

In Vivo Biodistribution and Clearance Studies Using Multimodal Organically Modified Silica Nanoparticles

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Nanomaterials promise to address the current limitations of sensitivity and specificity of medical diagnostics as well as significantly improve the outcome of existing and emerging therapeutics *via* the introduction of a new generation of multimodal nanoprobes.^{1–6} In this regard, it is critical to design nanoprobes with desired composition, size, and surface functionalities, and rigorously test them *in vitro* and *in vivo* for their safety and efficiency. Also biocompatibility and biodegradation of the nanoprobe materials play an important role in the use of the nanoprobes in the field of diagnostic and therapeutic applications.

The rise in nanoprobes development has bolstered the prospects of *in vivo* optical imaging through the development of a variety of NIR-luminescent nanoformulations, which include quantum dots,⁷ upconverting nanophosphors,⁸ and luminophore-containing nanoparticulate carriers such as liposomes,⁹ polymersomes,¹⁰ ceramic,^{11,12} or polymeric^{13,14} nanoparticles, *etc.* Silica and organically modified silica (ORMOSIL) nanoparticles have several attributes that facilitate their use as a platform for the fabrication of an ideal nanoprobe. Ultrafine (diameter below 20 nm) nanoparticles of silica/ORMOSIL can be easily synthesized in a user-friendly and cost-effective manner, using microemulsion medium and ambient conditions. These materials are inert, nonantigenic, and have excellent storage stability and resistance to biofouling. They are optically transparent, and can serve as excellent optical probes, following the encapsulation/conjugation of fluorophores such as organic dyes or quantum dots.^{15,16} Such nanoincorporation protects the fluorophores from photobleaching, and pre-

ABSTRACT Successful translation of the use of nanoparticles from laboratories to clinics requires exhaustive and elaborate studies involving the biodistribution, clearance, and biocompatibility of nanoparticles for *in vivo* biomedical applications. We report here the use of multimodal organically modified silica (ORMOSIL) nanoparticles for *in vivo* bioimaging, biodistribution, clearance, and toxicity studies. We have synthesized ORMOSIL nanoparticles with diameters of 20–25 nm, conjugated with near-infrared (NIR) fluorophores and radiolabeled them with ¹²⁴I, for optical and PET imaging *in vivo*. The biodistribution of the nontargeted nanoparticles was studied in nontumored nude mice by optical fluorescence imaging, as well by measuring the radioactivity from harvested organs. Biodistribution studies showed a greater accumulation of nanoparticles in liver, spleen, and stomach than in kidney, heart, and lungs. The clearance studies carried out over a period of 15 days indicated hepatobiliary excretion of the nanoparticles. Selected tissues were analyzed for any potential toxicity by histological analysis, which confirmed the absence of any adverse effect or any other abnormalities in the tissues. The results demonstrate that these multimodal nanoparticles have potentially ideal attributes for use as biocompatible probes for *in vivo* imaging.

KEYWORDS: ORMOSIL nanoparticles · optical and PET imaging · NIR fluorophore · ¹²⁴I radiolabeling · biodistribution · clearance and toxicity.

vents their interaction with the biological environment, thus potentially allowing a safe use of even toxic fluorophores.¹⁷ In addition, the rich surface chemistry of silica/ORMOSIL allows the co-incorporation of other diagnostic probes for multimodal bioimaging, as well as suitable biorecognition molecules for target-specific delivery.¹⁸

Using silica/ORMOSIL nanoparticles encapsulated/conjugated with fluorophores and targeting ligands, our group and others have already demonstrated efficient optical imaging of tumor cells *in vitro*.^{15,19} In addition, we have synthesized ORMOSIL nanoparticles coencapsulating quantum dots and iron oxide nanoparticles, and have shown their *in vitro* target specificity by using a synergistic combination of receptor-mediated targeting and magnetic guidance.²⁰ Finally, we have also developed ORMOSIL nanoparticles for a variety of therapeutic applications, which includes

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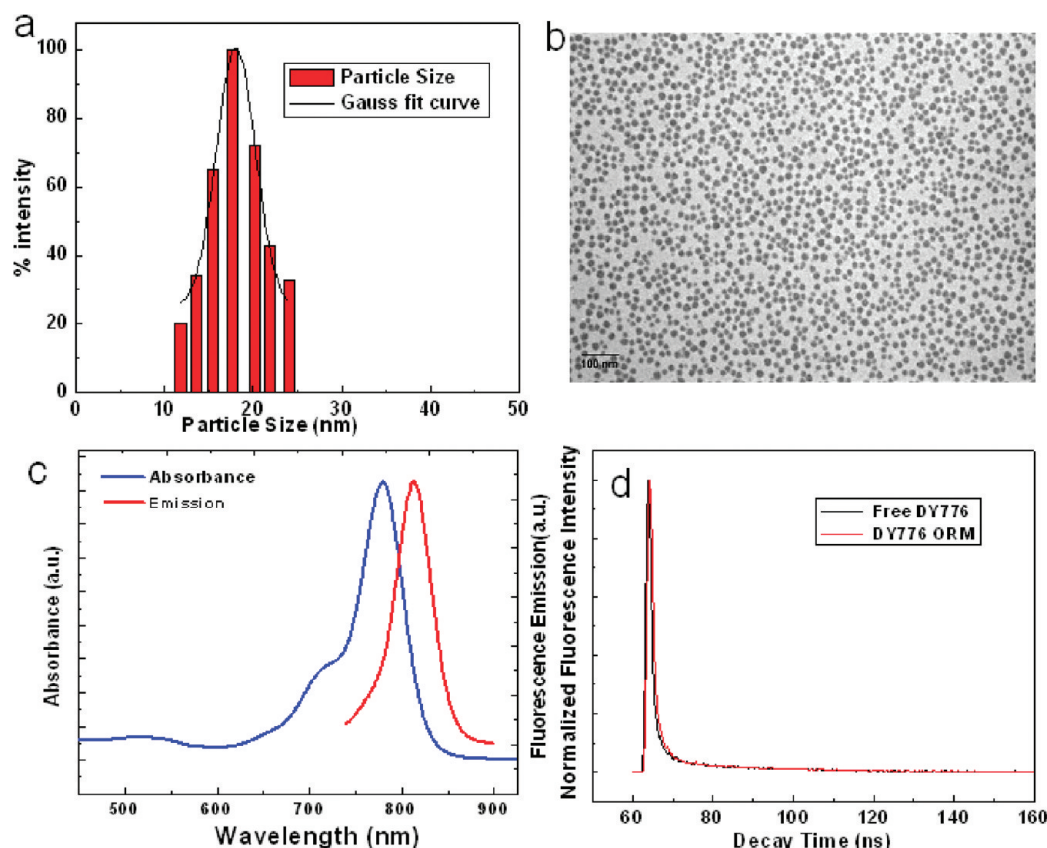


