Control of the in vivo Biodistribution of Hybrid Nanoparticles with Different Poly(ethylene glycol) Coatings

Anne-Charlotte Faure, Sandrine Dufort, Véronique Josserand, Pascal Perriat, Jean-Luc Coll,* Stéphane Roux,* and Olivier Tillement

Fluorescent nanoparticles containing a gadolinium oxide core are very attractive because they are able to combine both imaging (fluorescence imaging, magnetic resonance imaging) and therapy (X-ray therapy and neutron-capture therapy) techniques. The exploitation of these multifunctional particles for in vivo applications requires accurate control of their biodistribution. The postfunctionalization of these particles by four different poly(ethylene glycol) derivatives, which differ by chain length and end group, exerts a great influence on the ζ potential of the nanoparticles and on their biodistribution after intravenous injection to HEK-β3-tumor-bearing mice. This study reveals that the behavior of PEGylated nanoparticles, which was monitored by in vivo fluorescence imaging, depends on both the chain length and the end group of the PEG chain.

1. Introduction

The intense research activities devoted to nanotechnology have led to the development of a variety of multifunctional nanoparticles. Based on a physical and chemical point of view, some of these particles appear very well suited for imaging and/or therapy for at least two reasons. The first is their small size, which allows their use for exploring living machinery from the subcellular scale up to the whole living organism. The second reason is based on the possibility of gathering a large range of properties that can be accurately tuned by a convenient choice of components despite the small volume of the nanoparticles. Moreover, the behavior of the particles (e.g., colloidal stability in biological fluids) can be controlled by postfunctionalization of the nanoparticles. The potential of the multifunctional nanoparticles can be fully exploited for in vivo applications only if they do not nonspecifically accumulate, and if they are rather quickly eliminated by natural means. Frangioni et al. emphasized the necessity of rapid clearance and clearly pointed out the problems related to the accumulation of the nanoparticles in a living body, since their presence can interfere with contrast agents used for monitoring the effects of a therapy and induce unexpected long-term injury. The principal conclusions of their study focused on the biodistribution and clearance of quantum dots distinguished by their size, and the organic coating indicated the criteria required for the clinical use of nanoparticles. They demonstrated that a rapid clearance of quantum dots (QDs) is observed only when the hydrodynamic diameter (HD) is smaller than 5.5 nm. However, nonspecific accumulation of cysteine-coated QDs was observed in the liver even for the smallest particles, while the largest ones (HD = 8.65 nm) are mostly present in the liver, spleen, and lungs. Nonspecific accumulation in the liver and the spleen is...
2. Results and Discussion

Gadolinium oxide nanoparticles embedded in a fluorescent polysiloxane shell constitute a very attractive tunable platform combining several in vivo imaging techniques and therapies. Owing to the paramagnetic character of Gd\(^{3+}\) ions, the high-neutron-capture cross section of two gadolinium isotopes (\(^{155}\)Gd and \(^{157}\)Gd), and the relatively high atomic number of this element, a gadolinium oxide core gives the particle the ability to enhance the positive contrast of MRI and the dose effect of X-ray radiotherapy, and renders a harmless thermal neutron beam cytotoxic.\(^7,19–21\) However, these attractive features can be exploited only if colloidal stability in biological fluids is ensured. These cores are encapsulated in a polysiloxane shell, which can be designed sequentially in order to render the resulting particles fluorescent and highly hydrophilic (Scheme 1).\(^7,18\) In order to entrap dyes within the polysiloxane shell and graft hydrophilic groups onto the surface, the polysiloxane shell was obtained by hydrolysis-condensation of a mixture of tetraethyl orthosilicate (TEOS), aminopropyltriethoxysilane (APTES), and Cyanine 5-conjugated APTES (Cy5–APTES) (40%, 59.2%, and 0.8%, respectively). Due to the large proportion of unmodified amino groups of APTES (59.2% versus 0.8% for Cy5–APTES), –NH\(_2\) groups are likely to be accessible on the surface. The presence of unmodified amino groups at the surface was confirmed by zeta-potential measurements and the behavior of these particles in water at various pHs. As revealed by the value of the zeta potential at pH 4.4 (\(\xi = +25\text{ mV}\)), the surface charge of the particle is positive for pH < 5. This positive charge ensures colloidal stability in water due to the resulting electrostatic repulsion between the particles. However, an initial flocculation followed by precipitation of the particles is observed after 24 h when the pH is around pH = 7. This is not acceptable for in vivo applications as the pH of biological fluid is 7.4. The precipitation indicates the loss of the positive charge of the particles when the pH increases. This pH-dependent behavior reflects the presence of amino groups, whose protonation at low pH confers a positive charge to the particles. The postfunctionalization (i.e., the chemical modification of the surface of the particle after the hydrolysis/condensation step) therefore depends on the presence of these unmodified amino groups. Previous studies have demonstrated that amino groups are accessible on the surface of the polysiloxane shell since biotin, streptavidin, or an oligonucleotide can be grafted onto the resulting particles. This reaction can be direct between N-hydroxysuccinimide (NHS) moieties of the molecules and surface amino groups,\(^9\) or may use a cross linker (p-phenylene dithiothreitol (DITC)), which allows the grafting of aminated molecules onto aminated surfaces.\(^{26}\) Besides preventing nonspecific accumulation of particles in living bodies, derivatization of the particle surface by hydrophilic PEG chains

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Table 1. Characteristics of the four PEG derivatives used for the functionalization of gadolinium-oxide–fluorescent-polysiloxane core–shell nanoparticles.

