensity maps of the Si-L$_{2,3}$, Gd-N$_{4,5}$, and C-K edges, respectively. The blue circle highlights one polysiloxane core. The red circles indicate the position of individual Gd atoms.

Figure 12. a) Speciation diagram of Gd$^{3+}$/[DOTAGA, Si-RH] system as a function of pH under the synthesis conditions: [Gd$^{3+}$] = 0.80 mM, [DOTAGA] = 1.32 mM and [Si-RH] = 5.92 mM. (L stands for the DOTAGA ligands grafted on the SRP, SiRH stands for other surface protons). b) Comparison of Gd$^{3+}$ affinities for, on the one hand, free (i.e., not attached to particles) DOTA and, on the other hand, DOTAGA ligands grafted to SRPs, as a function of pH.
complement system that is part of the innate immune system, particles (at 10 mM Gd) were incubated with human serum at 37 °C for the different times indicated in Figure 13b. The figure shows that the complement activity is similar in all the samples regardless of the presence or absence of nanoparticles. This indicates a total absence of complement activation in presence of nanoparticles and demonstrates that the complement system is absolutely not modified, even for large doses of particles.

First theranostic applications: The ability of SRPs to behave as efficient positive contrast agents has been exploited for the first theranostic application involving SRPs. SRPs were injected intravenously ([Gd]3+ = 80 mM, 1.4 mL) in gliosarcoma (9LGS)-bearing rats and their accumulation in the tumour, due to the well-known enhanced permeability and retention (EPR) effect, was followed by MRI, as shown in Figure 14a, b.

Strong highlighting of the tumour could be easily observed in the right hemisphere only a few minutes after the injection of SRPs. The evolution of the MRI signal has also been compared in Figure 14b over time for the left (healthy zone) and right (tumour) hemispheres. These temporal evolutions showed only small values for the Gd content in the left hemisphere a few minutes after the injection. In the right hemisphere, the signal was approximately one order of magnitude greater, and maximal at about 5 min after the injection, with a very small decrease observed 5 min later. The absence of retention in the healthy zone confirmed previous experiments performed using different imaging techniques. These experiments also showed that, due to the ultra-small size of the SRPs, the removal of the SRPs is almost entirely ensured via renal excretion.

After having determined by MRI studies the time evolution of the Gd in each hemisphere, we decided to proceed with a first irradiation test 1 h after the injection of the particles, in order to have a very high ratio between the Gd content in the right hemisphere (with tumour) and in the contralateral hemisphere (without tumour). Rats bearing 9LGS were then exposed to microbeam radiation therapy (MRT) at ESRF 1 h after the injection and their survival...
was compared to that of rats that were either not treated or treated with MRT only. The untreated rats \((n = 5)\) had a median survival time of 20 days and the rats treated only with MRT \((n = 7)\) presented a median survival time of 48 days. On the other hand, the median survival time was extended to 58 days \((n = 7)\) when MRT was combined with SRP injection (Figure 14c). This experiment suggests an efficient radiosensitising effect of the SRPs, in good agreement with other studies (in vitro and in vivo) performed on Gd-based particles.\(^{[16,47]}\) The nanoparticles have proven their effect at even very small Gd concentrations (less than 1 ppm in the tumour). Finally, the possibility to guide the therapy by MRI is essential here to determine the best conditions for launching the X-rays. Indeed, we confirmed here that it is necessary to have the lowest dose as possible in the healthy region, while simultaneously keeping the highest Gd content in the tumour zone to obtain the best therapeutic results. This compromise, obtained by imaging, makes it possible to optimise the therapy conditions.

### Conclusion

This study describes the synthesis and characterisation of ultra-small polysiloxane nanoparticles displaying DOTAGA–Gd\(^{3+}\) groups on their surface. From previous work, it was already emphasised that these platforms were the first multifunctional silica-based particles that, in spite of some kidney retention, are sufficiently small to escape hepatic clearance and enable animal imaging by four complementary techniques. Here, it is carefully demonstrated that these nanoconstructs are obtained by a new top-down process; this method makes it possible to decrease the particle size and simultaneously conserve multimodality. The process consists of 1) formation of a gadolinium oxide Gd\(_2\)O\(_3\) core, 2) encapsulation in a polysiloxane shell grafted with DOTAGA ligands, 3) dissolution of the gadolinium oxide core by chelation of the Gd\(^{3+}\) with DOTAGA ligands, 4) dissolution of the gadolinium oxide core by chelation of the Gd\(^{3+}\) by the DOTAGA ligands and 4) polysiloxane fragmentation. We were able to optimise the synthesis to obtain a narrow size distribution and complete core dissolution. Also, the SRPs can be reinforced with rare-earth cations or radionuclides by further chelation from surface DOTAGA to enhance their contrast properties. After detailed characterisation by several complementary techniques, we were able to set up the precise molecular structure of SRPs. Potentiometric titrations showed that no leaching of Gd\(^{3+}\) could occur (complexation constant \(\log K_{1/2} = 24.78\)) and cellular tests confirmed a very low toxicity of the nanoconstructs. Finally, in vivo experiments with rats showed that the particles can accumulate in tumours by the EPR effect, allowing for efficient radiotherapy guided by MRI. For all these reasons, SRPs certainly constitute a major advance for more selective therapy and personalised medicine.