Figure 1. Showing the basic characterization of the DY776 conjugated ORMOSIL nanoparticles: (a) Nanoparticles size distribution profile as measured by DLS, (b) representative TEM image, (c) Absorption and emission spectra, (d) fluorescence lifetime decay curves of the DY776, either free or conjugated with ORMOSIL nanoparticles.

photodynamic therapy (PDT) of cancer and nonviral gene delivery.^{21,22} No adverse effects were observed when these nanoparticles have been directly introduced in the brain of live mice and rats. These promising observations have prompted us to investigate in detail the biodistribution and eventual fate of multimodal ORMOSIL nanoparticles *in vivo*, following systemic delivery. Such an investigation is critically important for the eventual application of these nanoprobe for routine clinical use in diagnostics and therapeutics.

Herein, we report the synthesis of ultrafine ORMOSIL nanoparticles (diameter \sim 20 nm), conjugated with a near-infrared (NIR) fluorophore, as optical probes. The resulting NIR-nanoparticles will facilitate optical bioimaging in the NIR window, with maximum tissue penetration of light and minimum background signal.^{23,24} Furthermore, we have also conjugated the well-known positron emission tomographic (PET) imaging probe iodine-124 with the nanoparticle, which will allow bioimaging independent of tissue-depth, as well as more accurate quantification of accumulation of nanoparticles in various major organs *in vivo*. These multimodal nanoprobe have been injected systemically in mice, and their *in vivo* biodistribution studies have been carried out, based on the acquisition of fluorescence emission of the conjugated fluorophore and the gamma emission of the conjugated ¹²⁴I. The clearance of the

nanoparticles from the animal body, as well as long-term toxicity involving the effect of the nanoparticles on the major organs, has also been studied. The results indicate that these nanoparticles slowly clear out of the animal body *via* the hepatobiliary excretion, without causing any overt toxicity and tissue damage. Thus, it can be concluded that these multimodal nanoparticles have potentially ideal attributes for use as safe and efficient probes for *in vivo* bioimaging.

RESULTS AND DISCUSSION

The nanoparticles characterization results are shown in Figure 1. Figure 1 panels a and b show the size and distribution of the nanoparticles by dynamic light scattering (DLS) and transmission electron microscopy (TEM), respectively. DLS has shown the nanoparticles to be of unimodal distribution with the maximum percentage intensity to be of 20 nm in size. TEM shows a good correlation with DLS showing the nanoparticle diameter to be around 20 nm. As can be visualized from the TEM image, the nanoparticles have a spherical morphology with a narrow size distribution. The overall surface charge of the nanoparticles was found to be negative with an absolute zeta potential value of -25.5 mV. The negative surface charge can be attributed to the presence of the PEG molecules and a small amount of remnant anionic surfactant, AOT, on the surface of the