<table>
<thead>
<tr>
<th>PEG</th>
<th>Anchoring group</th>
<th>End group</th>
<th>(M_w) [g mol(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG250–COOH</td>
<td>COOH</td>
<td>COOH</td>
<td>250</td>
</tr>
<tr>
<td>PEG2000–COOH</td>
<td>COOH</td>
<td>COOH</td>
<td>2000</td>
</tr>
<tr>
<td>PEG2000–NH(_2)</td>
<td>NH(_2)</td>
<td>NH(_2)</td>
<td>2000</td>
</tr>
<tr>
<td>PEG2000–OCH(_3)</td>
<td>COOH</td>
<td>OCH(_3)</td>
<td>2000</td>
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apply.\(^{22,23}\) The main limitation of fluorescence imaging (i.e., the strong absorption of visible light) can be overcome by the use of a fluorophore that can be excited in the 600–900-nm range since biological tissues are relatively transparent in this spectral region.\(^{24,25}\)

generally ascribed to uptake by the reticuloendothelial system (RES), like macrophage cells,\(^{11}\) whereas the presence of particles in the lungs results from their agglomeration caused by the adsorption of plasma proteins.\(^{13}\) The functionalization of nanoparticles by a PEG shell is probably the most efficient way to avoid nonspecific accumulation, which can be detrimental for the in vivo application of nanoparticles. Many studies, which were focused on liposomes or polymeric particles, have indeed demonstrated the positive effect of PEG on the biodistribution of such particles.\(^{15,16}\) Derivatization by PEG chains limits the uptake by resident phagocytes in the liver (Kupfer cells) and in the spleen (macrophages and B cells).\(^{14}\) Because of steric shielding, the PEG chains impede the adsorption of plasma proteins onto the surface of the nanoparticles (opsonisation), which can occur when particles are injected into the bloodstream. However, the effect of PEG chains on the biodistribution can be counterbalanced by the charge of the particle since negatively and positively charged liposomes showed increased

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should improve the colloidal stability in biological fluids. In order to evaluate the influence of the chain length and end group, four different PEG molecules (PEG250-COOH, PEG2000-COOH, PEG2000-NH₂, PEG2000-OCH₃) were studied (Table 1). All these PEG chains carry a reactive group at one end (a carboxylic acid group for PEG250-COOH, PEG2000-COOH, and PEG2000-OCH₃), and an amine function for PEG2000-NH₂) for grafting onto the aminated surface of the particles, and a carboxylic acid at the other extremity (PEG250-COOH, PEG2000-COOH), an amine group (PEG2000-NH₂), and a methoxy group (PEG2000-OCH₃), whose influence on the colloidal stability and biodistribution was investigated. Three of the PEGs (PEG2000-COOH, PEG2000-NH₂, PEG2000-OCH₃) have the same molecular weight (Mₑ = 2000 g mol⁻¹), and we can assume that they have similar chain lengths but differ by their terminal group. PEG250-COOH and PEG2000-COOH differ only in length. PEGs with a COOH moiety as the grafting group were covalently tethered to the surface of the particles through a reaction with amine promoted by 1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (EDC) and penta-fluorophenol (PFP) in order to yield the amide linkage (see Experimental Section and Scheme 1). The grafting of PEG2000-NH₂ to the aminated particles was performed thanks to the use of DITC (Scheme 1). DITC is a rigid cross linker containing two reactive isothiocyanate groups, which are well known for their ability to yield a thiourea linkage after reacting with the amine function.

