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Pharmacokinetics and biodistribution of near-infrared fluorescence polymeric nanoparticles

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Abstract

There has been increased interest in the use of polymeric nanoparticles as carriers for near-infrared (NIR) fluorescence dyes for cancer diagnosis. However, efficient delivery of nanoparticles to the tumors after systemic administration is limited by various biobarriers. In this study, we investigated the pharmacokinetics, biodistribution, and tumor uptake of sub-nanometer sized polymeric nanoparticles (<100 nm in diameter) coated with polyethylene glycol in tumor-bearing mice. To facilitate our studies, these particles were labeled with gamma emitter indium-111. We found that two NIRF nanoparticles having the same size (~20 nm) and chemical composition but different structures (i.e., hydrogel vs. core-shell nanolatex), or the same core-shell nanolatex particles with different sizes (20, 30, and 60 nm), had different blood circulation times, biodistribution, and tumor uptake. Interestingly, the tumor uptake of the nanolatex particles correlated well with their blood residence times ($R^2 = 0.95$), but similar correlations were not found between nanogel and nanolatex particles ($R^2 = 0.05$). These results suggest that both the blood circulation time and the extent of hydration of the nanoparticles play an important role in the tumor uptake of nanoparticles. Prolonged blood circulation of these NIRF nanoparticles allowed clear visualization of tumors with γ -scintigraphy and optical imaging after intravenous administration. A better understanding with regard to how the characteristics of nanoparticles influence their *in vivo* behavior is an important step towards designing NIRF nanoparticles suitable for molecular imaging applications and for efficient tumor delivery.

Keywords

Fluorescence; Optical imaging; Nanoparticle; Biodistribution; Pharmacokinetics

1. Introduction

Optical imaging encompasses many different techniques that use the absorption, scattering, or fluorescence of light to visualize the characteristics of tissues [1–3]. Significant advances in this field include the development of NIRF imaging, an optical imaging method capable of visualizing up to several centimeters of tissues, enabling highly sensitive detection of early-stage disease and the assessment of treatment response. One prerequisite for NIRF imaging is

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the development of fluorophores that emit light in the wavelength range of 700–850 nm (the near-infrared window), have a high molar absorption coefficient and fluorescence quantum yield, and possess adequate photostability and low cytotoxicity. In recent years, nanoparticles containing near-infrared fluorophores have been explored for noninvasive molecular imaging [4–7]. Advantages of NIRF nanoparticles include their versatility, stability, and the ease with which their surface can be modified.

Despite the recognized potential of NIRF nanoparticles in molecular imaging, only few polymeric nanoparticles loaded with NIRF dyes are suitable for *in vivo* imaging applications. One of the critical challenges in designing these nanoparticles is ensuring their efficient delivery to the tumors. The current problem with most nanoparticles is that they are rapidly removed by the cells of the reticuloendothelial system (RES) upon systemic administration, which diminishes the chances of the nanoparticles being directed to sites other than the RES. A number of factors, such as size, composition, and surface hydrophilicity, can influence the pharmacokinetics of nanoparticles. In particular, modification with polyethylene glycol (PEG) has been shown to be an efficient means of reducing the uptake of nanoparticles by the RES, which consequently increases the nanoparticles' blood circulation time [8–13]. In addition to blood circulation half-life, another key determinant that governs the uptake of nanoparticles by tumors is their efficient extravasation from tumor blood vessels. Here, the size of nanoparticles is considered one of the most important factors. Specifically, nanoparticles must not be larger than 100–400 nm in diameter to cross the tumor vessel wall, depending on the tumor type and microenvironment [14–17]. In our previous study with NIRF core-crosslinked polymeric micelles based on poly(PEG-methacrylate)-*b*-poly(triethoxysilyl propylmethacrylate) block copolymer, we showed that long-circulating nanoparticles with average diameters of ~20 nm can readily extravasate from tumor vessels [5]. However, it is not clear how variation in size for particles in the sub-nanometer range (particles with diameters of 20 to 100 nm) may affect their pharmacokinetic and biodistribution pattern. Increasing the size of the nanoparticles without adversely affecting tumor targeting capability should allow higher loading of dyes for each particle and thus fabrication of brighter NIRF nanoparticles. In this study, we investigated how the size and variation in surface chemistry of nanoparticles influence their pharmacokinetics and delivery to solid tumors in a subcutaneous human breast tumor xenograft model. Toward this end, we used core-shell nanolatex particles that are structurally similar to core-crosslinked polymeric micelles, but with the added ability to control particle size at will. In addition, a highly swollable NIRF nanogel particles without hydrophobic core was included in the current study to examine how variation in surface chemistry and/or degree of hydration affect nanoparticles' *in vivo* behavior.