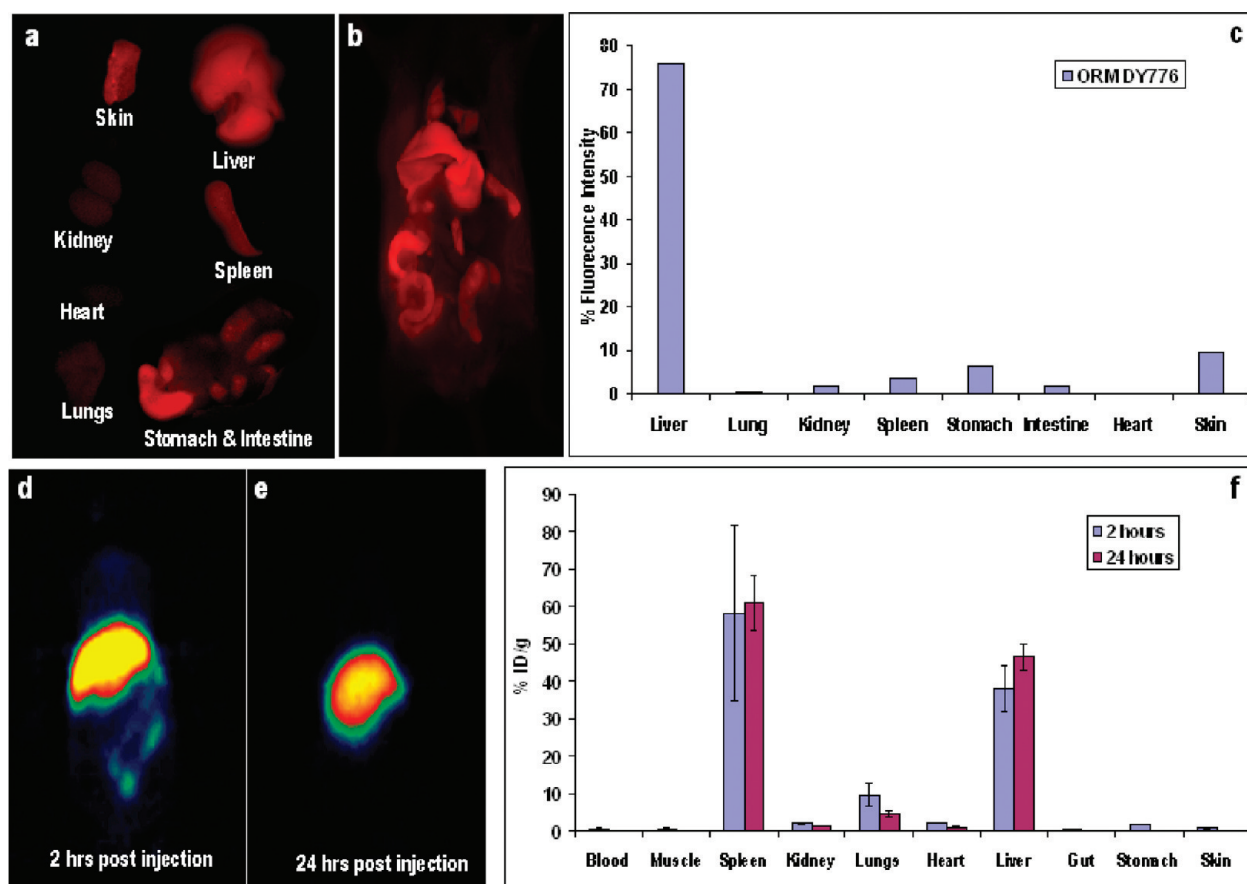


Figure 2. Biodistribution of ORMOSIL nanoparticles by the measurement of the fluorescence (a, b, c) and radioactivity (d, e, f) from different organs of the mice injected with DY776 conjugated and ^{124}I labeled ORMOSIL nanoparticles respectively: (a) fluorescence acquired from the individual organs, (b) whole body fluorescence imaging of the mice 24 h postinjection, (c) quantitative estimation of fluorescence acquired from different organs of the mice, (d and e) PET images of the mice injected with ^{124}I -ORMOSIL nanoparticles 2 and 24 h postinjection, respectively, and (f) quantitative radioactivity measurements from the individual organs of the mice.

nanoparticles. The net surface charge of the nanoparticles have a pronounced effect on the adsorption of different physiological lipoproteins in systemic circulation and plays a critical role in the clearance of the nanoparticles from the animal body.³ It was also observed that upon incubation with the serum media, the nanoparticle size increased by more than 15 nm in diameter, suggesting the binding of the serum proteins to the nanoparticles (data not shown).

Figure 1c shows the photophysical characterization of the ORMOSIL nanoparticles with conjugated NIR dye DY776. The optical absorbance and fluorescence spectra of the nanoparticles showed an absorbance peak with λ_{max} at 780 nm and emission peak at 815 nm. No considerable shift in the emission wavelength was observed for the DY776-NHS ester, DY776-silane, and DY776 conjugated nanoparticles, suggesting that the dye remained stable even after conjugation with the nanoparticle. Since both the excitation and emission wavelengths of the DY776 lie in the NIR region, the nanoparticle formulation offers an added advantage of autofluorescence-free *in vivo* imaging, as well as deeper penetration of the excitation and emission light. Figure 1d shows the normalized fluorescence decay curve

of the aqueous suspension of the DY776 conjugated nanoparticles. From the fluorescence lifetime decay studies the average decay time was estimated to be 62.0 ns, which indicates that decay time of the DY776 conjugated ORMOSIL nanoparticles is comparable with other optical-based diagnostic probes frequently used for real time and long-term imaging studies.

The biodistribution of ORMOSIL nanoparticles has been studied by two methods, one involving the fluorescence of the NIR fluorophore DY776 conjugated with the nanoparticles, and the other by radioactivity measured from the ^{124}I -labeled with the nanoparticles. For the *in vivo* fluorescence imaging a semiquantitative approach was used, involving fluorescence quantification acquired from the dissected tissues as a percentage of the fluorescence from the injected dose (details in Supporting Information). Figure 2 shows the biodistribution profile of the ORMOSIL nanoparticles in nude mice. As can be seen from Figure 2a, almost 75% of the injected dose accumulated in the liver and spleen, whereas the lung, kidney, and heart accounted for minimum accumulation (less than 5%). High fluorescence recorded from the highly perfused organs, such as liver and spleen, could be accounted for as the combined ac-

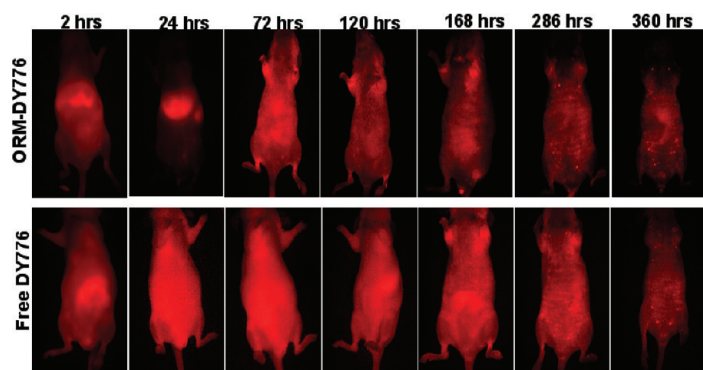


Figure 3. Clearance of the DY776 conjugated ORMOSIL nanoparticles injected intravenously into the mice (upper panel) and a comparison with the free DY776 injected mice as control (lower panel). Same acquisition time was maintained for all the time points of imaging.

tivity of the circulating blood passing through organs, as well as that due to particle uptake by cells of the reticuloendothelial system (RES) recruited by these organs. It is interesting to note that a considerable amount of the nanoparticles go to the stomach and intestine (~8%), which can be attributed to the excretion of the nanoparticles over a period of 24 h. It is also observed from the Figure that almost 9% of fluorescence was acquired from the skin, which can be corroborated to the possible fact that DY776 can direct the nanoparticles to the skin. This is further confirmed when the free DY776 was injected and a major percentage of fluorescence was observed from the skin (Supporting Information).