The encapsulation of the gadolinium oxide cores into a fluorescent, hydrophilic shell was reflected by an increase of the HD of the particles (from 11–14 nm, except for nanoparticles coated by PEG2000-COOH (8 nm), whereas the Gd₂O₃ cores have a HD of 3.8 nm (Figure 1). The conjugation of the fluorophore Cy5-NHS to APTES through the formation of an amide linkage before the hydrolysis/condensation allows distribution of the dyes in the whole thickness of the shell (Scheme 1). The coupling reaction between the organic fluorophores and APTES was certified by mass spectrometry, with complete disappearance of the molecular peak of Cy5-NHS (m/z = 792.0) and the appearance of the molecular peak of the fluoro-phores-conjugated APTES (Cy5–APTES, m/z = 858.3). As a result, the gadolinium oxide cores were coated by a fluorescent polysiloxane shell with excitation/emission wavelengths adapted to in vivo imaging. The incorporation of the fluorophores (Cy5–NHS) inside the polysiloxane shell is confirmed by the absorption and emission spectra of the colloids (Figure 1). The spectra of colloids display a similar shape to that of Cy5 in solution. In the case of nanoparticles functionalized by PEG250-COOH, PEG2000-COOH, and PEG2000-OCH₃, the position of the absorption and emission maxima are slightly shifted towards higher wavelengths (Table 2), whereas the shift is more pronounced in the case of nanoparticles coated by PEG2000-NH₂. These shifts arise from changes in the environment of fluorophores and can be attributed to the incorporation of the organic dyes in the polysiloxane shell. This strategy increases the photostability of the fluorophores and makes a large proportion of the surface available for further derivatization by hydrophilic groups since only a small portion of APTES was modified. The reaction of hybrid particles with the various PEGs significantly affects their colloidal stability in water. Whatever the PEG molecule, improved stability is observed in a larger pH range, including the pH of biological fluids (7.4), although zeta-potential measurements revealed that the influence of each PEG on the surface charge is different (Figure 2). Even if the methoxy group is neutral whatever the pH, the particles exhibit a weak positive charge until pH ≈ 5, which decreases to a relatively strong negative value when the pH is elevated (Figure 2a). An interesting result is the point of zero charge (PZC) is located between pH 5 and 6. The evolution of the charge with the pH reflects actually the behavior of ionisable groups like silanol and amine groups present in the polysiloxane shell. Since pKₐ(SiOH/SiO⁻) ≈ 3 and pKₐ(NH₂⁻/NH₃) ≈ 8, the positive charge is attributed to the presence of ammonium groups. From this data, we can deduce that some amine groups remain unchanged after the grafting of PEG–OCH₃, but a large part of amine groups acted as anchorage sites since the value of the ζ-potential is weaker than that of uncoated nanoparticles (3.3 mV at pH 3.2 versus 25 mV at pH 4.4). The increase of the pH induces the deprotonation of nonionic silanol into negatively charged silanolate first, and of positively charged ammonium into nonionic amine. As a result, for pH > 6, hybrid particles coated by neutral PEG2000–OCH₃ exhibit a negative charge. It must be pointed out that the colloid remains stable...
when the pH is between 3 and 8 despite the low values of the zeta potential (−5 mV; +5 mV). In this pH range (including the pH of biological fluids), the colloidal stability is unlikely to arise from electrostatic repulsion, but should be attributed to steric hindrance and the hydrophilic character of the PEG chains. This was corroborated by the fact that the derivatization of the hybrid nanoparticles by PEG750–OCH₃ chains, which are shorter and consequently less bulky and hydrophilic than PEG2000–OCH₃ chains, induces an irreversible precipitation. As expected, the particles carry a positive charge in a large pH range (until pH 9) when they are coated by PEG2000–NH₂ because amine groups are protonated into ammonium groups in this pH range (Figure 2b). Moreover, the amount of ammonium is sufficiently high to compensate the negative charge of the silanolate groups. However, the ζ potential of PEG2000–NH₂-coated particles is weaker than that of uncoated nanoparticles, whereas it was expected that the values would be similar since the amount of amine groups should be identical. Indeed, each amine group on the surface of the nanoparticles engaged in the linkage with PEG via DITC is replaced by the free amine group of PEG. This difference can probably be explained by the bridging of PEG2000–NH₂ with amine of the same particle and/or by the shielding effect of PEG chains, which has been reported previously.[15] In contrast to uncoated particles whose surface is covered by NH₂ groups (due to the use of APTES for the elaboration of the polysiloxane shell), PEG2000–NH₂-coated nanoparticles are very well dispersed in aqueous solution in a large pH range, although their positive charge is weaker. This confirms the role of the PEG chain in the colloidal stability, as seen in the case of nanoparticles coated with PEG–OCH₃.

The grafting of PEG250–COOH and PEG2000–COOH onto the hybrid particles renders them water soluble in a large pH range, including the pH of biological fluid, whereas the colloidal stability of uncoated nanoparticles is not sufficient. This behavior is attributed to the negative charge of carboxylate groups, which results from the deprotonation of COOH (pKa(COOH/COO⁻)=5) as well as the negative charge of silanolate, and to the steric hindrance and hydrophilic character of the PEG chains. However, the relative contribution of the end group and of the PEG chain to the colloidal stability seems different for both carboxylated PEGs. In both cases, which are similar to that of particles coated by PEG–OCH₃, the particles exhibit a

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**Table 2.** Wavelengths of maximum absorption (λ_{abs}) and emission (λ_{em}) of Cy5–NHS in solution (Cy5–NHS) and entrapped in the polysiloxane shell of nanoparticles coated by various PEG chains.