2. Experimental details

2.1. Materials

NIRF nanoparticles derived from the free radical copolymerization of amine-terminated PEG methacrylate macromonomer and alkyl methacrylate were obtained from Carestream Health Inc. (Rochester, NY, USA). The hydrogel nanoparticulate system, designated as “nanogel,” was synthesized using hydrophilic hydroxyethyl methacrylate as a co-monomer and methylenebisacrylamide as the crosslinker. The core-shell nanoparticulate system, designated as “nanolatex,” used hydrophobic methoxyethylmethacrylate as a co-monomer and divinylbenzene as the crosslinker. In nanogel, a water-soluble Cy7-like NIRF dye (Carestream Health) was chemically conjugated to the particles, while in nanolatex, a liposoluble Cy7-like NIRF dye (Carestream Health) was physically entrapped in the hydrophobic core of the particles. Detailed description of the synthetic procedures is disclosed in the patent literature [18]. Both nanogel and nanolatex had amine-terminated PEG chains covering the surface of the particles. The amine groups at the terminus of PEG chains acted as an anchor for the attachment of radiometal chelator diethylenetriaminepentaacetic acid (DTPA). DTPA was

conjugated to surface amines by reacting the nanoparticles with *p*-isothiocyanatobenzyl-DTPA (DTPA-Bz-NCS, Macrocylics, Inc., Dallas, TX, USA). Figure 1 depicts the reaction scheme and hypothetical structures of nanogel and nanolatex.

2.2. Analytical methods

The molecular weight of polymeric nanoparticles was characterized using gel permeation chromatography (GPC) according to the methods of Mourey et al. [19]. Briefly, GPC was performed on a system consisting of an Alliance 2695 separations module, a model 2487 spectrophotometric detector, and a model 2410 differential refractometer detector (all from Waters Corp., Milford, MA, USA); a PD2020 two-angle dynamic light scattering detector (Precision Detectors, Bellingham, MA, USA); and a model H502A differential viscometry detector (Viscotek, Porter, TX, USA). The samples were analyzed in 1,1,1,3,3,3-hexafluoroisopropanol containing 0.01 M tetraethylammonium nitrate at 40.0 °C using two PLgel mixed-C columns (Polymer Laboratories, Church Stretton, UK) at a flow rate of 0.5 mL/min. Absolute molecular weights were calculated from the viscosity data, and a universal calibration curve was constructed from narrow-molecular-weight-distribution poly(methyl methacrylate) standards. Although the number-average molecular weight (M_n) is the correct quantity to use for estimating the quantities of dye, PEG, or DTPA per particle, we found that it was difficult to determine M_n accurately, because small changes in the baseline of the GPC plot (that is, in how a peak is defined) could translate into large changes in the M_n value. Similarly, very small changes in the low-molecular-weight region of the curve (for example, resulting from the presence of residual soluble PEG) could produce a very large change in M_n . We therefore used the peak average molecular weight (M_p), which can be directly measured by size-exclusion chromatography with viscometry and light scattering detection, to estimate the quantities of dye, PEG, or DTPA per particle (see the following section).

Dynamic light scattering was performed on the ZetaSizer Nano-ZS, ZEN9061 (Malvern Instruments Ltd., Worcestershire, UK). The zeta potential was determined using a ZetaPlus Analyzer (Brookhaven Instruments Corp., Holtsville, NY, USA). Fluorescence properties were measured on a Fluorolog-3 spectrophotometer (Jobin Yvon Inc., Edison, NJ, USA). Transmission electron microscopy (TEM) was performed with a JEM 1010 transmission electron microscope (JEOL USA, Inc., Peabody, MA, USA) at an accelerating voltage of 80 kV. For sample analysis, a drop of the sample solution was placed on a 400 mesh copper grid coated with a 0.5 wt% poly(vinyl formal) aqueous solution. About 5 min after deposition, the sample solution was removed by using filter paper. Negative staining was performed using a droplet of 1% uranyl acetate solution.

2.3. Estimation of PEG, DTPA, and Cy7-like dyes per particle

The number of PEGs per particle was estimated using the molecular weight (MW) of a particle (M_p), as determined by GPC, and the molar ratios in the monomer feeds: number of PEG/particle = $M_p / (\text{MW of PEG} + \text{MW of methacrylate} \times \text{molar ratio of PEG to methacrylate})$.

DTPA loading per particle was estimated from the amount of total DTPA-Bz-NCS added before the reaction and the amount of unreacted DTPA-Bz-NCS after the reaction. The unreacted DTPA-Bz-NCS was quantified by capillary electrophoresis using a Beckman Coulter P/ACE MDQ system (Fullerton, CA, USA). Data acquisition was performed using a Dionex Universal Chromatography Interface, UCI-100, employing Dionex Chromeleon software, version 6.6 (Dionex Corp., Sunnyvale, CA, USA). The system was eluted with 10.0 mM acetate buffer (pH 4.75) containing 10 mM copper sulfate and 3.0 mM myristyltrimethylammonium bromide at 25 °C. The DTPA-Cu complex that formed permitted direct ultraviolet detection at 254 nm. DTPA was added as an internal standard at a concentration of 5.0 mg/L. Both hydrolyzed product DTPA and unhydrolyzed product DTPA-

Bz-NCS were quantified, and the sum of both products was used in the calculation. Attachment rates of ~37% for the nanogel and ~28% for the nanolatex were obtained. These values, along with the Mp, were used to calculate the number of DTPA units per particle: number of DTPA/particle = reacted DTPA in moles/(dry particle weight/Mp).