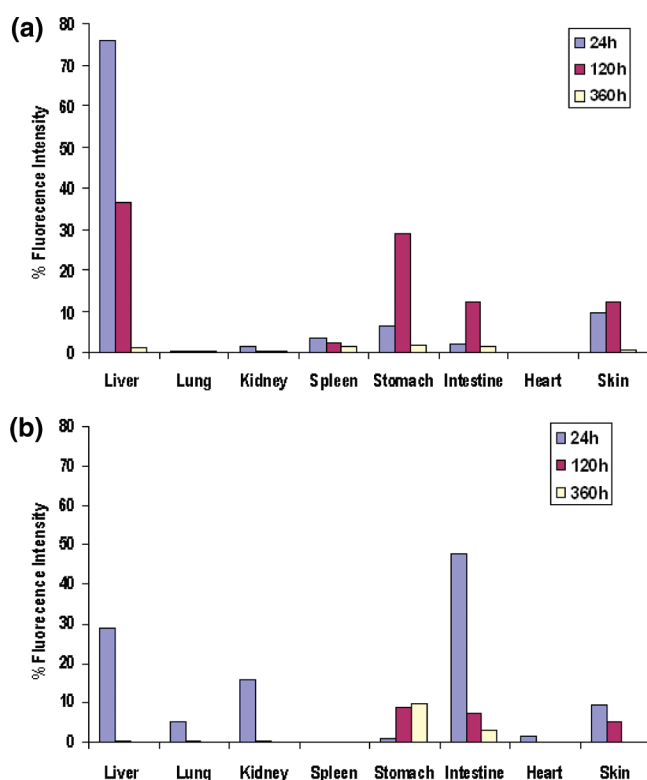


Figure 4. Quantitative estimation of fluorescence acquired from various organs of the mice injected with (a) the DY776 conjugated ORMOSIL nanoparticles and (b) free DY776, at 24, 120, and 360 h.

Figure 2b shows *via* PET imaging the biodistribution profile of the mice, intravenously injected with ^{124}I labeled nanoparticles over a period of 2–24 h. The PET images showed a very similar pattern of uptake of the nanoparticles as was obtained from the fluorescence imaging of the mice. From the images, it can be easily visualized that major percentage of nanoparticles accumulated in the liver and spleen. A quantitative assessment of radioactivity of the injected ^{124}I labeled OMRO-SIL nanoparticles is shown in Figure 2c. As shown in the Figure, liver and spleen were the major host organs for the ^{124}I ORMOSIL nanoparticles. Quantitatively, a majority of the nanoparticles accumulated in spleen (from 58% of the injected dose per gram of the organ, at the first 5 min after intravenously injection to 61% at 24 h), followed by liver (from 37% to 46%), lungs (from 9.6% to 4.7%), and kidney (from 1.93% to 1.22%).

The comparison between the biodistribution profile obtained from the two methods showed a contrast in liver to spleen uptake which can be attributed to a change in surface properties of the ^{124}I conjugated ORMOSIL nanoparticles. In the case of radiolabeled nanoparticles, the ^{124}I was conjugated on the outer surface of the nanoparticles *via* the surface NH_2 groups coupled to the Bolton hunter reagent. This might have resulted in the overall charge neutralization of the ORMOSIL nanoparticles and thus a different zeta potential value for the ^{124}I tagged ORMOSIL nanoparticles and thus different surface properties.

It is also worthwhile to note that the half-life of the ^{124}I is sufficient to perform decay-corrected gamma counting until 2 weeks post injection. However, the ^{124}I was tagged on the surface of the nanoparticles *via* the amide bond, which is pretty much more susceptible to cleavage by the various enzymes (esterases/hydrolases) and thus more labile in *in vivo* conditions. Also as we have seen from Figure 2, the major accumulation of these ORMOSIL nanoparticles occurs predominantly in liver, and it is well-known that amides are hydrolyzed in the liver.²⁵ There are several other reports in the literature, which indicate the instability of the amide bonds in *in vivo* environment.^{26,27} So the stability of the amide bond is the key concern for us to carry out the long-term biodistribution studies with the ^{124}I tagged ORMOSIL nanoparticles. Once the bond will be cleaved it will be difficult to distinguish between the ^{124}I tagged to nanoparticles from free ^{124}I . On the other hand, in the case of DY776, it is covalently conjugated inside the matrix of the ORMOSIL nanoparticles, which results in their reduced access to degrading enzymes and in the consequent higher physiological stability of these bonds. However, the rate of detachment of the linked probes may vary from species to species and also depends on the surrounding environment. More rigorous analysis is required to further understand the precise mechanism involved in the detachment of these molecules from the nanoparticles.

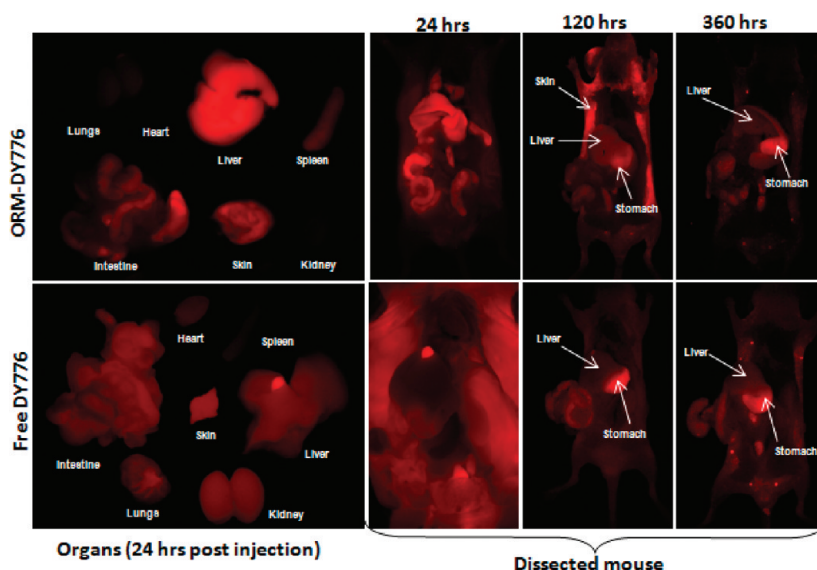


Figure 5. Fluorescence images of the organs as well as dissected mice injected with the DY776 conjugated ORMOSIL nanoparticles (upper panel) and free DY776 (lower panel) at 24, 120, and 360 h postinjection. A gradual reduction in the fluorescence intensity indicates excretion of the nanoparticles by the hepatobiliary route.