### Experimental Section

**Chemicals**: Gadolinium chloride hexahydrate (GdCl\(_3\)-6H\(_2\)O, 99.9 %), europium chloride hexahydrate (EuCl\(_3\)-6H\(_2\)O, 99 %), lutetium chloride hexahydrate (LuCl\(_3\)-6H\(_2\)O, 99 %), sodium hydroxide (NaOH, 99.99 %), tetraethyl orthosilicate (Si(OC\(_2\)H\(_5\))\(_4\)), TEOS, 98 %, amorphous p-toluylenediisoxoyslane (H\(_2\)N(CH\(_3\))\(_2\)-Si(OCH\(_3\))\(_3\)), APTES, 99 %, triethylamine (TEA, 99.5 %), and anhydrous dimethyl sulfoxide (DMSO, 99.5 %) were purchased from Aldrich Chemicals (France). Diethylene glycol (DEG, 99 %) was purchased from SDS Carlo Erba (France). Acetone (reagent grade) was purchased from Sodipro (France) and was used as received. 1,4,7,10-Tetraazacyclododecane-1-glutaric anhydride-4,7,10-triacetic acid (DOTAGA) was provided by Chemetach (France). For the preparation of aqueous nanoparticle suspensions, milli-Q water \((\rho > 18 \text{ M}\Omega)\) was used.

**Synthesis of gadolinium oxide cores embedded in a polysiloxane shell**: These particles were obtained by a two-step route. First, gadolinium oxide cores were synthesised by addition of soda on gadolinium salts, and then polysiloxane shell growth was induced by hydrolysis-condensation of convenient silane precursors.

**Preparation of gadolinium oxide cores**: A first solution was prepared by dissolving GdCl\(_3\)-6H\(_2\)O \((5.58 \text{ g, } 15 \text{ mmol})\) in DEG \((500 \text{ mL})\) at room temperature \((\text{RT})\), leading to a Gd concentration of \(30 \text{ mmol}\). A second solution was prepared by adding 4.95 mL of a 10 mmolar solution of NaOH \((49.5 \text{ mmol})\) to 500 mL DEG. The second solution was progressively added to the first one, at RT, for 24 h. A transparent colloid of gadolinium oxide nanoparticles in DEG was obtained. The Gd concentration of this colloidal solution was 15 mm.

**Encapsulation of Gd\(_2\)O\(_3\) cores by polysiloxane shell**: Polysiloxane shells were grown around the Gd cores, by a sol–gel route, using two silane precursors, APTES and TEOS in a 60:40 molar ratio (TEOS leads to SiO\(_2\) and APTES to SiO\(_2\)-(CH\(_2\))\(_4\)-(NH\(_2\)). The reaction was performed in DEG at 40 °C by several consecutive additions of APTES and TEOS, and was catalysed by TEA. Namely, APTES \((1050 \text{ mL, } 4.5 \text{ mmol})\) and TEOS \((670 \text{ mL, } 3 \text{ mmol})\) were added to the previously prepared solution of Gd\(_2\)O\(_3\) \((1 \text{ L})\) in DEG under stirring at 40°C. After 1 h, a DEG solution \((2550 \text{ mL, } 0.1 \text{ m of TEA, } 10 \text{ mL of water})\) was added. The whole coating procedure was repeated three more times, every 24 h. The final mixture was stirred for 48 h at 40°C. At this stage, 1.2 APTES per Gd and 0.8 TEOS per Gd were added in total. The obtained solution could be stored at RT for weeks without alteration. The Gd concentration of this colloidal solution stayed approximately equal to 15 mm.

**Covalent grafting of DOTAGA on hybrid nanoparticles**: Finally, a large excess of anhydrous DOTAGA was added to the core–shell particles for covalent grafting, with a ratio of 2DOTAGA per Gd atom. Namely, DOTAGA \((13.76 \text{ g, } 30 \text{ mmol})\) was dispersed in DMSO \((200 \text{ mL})\). The suspension of nanoparticles was added to this DOTAGA solution and the resulting mixture was stirred for 48 h at RT.