From the absorbance of the unreacted dye in the permeate of centrifugal filtration, the attachment yield of the Cy7-like dye to the amine-functionalized nanogel was determined to be 81.7%. The number of Cy7-like dye per nanogel particle was determined using this conversion value. For nanolatex, the ratio of Cy7-like dye per particle was determined using the initial quantities of dye and nanolatex that were added, assuming that 100% of the dye was incorporated into nanolatex.

2.4. Nanoparticle stability

Each nanoparticle preparation at a concentration of 4.9×10^{14} particles/mL was stored at 4 °C in phosphate-buffered saline (PBS) protected from light. Fluorescence spectra were obtained after 3 months of storage. The nanogel and nanolatex samples were excited at 753 nm and 760 nm, respectively. The peak intensities were quantified, and the data were expressed as a percentage of the peak area at day 0 of incubation.

2.5. Radiolabeling

Radiotracer counting is a more reliable method than measurement of fluorescence intensity, and therefore the preferred method for subsequent quantification [5]. For this reason, biodistribution studies were performed using ^{111}In -labeled NIRF nanoparticles. Aliquots of DTPA-nanogel and DTPA-nanolatex (4×10^{15} particles/mL) in 0.1 M sodium acetate solution (pH 5.2) were mixed with an aqueous solution of $^{111}\text{InCl}_3$ (~860 μCi) (Iso-Tex Diagnostics, Inc., Houston, TX, USA) for 30 min. Radiolabeled nanoparticles were purified by GPC using a PD-10 column. The purified nanoparticles were analyzed using instant thin-layer chromatography (ITLC). The ITLC strips were developed with PBS (pH 7.4) containing 4 mM ethylenediaminetetraacetic acid (EDTA) and quantified using a Bioscan IAR-2000 TLC imaging scanner (Washington, DC, USA). Free $^{111}\text{In}^{3+}$ moved to the solvent front ($R_f = 0.9$ – 1.0), and the nanoparticles remained at the original spot ($R_f = 0.0$). The efficiency of radiolabeling was 50%–70%, and the radiochemical purity of all nanoparticles after GPC purification was >95%.

2.6. Cell lines

Human breast cancer cell line MDA-MB-468 and mouse macrophage and monocyte cell line (RAW264.7) were obtained from American Type Cell Culture (Rockville, MD, USA). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium and nutrient mixture F-12 Ham containing 10% fetal bovine serum (GIBCO, Grand Island, NY, USA).

2.7. Cell uptake

About 30 000 RAW264.7 cells per well were seeded in 96-well plates. Two days later, after pre-incubation with Hanks' balance salt solution (HBSS) without phenol red for 15 min, wells were replaced with various NIRF nanoparticles in HBSS (100 μL) at a concentration of 8.4×10^{13} /mL. After 1 or 2 h of incubation at 37 °C (five wells for each sample at each time point), the cells were washed three times with ice-cold HBSS. After decanting the washing solution, 50 μL of CellLytic M cell lysis reagent (Sigma, MO, USA) was added and lysis allowed to occur for 15 min at room temperature. After the addition of 100 μL of H_2O , the whole solution was transferred into white 96-well polystyrene plates. The fluorescent intensity was measured using a TECAN microplate reader (San Jose, CA). The measurement parameters were set as

follows: fluorescence excitation wavelength: 755 nm; emission wavelength: 781 nm; gain (manual): 120. A standard curve for each nanoparticle was constructed by adding different numbers of nanoparticles in HBSS into blank cells. The standard was run at the same time and treated in a similar way as the samples. Afterwards, 20 μ L of RAW264.7 cell lysates were analyzed to determine the total cell protein content using the Bio-Rad protein assay kit (200 μ L, 1:5 dilution) (Hercules, CA, USA). The absorbance was measured at 595 nm after an 10-min incubation at room temperature (in Bradford reading mode). A standard curve was obtained with a bovine serum albumin solution. The uptake of nanoparticles was calculated from the standard curve and expressed as the number of nanoparticles taken up per μ g of cell protein. NIRF silica nanoparticles (Carestream Health) with an average diameter of $\sim$ 100 nm were used as a positive control.

2.8. Pharmacokinetic studies

All animal work was performed in the Small Animal Imaging Facility at The University of Texas M. D. Anderson Cancer Center in accordance with institutional guidelines. Healthy female BALB/c mice (22–25 g; Charles River Laboratories, Wilmington, MA, USA) were divided into 4 groups with each consisting of 5–7 mice. Mice in each group were injected with each radiolabeled nanoparticles intravenously at a dose of 5×10^{14} particles per mouse (65–80 μ Ci/mouse). At predetermined intervals, 10 μ L of blood samples was taken from the tail vein, and the radioactivity of each sample was measured with a Cobra Autogamma counter (Packard, Downers Grove, IL, USA). The blood pharmacokinetic parameters for the radiotracer were analyzed with a two-compartmental model using WinNonlin 4.1 software (Pharsight Corp., Palo Alto, CA, USA). Animals were euthanized by CO₂ exposure 48 h after injection.