<table>
<thead>
<tr>
<th></th>
<th>λ_{abs} [nm]</th>
<th>λ_{em} [nm]</th>
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<tbody>
<tr>
<td>Cy5–NHS</td>
<td>646</td>
<td>663</td>
</tr>
<tr>
<td>PEG250–COOH</td>
<td>650</td>
<td>662</td>
</tr>
<tr>
<td>PEG2000–COOH</td>
<td>653</td>
<td>666</td>
</tr>
<tr>
<td>PEG2000–OCH₃</td>
<td>651</td>
<td>665</td>
</tr>
<tr>
<td>PEG2000–NH₂</td>
<td>661</td>
<td>670</td>
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</tbody>
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**Figure 1.** Size distribution determined by photon correlation spectroscopy (left column) and by absorption and emission spectra (right column, red and black curves, respectively) of hybrid nanoparticles coated by a) PEG250–COOH, b) PEG2000–COOH, c) PEG2000–OCH₃, and d) PEG2000–NH₂.
positive charge at low pH owing to the presence of ammonium. However, inversion of the charge, which results from the deprotonation of silanol and of carboxylic acid groups accentuated by the deprotonation of ammonium groups at higher pH values, occurs at lower pH for particles coated by PEG250–COOH (pH 4 versus pH 7). The particles coated by the shorter carboxylated PEG chains (PEG250–COOH) consequently exhibit a strong negative charge at pH 7.4 (Figure 2c), whereas the charge of the surface of the particles coated by PEG2000–COOH is only slightly negative (Figure 2d). This difference probably stems from the folding of the long PEG2000–COOH chains. This folding allows the interaction of negatively charged carboxylate ends with ammonium groups and leads, therefore, to the neutralization of the negative charge of carboxylate groups until ammonium is deprotonated when pH increases. Such folding was not observed with PEG250–COOH because the PEG chain is too short. The folding of PEG2000–COOH limits the influence of electrostatic repulsion between negative charges on the colloidal stability, which is largely due to the steric hindrance and hydrophobic character of PEG chains, whereas colloidal stability of particles coated by PEG250–COOH is mainly ensured by the electrostatic repulsion. According to the ζ-potential measurements, nanoparticles coated by PEG250–COOH are negatively charged, whereas the derivatization by PEG2000–NH₂ confers a positive charge on the nanoparticles at pH 7.4. After functionalization by PEG2000–COOH and PEG2000–OCH₃, the nanoparticles exhibit such a weakly negative ζ-potential value at pH 7.4 that the particles are considered neutral. Visual examination, PCS, and ζ-potential measurements show that the derivatization of the particles by various PEG chains confers a high colloidal stability in aqueous solutions on them. The preparation of an injectable solution can be envisaged with each set of particles.

Figure 3 depicts the biodistribution of nanoparticles coated by PEG250–COOH at various durations after the intravenous injection to NMRI nude mice bearing a HEK-β3 tumor on the right side. It appears that only the kidneys and the bladder, which are involved in renal excretion, are fluorescent 15 min after the injection. Moreover, the light emitted from the bladder vanishes 1 h after the intravenous injection. The absence of fluorescence from the tumor indicates that the amount of nanoparticles in this zone is not sufficient to be detected, whereas HEK-β3 tumors are surrounded by a denser vascularization than healthy tissues. This can be explained not only by the rapid elimination of the particles from the body reflected by the biodistribution (Figure 3a and b), but also by the imaging of plasma (Figure 3c) that does not present fluorescence 24 h after the intravenous injection. The amount of particles in the blood decreases dramatically because they are very quickly accumulated in the kidneys and are then eliminated by urine (Figure 3a and b). Ex vivo examination of the organs 24 h after the intravenous injection of the nanoparticles unambiguously confirms that the nanoparticles freely circulate and do not accumulate in organs other than the kidneys, which are strongly fluorescent (Figure 3d). Although they have the same terminal group (carboxylic acid), the biodistributions of particles coated by PEG250–COOH and by PEG2000–COOH display some differences (Figure 4). These differences can be attributed to the longer PEG chain, which generates a higher steric hindrance. Moreover, ζ-potential measurements demonstrated that nanoparticles coated by PEG250–COOH exhibited a negative charge at pH 7.4, whereas nanoparticles coated by PEG2000–COOH were
neutral. As in the case of PEG250–COOH, the bladder is strongly fluorescent. Consequently, particles coated by PEG2000–COOH are also eliminated by urine, but the elimination occurs for a longer time since the bladder remains fluorescent for at least 1 h instead of 15 min, as in the case of PEG250–COOH. Moreover, for most of the mice, the bladder again became strongly fluorescent after 4–5 h. This indicates that PEG2000–COOH-coated nanoparticles circulate for a longer time. This assertion is confirmed by the data of Figure 4c, which shows that the blood is still fluorescent 24 h after the injection. Despite the excretion of the particles by urine, the kidneys are not visible because their low fluorescence due to a more gradual elimination is masked by the moderate fluorescence observed on the whole body of the mouse. Figure 4a and b gives the impression that the distribution of the particles in the body is more homogeneous, but this is invalidated by the visualization of the organs after the sacrifice of the mice 24 and 72 h after the injection (Figure 4d). After 24 h, the kidneys, bladder, adrenal gland, ovaries, uterus, and tumor are moderately fluorescent, while the skin is strongly fluorescent. As fluorescence intensity decreases in blood, it was expected that a similar decrease would be observed in the aforementioned organs (Figure 4d). Except for the kidneys and bladder, the adrenal gland, ovaries, uterus, tumor, and skin remain at the same level of fluorescence 72 h after the injection. However, it must be pointed out that, as expected, the functionalization of the particles by PEG2000–COOH prevented accumulation in the liver, spleen, and lungs since the latter were almost not fluorescent. As previously observed for liposomes, the behavior of these nanoparticles confirms the absence of uptake by the RES and of agglomeration for neutral PEGylated particles.