**Purification**: The nanoparticles were then precipitated in acetone \((9 \text{ L})\). The acetone was removed; the nanoparticles were washed two more times in acetone \((3 \text{ L})\) and precipitated again by centrifugation. Finally, the nanoparticles were dispersed in water \((200 \text{ mL})\), the remaining acetone was removed with a rotary evaporator at RT and the solution was stirred for one night at RT. Purification of the nanoparticles was performed by filtration through Vivaspin membranes (MWCO = 5 kDa) purchased from Sartorius Stedim Biotech (France). The colloidal solution was introduced into 20 mL Vivaspin tubes, and centrifuged. This step was repeated several times, by filling the tubes with water and centrifuging again, until the desired purification rate was achieved (at least \(\times 1000\)). Then, the solution was filtered through 0.2 µm syringe filters to remove the largest impurities. The solution was freeze-dried for storage, using a Christ Alpha 1-2 lyophilisator.

**Electron spectroscopy and microscopy techniques**: Transmission electron microscope (TEM) investigations were carried out using a JEOL 2010 microscope operating at 200 kV to study the morphologies of the synthesised hybrid systems. The spatial resolution of the microscope is 1.7 Å. Studies of the SRPs were also performed on a scanning transmission...
electron microscope (STEM) using a VG-HB501 STEM operated at 100 kV with an electron probe size of approximately 1 nm. Core-level electron energy-loss spectroscopy (EELS) experiments were performed simultaneously to imaging the SRPs to chemically map the elements of interest. The EELS spectrometer provides an energy resolution of 0.3 eV per channel and an acquisition time of 1 ms per spectrum. The sample holder was maintained at 150 K using a liquid-nitrogen-cooled cryo-stage mainly to reduce carbon contamination and beam damage on the organic layer.

Photon correlation spectroscopy (PCS) size measurement: Direct measurements of the size distribution of the nanoparticles suspended in any medium were performed with a Zetasizer Nano-S PCS (633 nm He-Ne laser) from Malvern Instruments (resolution 0.5 nm). Measurements were directly taken on the colloids after core synthesis, shell synthesis and after surface modification with DOTAGA and purification. The Gd concentration in the measured solution was always 15 mM.

Inductively coupled plasma-atomic emission spectrometry (ICP-AES) analysis: Determination of the Gd content in a sample was performed by ICP-AES analysis (with a Varian 710-ES spectrometer). Before measuring the Gd concentration, samples of the colloidal suspension were desolvated in concentrated nitric acid, heated for 3 h at 80 °C, and left one night at RT. The samples were then diluted with water until the nitric acid concentration reached 5%. Chemical analyses were also performed on the as-prepared samples at the Service Central d’Analyses du CNRS (Solaize, France) using ICP-MS, and enabled determination of the Gd, C, N and Si contents to a precision of 0.3%.

Fluorescence measurements: Fluorescence measurements were carried out using a Varian Cary Eclipse fluorescence spectrophotometer, in the resolved time mode. The parameters used were: 395 nm excitation wavelength, which is a characteristic excitation for Eu³⁺ ions, excitation slit 20 nm, emission slit 10 nm, delay time 0.1 ms and gate time 5 ms.

Mass spectrometry: Full scan mass experiments were performed using a linear quadrupole ion trap mass spectrometer (LTQ, Thermo Fisher Scientific, San Jose, CA) with enlargement for the high 2000–4000 Th range. The nanoparticle suspension was electrospun at a flow rate of 5 µL/min in positive ion mode. For collision-induced dissociation (CID) experiments, He was used as collision gas. The m/z isolation window was ±25 Th. Isotopic distributions of fragment ions were recorded using the zoom scan mode of the LTQ ion trap mass spectrometer.

Solid-state ²³³Ni nuclear magnetic resonance (NMR) spectroscopy: Solid state ²³³Ni NMR experiments were performed with a Bruker Avance 500WB spectrometer, with a MAS 4 mm N/P-F/H probe, at a MAS rate of 5 kHz, a 2 s repetition delay and a flip angle of 30°. Spectra were collected for 4 days. To avoid magnetic interactions, Lu replaced Gd in the core synthesis.

¹H NMR and diffusion ordered spectroscopy (DOSY): All experiments were performed at 298 K without spinning. On a Bruker Avance 300 spectrometer equipped with a 5 mm BBFO probe, the z-gradient coil of which is able to provide a maximum gradient strength of 49.8 G/cm, 30 mg of lyophilised SRP, prepared with Lu instead of Gd to avoid paramagnetic effects, were dispersed in 1 mL of D₂O. Standard ¹H NMR experiments were acquired with a presaturation sequence (zgr) to minimise the residual signal of HOD (Δ = 4.68 ppm). ¹H DOSY experiments were acquired with a stimulated echo sequence that includes a longitudinal eddy current delay (T = 5 ms), bipolar sine-shaped gradient pulses of amplitude G, two sine-shaped spoil gradients and presaturation of the HOD signal (ledbgpppr2s). 32 increments, each with 128 transients, were taken between 1 and 45 G/cm (linear spacing). The diffusion delay (Δ) and gradient pulse length (δ/2) were chosen to achieve at least a fivefold attenuation. The following values (Δ, δ/2), both in ms, were used: (100, 2.75) and (200, 2.00). ¹H DOSY data were processed with the GigaMaxent software interfaced with Topspin. The reported hydrodynamic diameters (Dh) are simply derived from the diffusion coefficients (D) with the well-known Stokes–Einstein formula: Dh = k_BT/6πηD, in which k_B is the Boltzmann constant, T the absolute temperature, and η the viscosity of the solvent (1.13 cP for D₂O at 298 K).

