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Heterogeneity in nanoparticles influences biodistribution and targeting

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Abstract

Aim—A large fraction of the administered dose of nanoparticles (NPs) localizes into nontarget tissue, which could be due to the heterogeneous population of NPs.

Materials & methods—To investigate the impact of the above issue, we simultaneously tracked the biodistribution using optical imaging of two different sized poly (D,L-lactide *co*-glycolide) NPs, which also varied in their surface charge and texture, in a prostate tumor xenograft mouse model.

Results—Although formulated using the same polymer and emulsifier concentration, small NPs were neutral (S-neutral-NPs) whereas large NPs were anionic (L-anionic-NPs). Simultaneous injection of these NPs, representing heterogeneity, shows significantly different biodistribution. S-neutral-NPs demonstrated longer circulation time than L-anionic-NPs ($t_{1/2}$ = 96 vs 13 min); accounted for 75% of total NPs accumulated in the tumor, and showed 13-fold greater tumor to liver signal intensity ratio than L-anionic-NPs.

Conclusion—The data underscore the importance of formulating nanocarriers of specific properties to enhance their targeting efficacy.

Keywords

cancer therapy; drug delivery systems; drug targeting; medical imaging; nanomedicine; nanostructures

The biodistribution of nanoparticles (NPs) in target tissue versus in other body compartments can largely determine their efficacy as a targeted drug delivery system [1]. In addition to exploring different ligands to improve drug delivery to target tissue using NPs, significant effort is currently being devoted to understanding the effect of the physical

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Ethical conduct of research

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properties of NPs, such as size, shape, charge and surface hydrophobicity/hydrophilicity, on the pharmacodynamics of their biodistribution to develop targeted delivery systems based on the physical characteristics of NPs [2–5]. Despite these efforts, a significant fraction of the injected dose of NPs accumulates in nontarget tissue, thus reducing the overall efficacy of NPs for targeted delivery of therapeutics [6,7].

One aspect of the nonspecific biodistribution of NPs could be the heterogeneous nature of most NP formulations, which comprise different populations of NPs that have distinct physical properties and hence dissimilar *in vivo* behavior [8]. For example, a typical dendrimer formulation contains a mixture of multiple generations, with each generation exhibiting different physical, biological and toxicological properties [9]. Similarly, PEGylation, a technique commonly used to prolong the blood circulation time of NPs, produces populations with different hydrophobicities, with each population exhibiting distinct biological activities [10,11]. Prabha *et al.* have shown that the fraction of small NPs (mean diameter = 70 nm), but not large NPs (mean diameter = 202 nm), predominantly contributes to gene transfection [12]. The heterogeneity of nanocarriers and their efficacy in targeting of small interference RNAs (siRNAs) to tumors was recently discussed by Lee *et al.* [13]. That study suggested the use of nucleic acids, which self assemble into NPs of uniform size; however, use of such a system could evoke an immune response following repeated administration [14,15]. In addition, heterogeneity in the density of targeting ligands on nanocarriers can also significantly influence the biodistribution and targeting efficiency of different NP fractions [16].

The heterogeneity of NPs thus raises the question as to which of the many fractions present in a given formulation is indeed reaching the target tissue. To understand the implications of this issue, we developed an optical imaging technique to simultaneously track the biodistribution of poly(D,L-lactide *co*-glycolide) (PLGA) NPs of different physical properties. We tested new near infrared (NIR) dyes with emission wavelengths that can easily be separated from each other. Using the above technique, we monitored biodistribution of two different sized PLGA-NPs, which also vary in their surface charge, mixed at a 1:1 weight by weight (w/w) ratio to mimic heterogeneity in NP formulations in a prostate tumor xenograft mouse model. We show that different fractions in a heterogeneous preparation of NPs have different pharmacodynamics of biodistribution in this model.

Materials & methods

Materials

PLGA (50:50, inherent viscosity = 0.26–0.54 dl/g) was purchased from LACTEL Absorbable Polymers (AL, USA). Polyvinyl alcohol (PVA; 87–90% hydrolyzed, mol wt 30,000–70,000) and sucrose were purchased from Sigma-Aldrich (MO, USA). NIR dyes SDB6825, SDB5491, SDA5177 and SDB5700 were obtained from HW Sands Corporation (FL, USA). Chloroform, methanol (HPLC grade) and Cy5.5 (form of cyanine) were obtained from Fisher Scientific (PA, USA). Qdot® 800 ITK™ organic quantum dots were purchased from Invitrogen (NY, USA) and Float-A-Lyzer® G2 dialysis membrane (mol wt cutoff = 500 D) from Spectrum Laboratories (CA, USA).