The replacement of PEG2000–COOH by PEG2000–OCH₃ leads to only minor changes (Figure 5), whereas the functionalization of the particles by PEG2000–NH₂ induces striking differences in the biodistribution (Figure 6). Figure 5a shows that the fluorescence of the bladder after the IV injection of PEG2000–OCH₃-coated nanoparticles varies periodically during the first 5 h. The bladder is strongly fluorescent 15 min, 1 h, and 5 h after the injection, whereas the fluorescence is less intense 2 and 3 h after the injection (Figure 5a). This variation of fluorescence reflects the gradual elimination of the particles by urine. However, the elimination from the body takes a longer time than in the case of nanoparticles functionalized by short PEG250–COOH chains. The fluorescence of the plasma remains important 24 h after the injection of PEG2000–OCH₃-coated particles, as compared to the blood of a control mouse (Figure 5c). The elimination of the particles is confirmed by the low intensity of the fluorescence of the plasma and of most of the organs 72 h after the injection.
Between 24 and 72 h, the fluorescence of the plasma and of the organs decreases (Figure 5c and d). If the fluorescence is observed in the same organs as in the case of nanoparticles functionalized by PEG2000–COOH, it can be noticed that skin is less fluorescent, but that the ovaries and uterus are strongly fluorescent (Figure 5d). Figure 5d shows that the liver, spleen, and lungs are not fluorescent. In other words, the covalent grafting of PEG2000–OCH\(_3\) prevents the nanoparticles from uptake by the RES and from agglomeration. It must be pointed out that the coating of the hybrid nanoparticles by this hydrophilic polymer is very efficient because no accumulation and no agglomeration are observed, whereas these nanoparticles circulate for a longer time than PEG250–COOH-coated nanoparticles do. PEG2000–NH\(_2\) confers a behavior on the hybrid particles that is obviously different (Figure 6). Besides the accumulation in the kidneys, adrenal gland, ovaries, uterus, tumor, and skin, which was also observed when particles were functionalized by PEG2000–COOH and PEG2000–OCH\(_3\), a huge accumulation is also observed in the liver and the spleen, as revealed by the strong fluorescence from these organs (Figure 6a and d). Moreover, the elimination of the particles was not performed by urine since no fluorescence was detected during the first 5 h in the bladder (Figure 6a). This was confirmed by visualization of the organs 24 and 72 h after the injection (Figure 6d). However, the fluorescence of the intestines indicates that particles accumulated in the liver are excreted through feces. This elimination mode is largely slower than the elimination by urine. For this reason, fluorescence from the liver remains very strong (Figure 6d), even if its intensity from the zone containing the liver starts to decrease 24 h after the injection (Figure 6a). In contrast to PEG250–COOH, PEG2000–COOH, and PEG2000–OCH\(_3\), PEG2000–NH\(_2\) does not prevent the uptake of the particles by organs containing phagocytic cells (liver, spleen). Such a difference in behavior could be attributed to the positive charges of PEG2000–NH\(_2\)-coated nanoparticles since the other PEG chains with the same molecular weight do not accumulate in the liver and the spleen. Indeed, the positive charge of ammonium groups should favor the adsorption of negatively charged opsonins, which constitutes the first step of the uptake by phagocytic cells. If functionalization by PEG2000–NH\(_2\) does not prevent accumulation in the liver and the spleen, it succeeds, however, in preventing agglomeration in the lungs since the fluorescence of these organs is very weak 72 h after the injection. However, this organic shell is not as efficient as the other PEG because some fluorescent spots are visible on the lungs 24 h after the injection of PEG2000–NH\(_2\)-coated nanoparticles. Despite the large amount of nanoparticles accumulated in the liver and the spleen, the tumor seems more fluorescent when particles are coated by
PEG2000–NH₂ (Figure 6d). Figure 7 gathers the data on fluorescence intensity of organs measured ex vivo 72 h after the intravenous injection of the PEGylated nanoparticles. As suggested by Figure 3, nanoparticles functionalized by PEG250–COOH are only present in the kidneys 72 h after injection. The very high fluorescence intensity, in comparison with the case of nanoparticles coated by longer PEG chains, indicates that a large number of particles are present in the kidneys, whereas the amount in other organs, muscle, fat, and the HEK-β3 tumor is, in most cases, the lowest. From this data, we can deduce that PEG250–COOH chains confer on the nanoparticles the ability to freely circulate without any undesirable accumulation and ensure that they are removed rather quickly by urine. The replacement of PEG250–COOH by longer PEG chains induces a significantly higher accumulation in the skin, adrenal gland, ovaries, uterus, and tumor. Apart from the case of skin, which is more fluorescent after the injection of nanoparticles coated by PEG2000–COOH, the accumulation is more pronounced when PEG2000–NH₂ is grafted onto the nanoparticles. The propensity of PEG2000–NH₂ to favor the accumulation of the nanoparticles is even more obvious in the case of the intestine, liver, and spleen. These organs are effectively largely more fluorescent in the case of nanoparticles functionalized by PEG2000–NH₂. Even if the nonspecific accumulation of nanoparticles coated by PEG2000–X is moderate in tumors, it must be pointed out that the influence of the PEG chain length is not negligible since no fluorescence (i.e., no accumulation) is observed with the shorter chain. The absence of nanoparticles coated by PEG250–COOH in the HEK-β3 tumor was confirmed by in vivo fluorescence imaging 24 and 72 h after the intravenous injection of the nanoparticles (Figure 8). Because of a strong fluorescence from the skin when the nanoparticles are functionalized by PEG2000–COOH, the accumulation of these nanoparticles in the tumor, which was revealed by ex vivo fluorescence measurements (Figure 7), is not visible because the fluorescence coming from the tumor is masked by that of the skin (Figure 8). Despite the fluorescence of the skin, the accumulation of nanoparticles functionalized by PEG2000–NH₂ in the tumor is perceptible (Figure 8), probably because the fluorescence intensity from the skin is weaker than in the previous case, while the tumor is more fluorescent when PEG2000–NH₂ chains are tethered to the nanoparticles (Figure 7). In contrast to the nanoparticles coated by PEG2000–COOH and PEG2000–NH₂, the moderate accumulation in the tumor of the nanoparticles whose hydrophilic character is due to PEG2000–OCH₃ is more easily distinguishable, although the fluorescence intensity of the tumor is similar to that observed in the case of nanoparticles functionalized by PEG2000–COOH. Figure 5b, which displays the evolution of the fluorescence after intravenous injection of PEG2000–OCH₃-coated nanoparticles in a mouse lain on its belly, shows that the fluorescence contrast is positive in the tumor 5 h after the intravenous injection. Despite the decrease in fluorescence intensity observed 24 and 72 h after the administration of the fluorescent nanoparticles, the tumor stands out against the surrounding tissues more clearly because the fluorescence intensity of the latter decreases more quickly. The image acquired when the mouse was on the left side confirms the passive accumulation of PEG2000–OCH₃-coated nanoparticles (Figure 8). However, the amount of fluorescent nanoparticles in the tumor is relatively weak compared to the low fluorescence intensity of the tumor when it was examined ex vivo after the sacrifice of the animals (Figures 5d and 7). Although derivatization of the nanoparticles by PEG2000–NH₂ leads to the highest accumulation in the tumor zone (Figure 7), functionalization by PEG2000–OCH₃ seems to be better suited because this polymer prevents accumulation in the liver and spleen, in contrast to PEG2000–NH₂.