As reported previously by several authors, the nanoparticle size, composition, and surface characteristics determine their biodistribution profile and stability in the body.²⁸ It is well established that the particle size and surface composition play a significant role in various physiological parameters such as hepatic filtration, tissue extravasation, tissue diffusion, and kidney excretion.^{3,29–33} It has been shown that larger particles tend to accumulate more into the liver as compared to the smaller ones.³⁴ The enhanced uptake in the liver, spleen, and bone marrow is largely attributed to the macrophages residing in these tissues, which are responsible for capturing particulates and macromolecules circulating in the blood. When nanoparticles are systemically administered, a variety of serum proteins bind to their surface, which are recognized by the scavenger receptors on the macrophage cell surface and are removed from circulation.³⁵ The serum proteins binding to the nanoparticles are also termed “opsonins”, and the macrophages belong to the reticuloendothelial system (RES) or mononuclear phagocyte system (MPS) of the body.

The surface charge of the nanoparticles greatly affects the biodistribution of the nanoparticles. It is known that nanoparticles with neutral or zwitterionic behavior have a reduced plasma adsorption. Gbadamosi *et al.*³⁶ successfully established a linear correlation between the surface charge of the nanoparticles and the extent of phagocytosis by the macrophage cells. The surface charge of the nanoparticles can be easily modulated using hydrophilic polymer poly(ethylene glycol) (PEG). It has been well established that PEG is a classical polymer with uncharged, hydrophilic, and non-immunogenic properties, which can be physically adsorbed or covalently attached to the surface of nanoparticles.³⁷ Furthermore, PEG has been shown to

substantially reduce nonspecific interactions with proteins through its hydrophilicity and steric repulsion effects, reducing opsonization.^{38,39} The chain length, shape, and the density of PEG on the nanoparticle surface has been studied as the main parameters affecting nanoparticle’s surface hydrophilicity and phagocytosis, and thus the biodistribution of the nanoparticles in the systemic circulation.

Figure 3 shows the data on the clearance of the DY776 conjugated ORMOSIL nanoparticles from a small batch of injected mice. The injected animals were imaged daily over a period of 15 days. Free fluorophore DY776 was used as a control. It is quite evident from Figure 3 (upper panel) that animals injected with the nanoparticles initially showed a liver and spleen accumulation over a period of 24 h, whereas at 72 h post injection maximum fluorescence was acquired from the skin. Subsequent daily imaging with the same acquisition time revealed that fluorescence from the skin gradually kept on decreasing, beginning at 120 h post injection, reaching a minimum at 360 h post injection. In contrast, free DY776 injected under the same experimental conditions showed complete dominance of fluorescence from the skin as early as 24hrs post injection (Figure 3, lower panels). The reason for the high affinity of free DY776 for skin is not known. Also, here the fluorescence from the skin kept on decreasing with time, and at 360 h postinjection the fluorescence patterns for animals injected with both the free dye and the nanoconjugated dye appeared very similar. For a more quantitative estimation of fluorescence from the animals, three random time points (24, 120, and 360 h postinjection) were selected, where the mice were sacrificed and the fluorescence quantified from their harvested organs.