2.9. Biodistribution

MDA-MB-468 tumors were inoculated subcutaneously in the right thighs of nude mice (18–25 g; Harlan Sprague Dawley, Inc., Indianapolis, IN, USA) by injecting 1×10^6 viable tumor cells in suspension in PBS. When tumors had grown to 6–8 mm in diameter, mice were randomly assigned to 4 groups of 3 mice each. Mice in each group were injected intravenously with ¹¹¹In-labeled nanogel-20, nanolatex20, nanolatex-30, or nanolatex-60 at a dose of 5×10^{14} particles/mouse (15 μ Ci/mouse). Animals were euthanized by CO₂ exposure 48 h after injection. Blood, lung, muscle, spleen, kidney, liver, and tumor tissues were removed, weighed, and counted for radioactivity with a Packard Cobra gamma counter (Downers Grove, IL). Uptakes of nanoparticles in various tissues were calculated as the percentage of the injected dose per gram of tissue (%ID/g).

2.10. Imaging

Nude mice bearing subcutaneous MDA-MB-468 tumors on their upper arm, in two groups of three mice each, were injected intravenously with either ¹¹¹In-labeled nanogel or ¹¹¹In-labeled nanolatex at a dose of 5×10^{14} particles/mouse (65–80 μ Ci/mouse). For both nuclear and optical imaging, mice were placed in the anterior position. Prior to imaging, mice were anesthetized with 2% isoflurane gas (Iso-Thesia, Rockville, NY, USA) in oxygen. During imaging, mice were maintained in an anesthetized state with 0.5%–1.5% isoflurane in oxygen. Gamma images were acquired using an M-Cam gamma camera equipped with a medium-energy collimator and ICON software (Siemens, Hoffman Estates, IL, USA). Images were acquired with the following parameters: matrix: 512×512 pixels; zoom: 3.20; energy peak: 247 KeV (15%) and 172 KeV (15%); total counts: 500 000. Whole-body optical imaging was done at various time points up to 48 h after injection using a Kodak Molecular Imaging System (New Haven, CT, USA) equipped with Cy7 filter sets (excitation, 730; emission, 790 nm). The field of view was 12.0 cm in diameter. The camera was set at 4×4 binning. For *ex vivo* imaging, various organs resected at the end of the imaging study (48 h after injection) were imaged with a Xenogen

IVIS-200 imaging system (Alameda, CA, USA) with ICG filter sets (excitation, 710–760; emission, 810–875 nm). The camera settings included maximum gain, 2×2 binning, 640×480 pixel resolution, and an exposure time of 0.8 s.

2.11. Statistical Analysis

Statistical analysis of pharmacokinetic parameters was conducted using analysis of variance (ANOVA) with the post hoc Tukey test (SYSTAT[®] for Windows 11, SYSTAT Software, Inc., San Jose, CA). Differences in biodistribution data were analyzed using a two-tailed unpaired Student's *t*-test, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Physicochemical properties

The physicochemical properties of the NIRF nanoparticles used in this study are summarized in Table 1. To distinguish nanoparticles of different sizes in this report of our findings, the average diameter of each NIRF nanoparticle is added after its name. As shown in Figure 2A, both nanogel-20 and nanolatex-20 had the same volume-average diameter and similar size distributions. The size of nanolatex-30 and nanolatex-60 particles was further confirmed by TEM, which showed a relatively uniform size distribution of these nanoparticles (Fig. 2B). At pH 7.4, all NIRF nanoparticles were negatively charged.

The emission intensity of nanolatex-20 was about 3.8 times higher than that of nanogel-20 when both were measured at a nanoparticle concentration of 2.54×10^{13} particles/mL. Each nanogel-20 particle contained 16 Cy7-like molecules, and each nanolatex-20 particle contained seven Cy7-like molecules. Thus, the Cy7-like dye in a nanolatex-20 particle was about 8.7 times brighter than that in a nanogel-20 particle. It may be that the fluorescence quantum yield of Cy7-like dye was reduced upon chemical conjugation and that this caused a reduction in the fluorescent intensity of nanogel-20. Alternatively, the significant loss of fluorescence signal observed in nanogel may be due to the stronger self-quenching effect that Cy7-like dyes have in nanogel compared to the same effect in nanolatex. This effect is likely due to the Cy-7-like dye being attached to one terminus of the flexible PEG chain in the nanogel, giving those dye molecules more chances to interact with other dye molecules, while the Cy-7 dye molecules entrapped in the hydrophobic core of the nanolatex particles are less mobile.