This study, which focused on the biodistribution of hybrid nanoparticles (HD < 20 nm) that were designed for combining multimodal medical imaging techniques and therapy,
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shows that the retention time and biodistribution of these nanoparticles depends strongly on the chemical composition of the outer surface. If the grafting of hydrophilic PEG molecules is required to ensure circulation of the nanoparticles in the bloodstream, the biodistribution depends on the chain length and on the end group. For the same end group (COOH), clearance from the blood is significantly higher for the short chain (PEG250–COOH) than for the long chain (PEG2000–COOH). Nanoparticles coated by PEG250–COOH quickly accumulated in the kidneys before being removed from the body by urine. The fluorescence intensity of the blood is in the same magnitude range as that of the blood of a mouse that did not receive any nanoparticles 24 h after the injection.

For equal chain lengths, the biodistribution depends on the nature of the end group. When the PEG chain is terminated by an NH2 group, the particles are positively charged in the biological pH range. The undesirable accumulation of hybrid nanoparticles coated by PEG2000–NH2 in the liver and the spleen is probably correlated to the positive ζ potential, which favors, as previously reported, uptake by phagocytic cells of the RES (mainly present in these organs).[15] The absence of PEG2000–NH2-coated nanoparticles in the lungs reflects a satisfactory colloidal stability of these nanoparticles despite the presence of negatively charged serum proteins, which are well known for inducing the agglomeration of positively charged nanoparticles.[12] Such a colloidal stability can probably be ascribed to the steric hindrance of hydrophilic PEG chains since aminated nanoparticles without PEG accumulate quickly in the lungs.[7] In contrast to the NH2 end group, COOH and OCH3 functions carried at the extremity of PEG chains with the same length prevented accumulation of the nanoparticles in the liver and the spleen. The biodistribution and clearance of nanoparticles coated by PEG2000–COOH and PEG2000–OCH3 were similar, except for the accumulation in the skin. Tumoral uptake is based on the enhanced permeability and retention (EPR) effect due to the increased capillary permeability in the tumor vasculature. Among the four PEG derivatives, PEG2000–OCH3 seems the most attractive because it favored accumulation in the tumor without undesirable uptake in the liver and spleen, in contrast to PEG2000–NH2. The accumulation of the nanoparticles seems to be dependent on the PEG chain length since tumoral uptake based on the EPR effect was not observed for nanoparticles functionalized by the shortest PEG chain (PEG250–COOH). The absence of any accumulation of nanoparticles coated by PEG250–COOH in the tumor probably arises from too rapid a clearance, and also from the absence of interactions between the tumoral tissue and the nanoparticles, which can easily enter the tumor through its poorly organized and leaky endothelium, and come out again.