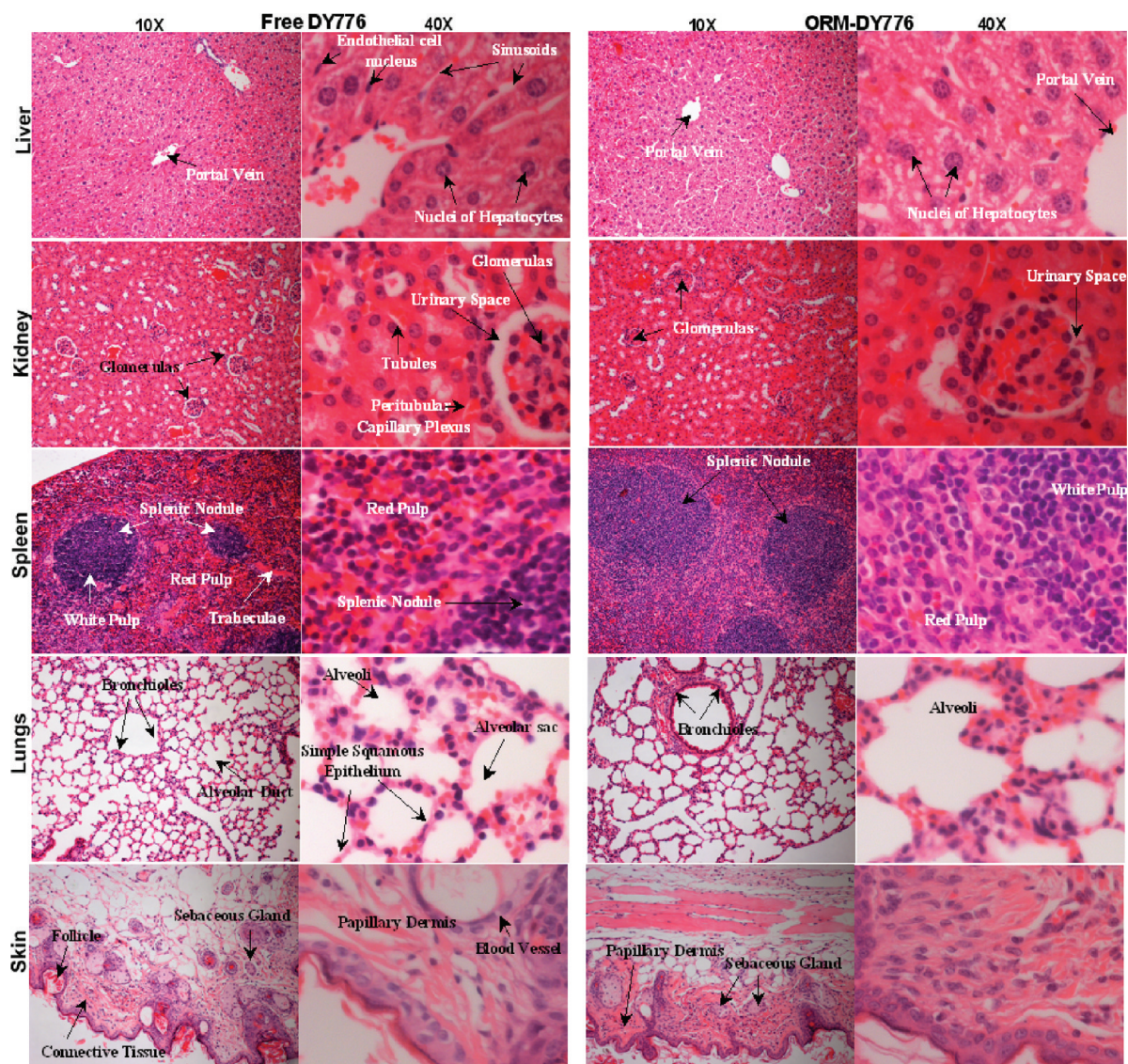
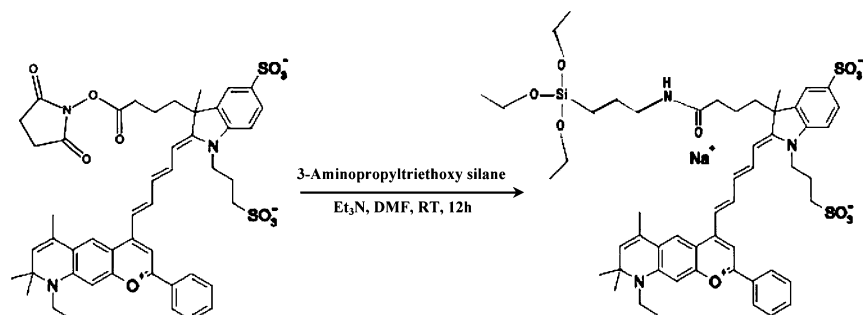


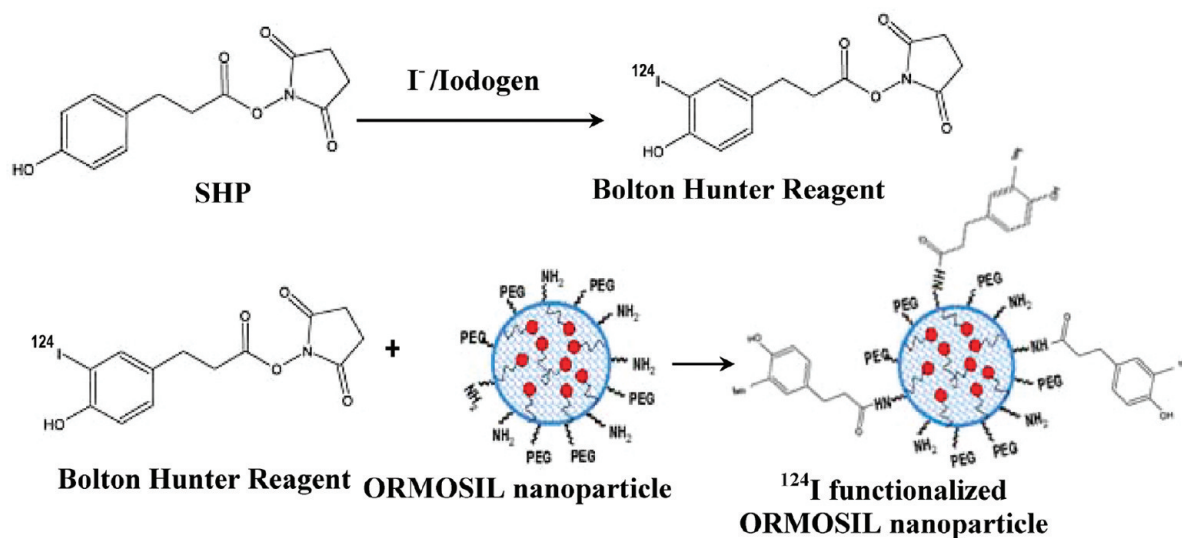
Figure 6. Histological sections of liver, kidney, spleen, lungs, and skin samples, collected after 15 days postinjections of the free DY776 (left) and DY776 conjugated ORMOSIL nanoparticles (right). Sections were stained with H&E and observed under a light microscope at 10 \times and 40 \times magnification.

It is interesting to note that in the mice injected with the DY776 conjugated nanoparticles (Figure 4a), the intensity of fluorescence (% of fluorescence of the injected dose) in the liver is reduced to almost half postinjection. This result indicates the excretion of the nanoparticles with the fecal matter *via* the hepatobiliary

transport mechanism through the stomach. Previously, Renaud *et al.* reported that low-density lipoprotein-coated gold nanoparticles at 20 nm diameter entered hepatocytes in the liver and were excreted through the fecal pathway.⁴⁰ Also, it is worthwhile to note that the intensity of fluorescence in the skin had also increased,



Scheme 1. Coupling of silane precursor, 3-aminopropyltriethoxy silane, to DY776 NHS ester to form DY776 silane precursor.