The dry weights of equal concentration of nanogel-20 and nanolatex-20 particles were 0.12 mg/nmol and 1.4 mg/nmol, respectively, indicating that nanogel-20 was significantly more hydrated than nanolatex-20. Both nanogel and nanolatex were optically stable when appropriately stored. About 94.2% of the fluorescence intensity for nanogel and 91.1% of the fluorescence intensity for nanolatex were retained after nanoparticle suspensions were stored at 4 °C in the dark for 3 months.

3.2. Cellular Uptake

All nanoparticles had significantly lower uptake than 100-nm silica-based NIRF nanoparticles, which served as a positive control. However, there was no significant difference in cell uptake between nanogel-20 and nanolatex-20 after 1 h and 2 h of incubation. In addition, we did not observe a strong size-dependent cellular uptake in the nanolatex series (Fig. 3). This contrasts with the results obtained from gold nanoparticles in the absence of PEG [20]. In that study, the cellular uptake of gold nanoparticles between 10–100 nm in diameter was strongly dependent upon particle size, with maximum uptake by a cell occurring at a nanoparticle size of 50 nm.

3.3. Pharmacokinetics

Figure 4 compares the activity-time profile of ^{111}In -labeled nanogel and nanolatex particles. A two-compartmental model was used to describe the blood pharmacokinetics of both agents. Model parameters were obtained by fitting the percentage of injected dose per mL of blood (% ID/mL) versus time data to the following equation:

$$C_t(\% \text{ID/mL blood}) = Ae^{-\alpha t} + Be^{-\beta t}$$

where A and B are the y intercepts of extrapolated lines for the central and tissue compartments, respectively, and α and β are first-order rate constants for the central and tissue compartments, respectively. The mean equations for ^{111}In -labeled nanogel-20, nanolatex-20, nanolatex-30, and nanolatex-60 were: $C_t = 25.69e^{-0.38t} + 11.68e^{-0.029t}$, $C_t = 26.04e^{-0.37t} + 9.81e^{-0.05t}$, $C_t = 19.98e^{-0.40t} + 47.18e^{-0.05t}$, and $C_t = 39.36e^{-1.48t} + 41.27e^{-0.04t}$, respectively. The other pharmacokinetic parameters are summarized in Table 2. The comparison of nanoparticles of the same size showed that nanogel-20 exhibited a longer blood terminal elimination half-life than nanolatex-20 in the clearance phase ($t_{1/2,\beta}$ 28.9 h vs. 15.2 h). The mean residence time (MRT) of nanogel-20 was also significantly longer than nanolatex-20 (34.1 h vs. 15.8 h). However, nanoparticle size does not have significant impact on the MRT among the nanolatex particles, despite an increased trend with the larger particle size. The mean volume of distribution of the central compartment (V_c) and at steady-state (V_{dss}) was similar between nanogel-20 and nanolatex-20 particles, but was significantly lower in larger particle sizes. The mean systemic clearance (CL) was significantly lower in nanogel-20 particles as compared to nanolatex-20 (0.21 vs. 0.35 % ID h/mL blood). Among nanolatex particles, the mean CL decreased significantly in nanolatex-30 and nanolatex-60 particles as compared with the nanolatex-20 particles (Table 2).

3.4. Biodistribution

Figure 5 compares the biodistributions of nanogel-20 and nanolatex-20, as well as the size series of nanolatex particles 48 h after injection. The %ID/g tissue values in the liver and the spleen were 25.4% and 4.98%, respectively, for nanogel-20, and 22.3% and 10.02%, respectively, for nanolatex-20. Nanogel-20 had significantly lower spleen uptake than that of the nanolatex-20 ($p = 0.034$). On the other hand, nanogel-20 had significantly higher activity in the blood ($p = 0.004$) and the lung ($p = 0.020$) than did nanolatex-20; nanogel-20 activity was also moderately, but not significantly, higher in the tumor ($p = 0.058$). The higher activity observed in the lung and tumor may have resulted from the higher blood pool activity of the nanogel-20 (Fig. 5A).

The distribution patterns for nanolatex particles of different sizes were similar, with the liver and the spleen being the major organs taking up the radiolabeled NIRF nanoparticles. However, nanolatex-20 had a significantly higher uptake in the liver than did nanolatex-30 ($p = 0.016$) and nanolatex-60 ($p = 0.0087$). Nanolatex-20 also had a significantly lower uptake in the kidney than nanolatex-30 ($p = 0.009$) and nanolatex-60 ($p = 0.0005$). Nanolatex-60 had significantly higher blood activity than nanolatex-30 ($p = 0.003$) and nanolatex-20 ($p = 0.019$). Tumor uptake of nanolatex particles increased with particle size (Fig. 5B). Nanolatex-60 had significantly higher uptake in the tumor than did nanolatex-20 ($p = 0.009$), although the difference in tumor uptake between nanolatex-60 and nanolatex-30 was not significant ($p = 0.099$).