3. Conclusions

In this study, fluorescent nanoparticles composed of a gadolinium oxide core and a fluorescent polysiloxane shell were derivatized by four different hydrophilic PEG chains. Whatever the nature of the hydrophilic polymer, PEGylation led to a higher colloidal stability in the biological pH range. However, the polymers exert an influence on the biodistribution and on the clearance of the fluorescent nanoparticles that depends on the length and the end group of the various PEG chains immobilized on the nanoparticles. Therefore, for a given nanoparticle, the clearance and biodistribution can be tuned. Among the four hydrophilic polymers, PEG2000–OCH3 appears the most interesting because this polymer favors the accumulation of the nanoparticles in the tumor by the EPR effect, whereas they freely circulate in the bloodstream without undesirable accumulation in the liver, spleen, and lungs. However, the postfunctionalization of these nanoparticles by biotargeting groups will be impeded by the absence of grafting sites. This could, however, be overcome by mixing PEG2000–OCH3 with PEG terminated by reactive groups (COOH, NH2). The data collected during this biodistribution study also confirms the deleterious effect of the positive charge, which favors accumulation in resident phagocyte-rich tissues (liver, spleen), despite the hydrophilic character of the PEG chain, which is well known for preventing plasma protein adsorption and avoiding subsequent uptake by phagocytes. The ability of PEG chains to reduce the uptake by macrophages is reflected by the behavior of nanoparticles coated by carboxylated and methoxy PEG. Although PEG250–COOH is the shortest hydrophilic polymer of the series, it confers the greatest ability to circulate freely in the blood pool on the nanoparticles since no accumulation is observed apart from organs involved in the natural elimination (kidneys, bladder). The next step of our work will consist of improving the selective uptake of these PEGylated nanoparticles by tumoral tissues by grafting various molecules onto the PEG corona that will favor cancerous-cell targeting and uptake.

4. Experimental Section

Chemicals: Gadolinium chloride hexahydrate (99.99%), sodium hydroxide (99.99%), tetraethyl orthosilicate (Si(OCH2)4), TEOS, 98%, (3-aminoxypropyl)triethoxysilane (H2N(CH2)3O)Si(OCH2)3, Si(OH)4, >99%, APTES, 99%, EDC (98% w/w), PFP (99%), poly(ethylene glycol)bis(carboxymethyl)ether (PEG250–COOH, 99%, 250 g mol⁻¹), α,ω-Bis[2-[(3-carboxy-1-oxopropyl)amino]ethyl]-poly(ethylene glycol) (PEG2000–COOH, 99%, 2000 g mol⁻¹), poly(oxethylene) bis(amine) (PEG2000–NH2, 99%, 2000 g mol⁻¹) 0-Methyl-O’-succinyl-poly(ethylene glycol) (PEG2000–OCH3, 99%, 2000 g mol⁻¹), hepes solution (solution in water, pH 7.0–7.6), sodium chloride, triethylamine (TEA, 99.5%), and hydrochloric acid (HCl, 37%) were all purchased from Aldrich Chemical (France). Cy5 mono-NHS ester (∼90%) was purchased from Amersham Bioscience. Ethanol, diethylether glycol (DEG, 99%), and other organic solvents (reagent grade) were purchased from SDS (France) and used as received. For the preparation of an aqueous solution of nanoparticles, only milliQ water (pH > 6.1 MΩ) was used. The dialysis of colloidal solutions was performed with a tubular membrane of cellulose (molecular-weight cut off (MWCO) 4–6 kDa) purchased from Roth (France). Purification and concentration of colloids by tangential filtration were carried out using Vivaspin (MWCO 10 000 Da).
Synthesis of gadolinium oxide nanoparticles embedded in a polysiloxane shell: These particles were obtained by a two-step route. Gadolinium oxide nanoparticles were first synthesized by applying a modified “polyol” protocol. Afterwards polysiloxane shell growth was induced by hydrolysis/condensation of convenient siloxane precursors in the presence of the nanoparticles. Preparation of gadolinium oxide core: Gadolinium chloride salt (11.53 g) was placed in DEG (200 mL) at 60 °C overnight under vigorous stirring. Sodium hydroxide solution (7.5 mL, 3 M) was added and the solution was heated at 140 °C for 1 h, and at 180 °C for 4 h. A transparent colloid of gadolinium oxide nanoparticles was obtained, which can be stored at room temperature for weeks without alteration.