Potentiometric measurements: Potentiometric titrations were carried out with an automatic titrator composed of a microprocessor burette Metrohm dosimat 665 and a Metrohm 713 pH meter connected to a computer. All measurements were performed within a thermo-regulated cell at (25.0 ± 0.1) °C under an Ar stream to prevent dissolution of the CO₂. The ionic strength was adjusted to 0.1 with NMe₄Cl. The combined Type “U” glass Metrohm electrode used had a very low alkaline error. The procedures and apparatus used for potentiometric measurements have been previously described. The ionic product of water was determined by titration of acetic acid with a CO₂-free NMe₄OH solution (pK_a = 13.78 at (25.0 ± 0.1) °C in 0.1 M of NMe₄Cl). The determination of the complexation ability of the SRP towards Gd³⁺ was performed according to the “batch method”. The mother solutions of SRP (0.4 mM in HCl) and Gd³⁺ (5 mM), were mixed according to different Gd³⁺/SRP stoichiometric ratios (I = 0.1). For each ratio, a series of 24 stopped flasks was prepared. Each flask corresponded to a pH value, obtained by micro-addition of NMe₄OH (50 mM, I = 0.1). All the flasks were stored under Ar and put for six weeks in a thermo-regulated enclosure at 37°C. All the colloidal suspensions were stable for this specified period. Before pH measurements, these solutions were finally allowed to reach equilibrium temperature (25°C) for 48 h. The potentiometric data were processed by using the PROTAF program, in order to obtain the best chemical model fit and refined overall constants kslow [Eq. (2)]

\[ \text{log}_10 M + \text{H}^+ \rightarrow M_\text{slow} \text{H}^+ \]

Electron paramagnetic resonance (EPR) spectroscopy: EPR measurements were carried out in H₂O-DEG (95.5, vol%) at 150 K using a Bruker Elexsys 500 spectrometer with X-band microwave (ν = 9.4 GHz). The modulation amplitude was 5 G and the microwave power 0.2 mW. The EPR line width ΔH_L/2 was determined as the peak–peak distance between the extreme points of the first derivative of the EPR signal.

Superconducting quantum interference device (SQUID): Magnetic susceptibility measurements were performed using a SQUID MPMS-XL5 Quantum Design magnetometer, with a temperature scale from 1.9 K to 350 K.

Dendritic cells: Dendritic cells were generated from bone marrow extracted from C57BL/6 mice (Janvier, Le Genest St Isle, France) as previously described. Briefly, bone marrow cells were isolated by flushing from the femurs. Erythrocytes and GR1 positive cells were removed by magnetic cell sorting and the remaining negatively sorted cells were resuspended at 5 × 10⁶ cells.mL⁻¹ in complete Iscove’s modified Dulbecco’s medium (IMDM) supplemented with growth factors (GM-CSF, FLT-3L and IL-6) and cultured at 37°C in the presence of 5% CO₂. The transformation of the progenitors into fully active dendritic cells occurs during 10 days culture.

Incubation with nanoparticles: After washing, dendritic cells (10⁵ cells.mL⁻¹) were incubated in culture medium in 24 well plates for 24 h; then nanoparticles were added and cells further incubated at 37°C. The impact on dendritic cells was tested for various parameters, such as necrosis or activation. Necrosis was analyzed by flow cytometry after 24 h incubation with nanoparticles; cells were stained in the presence of 7-aminometycin D (7AAD). The analyses were performed on a FACS LSRII (BD Bioscience) and the results recalculated using FCS Express V3 (De Novo Software). Each experiment was performed at least three times with cells issued from different cultures.

Measurement of classical pathway complement activity: The classical pathway complement activity was measured as previously described. Briefly, sheep erythrocytes were sensitised with rabbit antisera to sheep red blood cells. Human serum was incubated at 37°C in the presence of nanoparticles as indicated in the text, and then 20 µL of this serum were added to 3 mL of the medium containing the erythrocytes (4 × 10⁶ cells.mL⁻¹). The lysis of red blood cells by complement at 37°C was monitored at 660 nm using a spectrophotometer (Evolution201, Thermo Scientific). The time required for 50% hemolysis (TH50) was used for the determination of complement activity.
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