3.5. In vivo γ - and NIRF optical imaging

Figure 6A shows representative dynamic γ -images of a mouse injected with nanolatex-60. The relatively high radioactivity in the cardiac area at the early time point (up to 4 h after injection) indicates the high blood circulation activity of the nanoparticles. By 24 h after injection, the

nanoparticles were mostly deposited in the liver and the spleen. Tumor uptake of the nanoparticles was clearly visualized at 24 and 48 h after nanoparticle injection. Radioactivity in the bladder observed at earlier time points (15 min and 1 h after injection) suggests that a fraction of small nanoparticles was cleared through the kidney (Fig. 6A).

Whereas tumor uptake of nanolatex-60 could not be delineated with γ -imaging until 24 h after injection, the accumulation of these nanoparticles in the tumors was readily visualized by NIRF imaging as early as 4 h after injection (Fig. 6B). In agreement with the biodistribution data obtained using the radioactivity count method, *ex vivo* optical imaging of excised tissues showed that nanolatex-60 was primarily removed by the liver. The average signal intensity of nanolatex-60 in various organ decreased in the order of liver > lung > spleen ~ tumor ~ intestine > bone > kidney > blood ~ heart > muscle (Fig. 6C).

The imaging properties of the other NIRF nanoparticles used in the current study were similar to that observed with nanolatex-60.

4. Discussion

In this paper, two series of PEGylated polymeric nanoparticles containing NIRF dyes were evaluated with regard to their pharmacokinetics and biodistribution. Although the plasma half-life of unmodified (without PEG coating) polymeric nanoparticles usually ranges from 1–10 min [13,21], all NIRF nanogel and nanolatex particles exhibited extended blood half-lives, with distribution phase $t_{1/2,\alpha}$ values varying from 30–140 min and the clearance phase $t_{1/2,\beta}$ values varying from 15.0–28.9 h. The prolonged blood half-lives of nanogel and nanolatex particles were most likely due to the presence of PEG chains on the nanoparticles' surfaces, which decreased their recognition and uptake by macrophages (Fig. 3) and subsequent sequestration by RES organs.

Both nanogel-20 and nanolatex-20 were prepared with a similar free radical-mediated emulsion polymerization reaction, resulting in their surfaces' being coated with the same PEG chains chemically crosslinked to methacrylate. In spite of the similarity in size, size distribution, and surface chemistry, nanogel-20 exhibited a significantly longer terminal elimination half-life than nanolatex-20 ($t_{1/2,\beta}$ = 28.9 h vs 15.2 h). Because no changes in the volume of distribution were observed, the data suggest that nanogel formulations eliminated slower *in vivo* than nanolatex formulations. The classic biodistribution study, performed through the cut-and-count method, confirmed significantly higher blood activity for nanogel-20 than nanolatex-20 at 48 h after intravenous injection. In addition to blood activity, nanogel-20 and nanolatex-20 also differed significantly in their uptakes in such organs as spleen and lung (Fig. 5A). Several factors may have contributed to these differences. It is well established that surface charges of nanoparticles can significantly affect their pharmacokinetics and biodistribution. In nanogel, the NH_2 groups were used to conjugate both the NIR dye and DTPA, but in the nanolatex, NH_2 groups were only used for DTPA conjugation and thus more free NH_2 groups were left on the surface. NH_2 groups in water carry cationic charges, correspondingly, the nanogel's zeta-potential was -30 mV, but that of nanolatex was -5 to -7 mV. Negatively charged particles may have weaker interactions with the cells and thus a longer time in blood circulation. In addition, the dye molecules on the surface of nanogel may influence their pharmacokinetics and biodistribution. Lastly, nanogel-20 and nanolatex-20 displayed a striking difference in the extent of hydration. Nanogel-20 was thoroughly hydrophilic, whereas nanolatex-20 had a hydrophobic core and a hydrophilic shell. As a result, nanogel-20 had much higher water content and was highly deformable. The observed differences in the pharmacokinetics and biodistribution patterns between nanogel-20 and nanolatex-20 may simply arise from a difference in their mechanical deformability. To further clarify the relatively role of particles'

deformability on these parameters, it is necessary to use nanoparticles with well controlled surface chemistry.

Our results also indicate that particles size influences *in vivo* behavior of nanoparticles. All three nanolatex particles used in our studies possessed the same physicochemical properties. They differed only in their size, with average diameters varying from 20 nm to 60 nm. Yet, there were significant differences in pharmacokinetic parameters and in values of uptakes in the liver, kidney, and tumors among the three nanolatex particles studied (Fig. 4B and 5B). Both the systemic clearance and the volume of distribution were significantly decreased with an increase in particle size. This is in consistent with an observed lower liver uptake of larger particle size formulations (nanolatex-30 and nanolatex-60). It is unclear whether the decrease in systemic clearance was due to decreases in renal elimination or hepatic metabolism. With limited samples of nanoparticles investigated in the current study, no clear trend had emerged as to how particle size affected the biodistribution. Future work using series of nanoparticles having different composition, and with sizes ranging from 20- to 200-nm in diameter, is necessary to further clarify the role of particle size in governing pharmacological properties of nanoparticles.