Scheme 2. Synthesis of ^{124}I labeled DY776-conjugated ORMOSIL nanoparticles *via* the Bolton–Hunter method.

which can be attributed to the release of the free fluorophore, indicating the degradation of the nanoparticles. The fluorescence intensity from the mice injected with free DY776 showed a typical accumulation pattern for small molecules. The maximum intensity after 24 h postinjection was observed from intestine ($\sim 47\%$), whereas the liver showed an intensity of 28%, followed by kidney ($\sim 15\%$), and skin ($\sim 9\%$). The fluorescence intensity after 360 h in both cases was maximum from the stomach and the intestine, which was $\sim 3\%$ (combined) in the case of mice injected with the ORMOSIL nanoparticles and $\sim 12\%$ in control mice. Other organs like liver, spleen, and skin accounts for $\sim 1\%$ (each) fluorescence intensity. This observation clearly suggests an initial uptake of the ORMOSIL nanoparticles by the RES system, followed by their gradual degradation and excretion *via* the hepatobiliary mechanism over a period of 15 days.

Figure 5 shows the fluorescence images of the dissected mice injected with DY776, both free and conjugated with nanoparticles, recorded at 24, 120, and 360 h postinjection. It can be clearly visualized that fluorescence from the liver at 24 h was maximum, which decreased after 120 h and became negligible at 360 h, whereas in the stomach it followed a reverse trend. These observations indicate excretion of the nanoparticles *via* the hepatobiliary transport mechanism.

In a similar study on the clearance of silica-based nanoparticles from animals, Burns *et al* have shown that smaller sized nanoparticles (3–6 nm) can be effectively cleared out of the animal by renal excretion.²⁹ They have also seen the retention of the bigger sized (~ 30 nm) nanoparticles into the liver, which is quite similar to our biodistribution results. Since the particle size in our case is in the range of 20–25 nm, glomerular filtration in kidney, which is primarily dependent on the size of the metabolites, is ineffective in their clearance. For larger metabolites, the preferred route of ex-

cretion is through the fecal matter, *via* the liver and the bile. Protein binding and phagocytosis by RES plays a critical role in removing the xenobiotic metabolites from the plasma and delivering them to the liver, whereby they are degraded and excreted out as feces along with bile. The present observation of the fluorescence emission from the fecal matter suggests the degradation of the nanoparticles in the liver and spleen, which is similar to the studies done earlier using other nanoparticles.^{40,41}

For any of the existing clinically approved diagnostic formulations, the most favored route of excretion is still the renal route, as opposed to the hepatobiliary route. This is because the hepatobiliary excretion is a slow process, and long-term retention in the RES increases the likelihood of organ toxicity of the injected nanoparticles. Therefore, it is critical to ensure that the injected nanoparticles, prior to their fecal excretion, did not inflict any tissue/organ toxicity. For that, we have carried out histological analysis of the major organs (liver, spleen, kidney, and lung) at 360 h post injection of the DY776 conjugated ORMOSIL nanoparticles. Tissues were harvested and fixed in 10% neutral buffered formalin, processed routinely into paraffin, and 4 μm sections were cut and stained with hematoxylin and eosin (H&E) and examined using light microscopy. As seen in Figure 6, no apparent tissue/cellular damages were observed in the mice injected with the DY776 conjugated nanoparticles, when compared to that obtained from mice injected with free DY776. Normal liver macrophages showed no evidence of cell injury. No pathological changes were observed in the spleen, kidney, liver, and lungs. Tissue sections were also studied for fluorescence; absence of any fluorescence from the tissues confirmed the clearance of the nanoparticles from these organs at 15 days post injection. Furthermore, mice injected with the ORMOSIL nanoparticles underwent physical and neurological evaluations for

more than one month. No changes in eating, drinking, grooming, exploratory behavior, activity, physical features (e.g., weight and skin color), and neurological status were observed in these.

CONCLUSIONS

We report the synthesis of NIR fluorophore DY776 conjugated ORMOSIL nanoparticles by a simple normal micellar route, which can also be subsequently radiolabeled with iodine-124. The biodistribution of the nanoparticles by NIR optical and radiolabeling studies suggests accumulation of the nanoparticles

in the major RES organs in mice. The clearance studies of the injected nanoparticles have indicated that almost 100% of the nanoparticles were effectively cleared out of the animal *via* the hepatobiliary excretion, without any sign of organ toxicity. These studies provide preliminary answers to some of the key biological issues raised over the use and safety of the silica-based nanoparticles for diagnostic and therapeutic applications. Overall, the results demonstrate the use of these NIR dye and iodine-124 conjugated ORMOSIL nanoparticles as promising probes for safe *in vivo* bioimaging.

EXPERIMENTAL SECTION

Materials and Methods. ORMOSIL precursors vinyltriethoxysilane (VTES) and aminopropyltriethoxysilane (APTES) as well as the surfactant AOT were purchased from Sigma-Aldrich, and 2-[methoxy(polyethylenoxy)propyl]trimethoxy silane (PEG-silane) was procured from Gelest, Inc. The NIR fluorophore DY776-*N*-succinimidyl ester (DY776-NHS) was obtained from Dyomics GmbH. The pancreatic cancer cell line Panc 1 was obtained from ATCC (Manassas, VA) and cultured according to vendor's instructions. Unless otherwise mentioned, all cell culture products and antibodies were obtained from Invitrogen.

Synthesis of DY776 Conjugated ORMOSIL Nanoparticles. Amino-terminated ORMOSIL nanoparticles with covalently incorporated NIR fluorophore, DY776, were synthesized in the nonpolar core of an oil-in-water microemulsion, as per our earlier report but with a slight modification.¹⁵ Briefly, 0.2 g of surfactant AOT and 300 μ L of *n*-butanol and 100 μ L of DMSO were dissolved in 10 mL HPLC grade water by magnetic stirring. To this microemulsion system, 100 μ L of 1 mg/mL of DY776-silane precursor (DY776 NHS ester, conjugated to aminopropyltriethoxy silane; Scheme 1a) in DMSO was added and the mixture was stirred. This was followed by the addition of 100 μ L of neat VTES. The reaction mixture was stirred for another hour, and the polymerization reaction was started by the addition of 10 μ L of aminopropyltriethoxysilane. For incorporation of the PEG molecules on the surface of the nanoparticles, 10 μ L of PEG silane was added after 30 min of stirring. The final reaction mixture was allowed to stir overnight. The mixture was then dialyzed against distilled water for 48 h at room temperature, using a cellulose membrane with a cutoff size of 12–14 kD. Following dialysis, the nanoparticles were sterile filtered and stored at 4 °C for future use.