Encapsulation of Gd$_2$O$_3$ cores by polysiloxane shell: A solution containing organic dyes (Cy5 NHS ester, 5 mg) and APTES (1.4 μL) dissolved in dimethylsulfoxide (291 μL) was prepared as a precursor and stirred overnight. The coupling reaction between the organic fluorophores and APTES was certified by mass spectrometry with complete disappearance of the molecular peak of the fluorophore (m/z = 792.0 for Cy5–NHS) and the appearance of the molecular peak of the fluorophore-conjugated APTES (m/z = 858.3 for Cy5–APTES). Cy5–APTES solution and a portion of 327 μL of APTES and of 209 μL of TEOS were added to a solution containing Gd$_2$O$_3$ nanoparticles (1.5 mL) diluted in DEG (13.5 mL) under stirring at 40 °C. After 1 h, a portion of 792 μL of a DEG solution (0.1 M of TEA, 10 M of water) was added. The other portions of polysiloxane precursors and hydrolysis solution were sequentially and alternately added. The final mixture was stirred for 48 h at 40 °C.

Nanoparticles PEGylation: Particles were functionalized by three different homobifunctional PEGs carrying amino or carboxylic acid groups at both ends and one heterobifunctional PEG bearing a methoxy group at one end and a carboxylic acid function at the other end.

Covalent grafting of carboxylated PEG on hybrid nanoparticles: For grafting PEG via its carboxylic acid function, –COOH functions were activated by EDC and PFP. DEG solution (500 μL containing PEG250–COOH (0.091 μM) was added to an isoamyl alcohol solution (5 mL) containing a mixture of EDC and PFP (0.367 μM). In the case of PEG2000–COOH and PEG2000–OCH$_3$, the activation of the carboxylic acid function was performed by first dissolving the polymers in DMF (100 mg mL$^{-1}$). Afterwards the PEG solution (1.8 mL) was mixed with EDC/PFP in DMF solution (1.8 mL, 0.1 M). The reaction between COOH groups of the polymer and PFP, assisted by EDC, is expected to yield pentfluorophenyl esters. The resulting mixture was stirred for 1.5 h at room temperature and added to 1 mL of colloidal suspension composed of hybrid nanoparticles (Gd$_2$O$_3$ cores encapsulated in a fluorescent polysiloxane shell) dispersed in DEG. The covalent grafting of PEG onto the hybrid nanoparticles was performed overnight at room temperature with gentle stirring.

Covalent grafting of aminated PEG on hybrid nanoparticles: PEGs bearing amine functions as the grafting group were covalently bound to the aminated nanoparticles by using DITC as a cross linker. Prior to the reaction, a stock solution of DITC was prepared by dissolving DITC in DMF (10 mg mL$^{-1}$). The stock solution containing DITC (1.6 mL) was added to 1 mL of a colloidal suspension composed of hybrid nanoparticles (Gd$_2$O$_3$ cores encapsulated in a fluorescent polysiloxane shell) dispersed in DEG solution (7.5 μM). After 8 h of stirring at room temperature, PEG2000–NH$_2$ in DMF solution (1.8 mL, 100 mg mL$^{-1}$) was added. The resulting solution was allowed to react overnight at room temperature under stirring.

Purification: Whatever the nature of the PEG anchored onto the nanoparticles, the resulting colloids are purified in the same way. The colloids of PEGylated nanoparticles were introduced into a tubular dialysis membrane and immersed in water (250 mL), which was replaced four times every 12 h. Diluted purified solutions were finally concentrated by tangential filtration using Vivaspins until their concentration was equal to 7.5 μM.

Size measurement: Direct measurement of the size distribution of the nanoparticles was performed by PCS with a Zetasizer NanoZS from Malvern Instruments. Measurements were made on the colloids after purification by dialysis.

Zeta-potential measurements: Direct determination of the ζ potential of the hybrid PEGylated nanoparticles was performed using a Zetasizer NanoZS (laser He–Ne, 633 nm) from Malvern Instruments. Prior to the experiment, the aqueous colloid was diluted in an aqueous solution containing NaCl (0.01 M) and adjusted to the desired pH.

Absorption and emission spectra: Absorption and emission spectra were acquired on a WPA spectrophotometer and a fluorescence spectrometer (F2500, Hitachi), respectively.

Preparation of injectable solution: After tangential filtration, 160 μL of colloid (hybrid nanoparticles in water) was diluted with an aqueous solution (40 μL) containing NaCl (750 mM) and hepes (20 mM).

Biodistribution of nanoparticles: Female NMRI nude mice (6–8 weeks old, JANVIER) received a subcutaneous xenograft of HEK293 (6–8 weeks old, JANVIER) received a subcutaneous xenograft of HEK293/β3) cells (20 × 10$^6$ cells per mouse). After tumor growth, mice (n = 3 for each type of PEG, and in vivo imaging experiments were reproduced at least twice) were anesthetized (isoflurane/oxygen 4% for induction and 2% thereafter) and were injected intravenously with a nanoparticle suspension (200 μL). The mice were illuminated by 633-nm light-emitting diodes equipped with interference filters. Fluorescence images, as well as black-and-white pictures, were acquired by a back-thinned charge-coupled device cooled camera (ORCAII-BT-512G, HAMAMATSU). A colored-glass long-pass filter (RG 665) cut off all excitation light in the image. After imaging, the mice were sacrificed and dissected in order to image the organs and blood.

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Control of the in vivo Biodistribution of Hybrid Nanoparticles