A significant finding of this study is that tumor uptake of nanoparticles is not only a function of their blood circulation time, but also profoundly affected by the extent of hydration. As shown in Figure 7, there is a close correlation between the MRT and tumor uptake for nanolatex, with a correlation coefficient (R^2) of 0.95. The R^2 values for $t_{1/2,\alpha}$ vs. tumor uptake and $t_{1/2,\beta}$ vs. tumor uptake curves are 0.64 and 0.61, respectively, suggesting that MRT is a more reliable predictor of tumor uptake than $t_{1/2,\alpha}$ and $t_{1/2,\beta}$ for this series of nanoparticles. However, when nanogel-20 was added to the linear regression analysis, the correlation between the MRT and tumor uptake in %ID/g no longer held ($R^2 = 0.052$; Fig. 7). Thus, the highly hydrated nanogel-20 exhibited prolonged blood circulation, but unlike nanolatex particles, its longer blood resident time did not translate into higher uptake in the tumor. If the PEG chains in nanogel-20 is more flexible than nanolatex-20, it is conceivable that the “molecular extension” of PEG chain in nanogel-20 may have a significant effect on the extravasation of nanoparticles from tumor vessels.

The *in vivo* distribution of nanoparticles over a period of 2 days was carried out noninvasively with both nuclear and optical imaging techniques. All NIRF nanoparticles in the current study exhibited blood pool activity, and tumor uptakes were visualized with both γ - and optical imaging. As exemplified with nanolatex-60, significant activity was observed in the heart region, indicating prolonged blood pool activity. γ -Imaging with ^{111}In -labeled nanolatex-60 also revealed predominant uptake in the liver and spleen 24 h after nanoparticle injection and uptake in the tumor by 48 h after injection (Fig. 6A). Optical imaging, unlike γ -scintigraphy, clearly revealed the accumulation of NIRF nanolatex-60 in the tumors as early as 4 h after injection (Fig. 6B). This result is not surprising for subcutaneously implanted tumors because optical imaging is heavily surface-weighted. NIRF nanogel and nanolatex particles may find potential applications in *in vitro* and *in vivo* diagnosis of cancer and other diseases.

5. Conclusions

In the current study, both biodistribution analysis and noninvasive imaging were used to evaluate the fate of ^{111}In -labeled NIRF nanoparticles *in vivo*; attention was focused on blood retention and accumulation in the tumor. Pharmacokinetic and biodistribution studies revealed that charge, size, and even deformability of nanoparticles may play an important role in their *in vivo* behaviors, particularly the extravasation of nanoparticles into tumor matrix. Major findings of our studies are: 1) for core-shell polymeric nanolatex particles with sizes from 20- to 60-nm, blood circulation time increases with increasing particle size; 2) prolonged

circulation time is a prerequisite for enhanced permeability and for effective extravasation from tumor vessels, and 3) in addition to size and blood circulation time, the extent of hydration of the nanoparticles play an important role in the tumor uptake of nanoparticles. A better understanding of the influence of some variables on blood circulation time and tumor uptake should help in optimizing the delivery of NIRF nanoparticles for cancer diagnosis and therapy.

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Fig. 1.
(A) Schematic diagram of nanogel and nanolatex, and reaction scheme for the introduction of radiometal chelators.

A **B** **Fig. 2.**

(A) Size distribution of nanoparticles prior to DTPA conjugation measured by dynamic light scattering. Both nanogel and nanolates had similar size and size distribution pattern. (B) Transmission electron microscopy of nanolates-30 and nanolates-60. Bar, 20 nm.

Fig. 3. The uptake of NIRF nanoparticles loaded with Cy7-like dyes by RAW264.7 cells at 37°C. Values are means $\pm$ standard deviations (SDs) ($n = 5$). Significant differences, $p < 0.01$ **, were observed between silica-100 and other nanoparticles.

Fig. 4.

Blood activity-time profile of nanogel and nanolatex. The data represent the mean radioactivity expressed as a percentage of injected dose per gram of blood (%ID/g) $\pm$ standard deviation ($n = 5$). The solid and dotted lines are curve-fitted to a two-compartment model consisting of a central and a peripheral compartment.

Fig. 5. Biodistribution of ^{111}In -labeled nanogel and ^{111}In -labeled nanolatex at 48 h after intravenous injection of particles in nude mice bearing subcutaneous MDA-MB-468 tumors. Data were plotted as percentage of injected dose per gram of tissue (%ID/g). All data are expressed as mean $\pm$ SD ($n = 3$). Significant differences, $p < 0.05^*$ and $p < 0.005^{**}$, were observed between different groups.

Fig. 6.