¹²⁴I Radiolabeling of ORMOSIL Nanoparticles. The radiolabeling protocol is an adaptation of the well-known Bolton–Hunter method, whereby the free amino groups on the nanoparticle surface will form a covalent linkage with the *N*-hydroxysuccinimide (NHS) ester group linked with ¹²⁴I (¹²⁴I-NHS).⁴² For the synthesis of ¹²⁴I-NHS, ¹²⁴I was distilled and dried in a small reaction vial (0.3 mL, Wheaton, USA). A solution of NaI carrier (5 μ L, 89.2 μ M), HCl (14 μ L, 100 mM), and sodium phosphate buffer (10 μ L, 250 mM, pH 7.4) was added to ¹²⁴I (14 μ L, 370 MBq). The reaction was allowed to incubate at room temperature for 10 min. SHPP (*N*-succinimidyl-3-[4-hydroxyphenyl]propionate in 1,4-dioxane, 2 μ L, 3.8 mM) and chloramine-T (10 μ L, 22 mM), dissolved in sodium phosphate buffer (250 mM, pH 7.4), were added with mixing, and the reaction was terminated immediately (~15 s) with a sodium phosphate buffered solution (250 mM, pH 7.4) of Na₂S₂O₅ (10 μ L, 63 mM). The reaction product was extracted with benzene/dimethylfluoride (100 μ L, 40:1 v/v), dried with 30 mg Na₂SO₄, and transferred into another reaction vessel (Scheme 2).

For conjugation with the nanoparticles, the solvent was evaporated with a stream of nitrogen and 2 mL of amino-terminated ORMOSIL nanoparticle dispersion was added. The solution was left for stirring overnight at room temperature. The fi-

nal dispersion of radiolabeled ORMOSIL nanoparticles was purified from any nonreacted ¹²⁴I-NHS by dialysis using a cellulose membrane with a cutoff size of 12–14 kD for 24 h at room temperature.

Animal Model. Animal experiments were conducted in agreement with all relevant guidelines and regulations set by the University at Buffalo and with approved institutional protocols by the American Association of Laboratory Animal Care. Six-to-seven week old female athymic nude mice (Hsd:ATHymic Nude-Foxn1tm) were procured from Harlan Sprague–Dawley Inc., and were housed in a group of five in standard cages with free access to food and water and a 12 h light/dark cycle. All animals acclimated to the animal facility for at least 48 h before experimentation. All possible parameters that may cause social stress, like group size, type (treated and nontreated), etc., among the experimental animals were carefully monitored and avoided. Animals were observed daily for any behavioral abnormalities and weighed weekly.

ORMOSIL Nanoparticles Biodistribution and Clearance Studies. For biodistribution and clearance studies, isotonic dispersion (5% glucose) of the above-synthesized ORMOSIL nanoparticles (nonradiolabeled) were injected intravenously in nude mice ($n = 3$) at a concentration of 2.0 mg/kg body weight. For the control group ($n = 3$) of mice, DY776-NHS ester was injected *i.v.* after matching their absorbance with that of DY776 conjugated with ORMOSIL nanoparticles. Also, 100 μ L of sterile PBS was injected *i.v.* into a negative control mice group. The mice were imaged every 24 h until 15 days, using the Maestro GNIR FLEX fluorescence imaging system (CRI) (for details, see Supporting Information). The NIR fluorophore DY776 was excited using a "NIR" excitation filter (CRI), transmitting light from the source (Xe lamp) in the range of 710–760 nm. An NIR emission filter (800 LP) in front of the imaging CCD camera was used to cut off the excitation light. The images were acquired with the same acquisition time of 623 ms for all the experimental animals. A quantitative estimation of the fluorescence intensity was done using the Maestro software. For probing any potential *in vivo* toxicity of the injected nanoparticles, the mice were anesthetized and sacrificed at different intervals, and major organs such as liver, spleen, kidney, lungs, heart, and skin were dissected out for further histological analysis.

For biodistribution studies using the ¹²⁴I labeled and dyed-doped ORMOSIL nanoparticles, 200 μ Ci of radiolabeled nanoparticles were injected intravenously through tail vein in mice ($n = 3$). MicroPET imaging was carried out with a microPET camera, Focus 120 (Siemens Concorde Microsystems, previously CTI Concorde Microsystems). Before scanning, the mice were anesthetized with O₂/isoflurane (1%–3% isoflurane). The mouse was imaged in the prone position in the gantry of the microPET scanner and was scanned for 30 min. The emission scan window was set between 350 and 750 keV, and the analysis of the acquired data and image reconstruction was done with 2D filtered back projection, using the software microPET manager 2.2.4.0. The mice were imaged and sacrificed at 10 min and 24 h postinjection. Organs of interest (tumor, heart, liver, spleen, lungs, muscle, gut, kidney, blood, and stomach) were immediately dissected

out and weighed for quantitative estimation of gamma counts from the ^{124}I conjugated to ORMOSIL nanoparticles using a gamma scintillator. Radioactivity obtained from different organs was calculated as the percentage of the injected dose per gram of the tissue (%ID/g).

Histological Analysis. For histochemical studies, the major organs such as liver, spleen, kidney, heart, lung, and skin were harvested and fixed in 10% neutral buffered formalin. Following that, they were embedded in paraffin, and approximately 4 μm thick sections were cut and stained with hematoxylin and eosin (H&E). The histological sections were observed under an optical microscope with different combinations of magnification and objective lens. Micrographs of the sections were recorded for comparison.

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Supporting Information Available: Description of the experimental details and MTS assay results. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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