(A) Representative dynamic posterior γ -images of mice at different times after intravenous injection of NIRF nanolatex-60. MDA-MB-468 breast tumors (arrows) were inoculated subcutaneously in the hind legs of the mice. The radioactivity in the cardiac area is circled in red. (B) Representative NIRF optical images of the same mice at various times after intravenous injection of ^{111}In -labeled nanolatex-60. Arrows point to tumors. The scale bar shows signal intensity in arbitrary units. (C) Representative NIRF images of various tissues resected from mice at the end of the imaging sessions (48 h post-injection).

Fig. 7.
Correlation between tumor uptake and mean residence time for nanolatex and nanogel particles.

Table 1

Summary of the physicochemical properties of nanogel and nanolatex

Specification	Nanogel-20	Nanolatex-20	Nanolatex-30	Nanolatex-60
Diameter (nm)^a	22.3±7.8	23.3±6.7	33.3±6.9	62±10.8
Zeta potential (mV)^b	-30.7±3.5	-5.59±0.62	-6.99±2.08	-7.08±3.17
Mp^c	1.25 × 10 ⁵	1.26 × 10 ⁶	1.09 × 10 ⁷	7.04 × 10 ⁷
PEG/particle^d	83	637	4,240	22,800
Dye/particle^e	16	7	74	384
DTPA/particle^f	3.6	7.3	271	1767
Dry weight (mg/nmol particles)	0.12	1.4	14	76.3
Measured A_{max} (nm)	754	761	761	760
Measured EM_{max} (nm)	776	782	781	787
Emission Intensity^g	1.00 × 10 ⁵	3.80 × 10 ⁵	5.02 × 10 ⁵	1.20 × 10 ⁶

^aVolume-average mean diameter measured by dynamic light scattering.^bMeasured in phosphate-buffered saline at pH 7.4.^cMeasured by gel permeation chromatography (GPC), or calculated using molecular weight = $N \cdot 1.25\pi(0.5D)^3$ wherein D is the volume mean diameter (cm) obtained using dynamic light scattering. N is Avogadro's number.^dPolyethylene glycol (PEG) per particle; estimated on the basis of molecular weight of particle as determined by GPC and of molar ratios of monomers used in nanoparticle preparation.^eFor nanogel, the value was estimated spectrophotometrically. For nanolatex, the value was estimated assuming all dye molecules were incorporated into the nanoparticles.^fDiethylenetriaminepentaacetic acid (DTPA) loading per particle; estimated on the basis of amount of DTPA used and of conjugation efficiency as measured by capillary electrophoresis.^gMeasured at a nanoparticle concentration of 2.54×10^{13} particles/mL.

Table 2

Blood pharmacokinetic parameters for ^{111}In -labeled nanogel and ^{111}In -labeled nanolatex in five mice after intravenous administration

Pharmacokinetic Parameters	Nanogel-20	Nanolatex-20	Nanolatex-30	Nanolatex-60
Number of mice	7	7	5	5
$T_{1/2\alpha}$ (h)	2.31±1.29	2.32±1.01	2.35±1.21	0.51±0.14 [*]
$T_{1/2\beta}$ (h)	28.87±14.55 [*]	15.21±4.29	14.96±0.87	19.63±1.43
Vc (mL)	2.70 ± 0.26	2.81 ± 0.26	1.49 ± 0.046 ^{**}	1.24 ± 0.038 ^{**}
Vd(ss) (mL)	6.70±1.87	5.54±0.86	1.88±0.07 ^{**}	2.31±0.14 ^{**}
K_{10} (h ⁻¹)	0.078 + 0.017 ^{**}	0.126 + 0.016	0.062 + 0.002 ^{**}	0.068 + 0.007 ^{**}
K_{12} (h ⁻¹)	0.184 + 0.10	0.141 + 0.094	0.079 + 0.057	0.658 + 0.24 ^{**}
K_{21} (h ⁻¹)	0.146 + 0.087	0.146 + 0.092	0.306 + 0.23	0.775 + 0.31 ^{**}
AUC (%ID h/mL blood)	496.57±103.07 ^{**}	286.57±33.26	1086.2±13.8 ^{**}	1199.4±135.5 ^{**}
CL (mL/h)	0.21±0.04 ^{**}	0.35±0.04	0.09±0.00 ^{**}	0.08±0.01 ^{**}
MRT (h)	34.05±14.35 ^{**}	15.77±2.25	20.42±0.97	27.63±2.05

Values are the mean ± standard deviation (SD) obtained from 5–7 mice in each group after intravenous injection. $T_{1/2\alpha}$ and $T_{1/2\beta}$ are blood half-lives of the distribution and the clearance phases; Vc is the volume of distribution in the central compartment; Vd(ss) is the volume of distribution at steady-state; k_{10} represent the first-order elimination rate constant from the central compartment; k_{12} is the first-order distribution rate constant from the central to peripheral compartment; k_{21} is the first-order distribution rate constant from the peripheral to central compartment; AUC is the area under the %ID/mL blood-time curve; CL is the total body clearance; MRT is the mean residence time.

* indicates $p < 0.05$ and

** indicates $p < 0.01$ compared with the corresponding parameters of the Nanolatex-20 formulation.