NIR dyes & photostability—The NIR dyes to be incorporated into PLGA-NPs were first tested to determine how clearly their signals appeared under simultaneous optical imaging without the signals interfering with each other. We then tested for photostability against multiple exposures to the laser of the Optical *In vivo* Imaging System (Maestro Ex™ Optical Imaging System, Caliper Life Sciences [PerkinElmer], MA, USA), which utilizes epiillumination to generate 2D images. Since these dyes are hydrophobic, their methanolic solutions were used to determine spectral characteristics. A 100 µl aliquot of methanolic

solution (5 µg/ml) of each dye was added into white 96-well plates (Fisher Scientific). The bottom of each well was precoated by adding 100 µl of 1% agar. We had earlier determined that this agar coating provides an inert surface that prevents aggregation and ensures the uniform spread of NPs or dye in the wells. Hence, for imaging of the dye solution, we used an agar coating to keep the conditions consistent for imaging of dyes and dye-loaded NPs. The dye-loaded plates were immediately imaged with the optical imaging system using the NIR filter set to determine their maximum emission wavelengths. This step was performed independently, with the four NIR dyes tested in separate plates. Following the initial screening, pairs of NIR dyes with different emission wavelengths were mixed and imaged with filter/wavelength selection set at narrow in the 'acquire fluorescence' tab in the Optical Imaging System software (version 2.10.0). Separation of the two signals when mixed was performed using the real component analysis tool of the imaging software to distinguish between the different signals. Based on the ability to be separated from each other, NIR dyes SDB5700 and SDA5177 were selected for incorporation into NPs.

To determine the photostability of the dyes, the agar-coated plates with methanolic solution of dyes were imaged for ten successive images as above with 30 s intervals between image acquisitions, with the laser kept on during the entire period. This process results in continuous exposure of dyes to laser for 10 min. This length of time for exposure represents the cumulative time required for *in vivo* imaging. Regions of interest (ROIs) were created to quantify the signal intensities in dye-containing wells for successive images taken to determine photostability. All images were normalized to the first image taken to determine the extent of loss of signal with each subsequent exposure. To compare photostability, Qdot® 800 ITK™ organic quantum dots and Cy5.5, both either dispersed or dissolved in methanol, were imaged and the signal intensities quantified as above.

Formulation & characterization of NIR dye-loaded NPs—PLGA-NPs of two different sizes, each loaded with a different NIR dye, were formulated using an emulsion solvent evaporation method [17]. For large NPs, 90 mg PLGA was dissolved in 3 ml chloroform containing 100 µg of SDA5177 dye (400 µg/ml). The polymer solution was emulsified into 12 ml of 1% weight by volume (w/v) emulsifier using a probe sonicator (XL 2015 Sonicator Ultrasonic processor, Misonix Inc., NY, USA) with a stepped microtip (Qsonica LLC, CA, USA) set at 55 W energy output on ice for 3 min. The emulsion was stirred overnight under a fume hood with airflow set at >220 ft/min to evaporate chloroform, followed by vacuum desiccation to ensure complete evaporation of chloroform. The formed NPs were recovered by ultracentrifugation at 30,000 rpm (82,000 g, L-80 Ultracentrifuge, Rotor 50.2Ti, Beckman Coulter, Inc., CA, USA) at 4 °C for 30 min. The recovered NPs were washed twice with distilled water to remove excess PVA and unencapsulated dye by resuspending the NP pellet in water, sonicating for 30 s followed by ultracentrifugation as above. After the final wash, the NP pellet was resuspended by sonication for 30 s and centrifuged at 1000 rpm (100 g, Sorvall Legend RT centrifuge, Thermo Scientific, NC, USA). The supernatant was collected, frozen with 3% (w/v) sucrose by adding 500 µl of 9% (w/v) sucrose to 1 ml NP supernatant and lyophilized for 48 h (Freezone 4.5, Labconco, MO, USA). Small NPs were prepared as above using SDB5700 dye. The polymer solution was emulsified in 1% PVA solution and sonicated as above to form o/w emulsion. The emulsion was further passed through a high-pressure homogenizer (EmulsiFlex C5, Avestin, ON, Canada) at 5000–10,000 PSI pressure for ten cycles. The resulting emulsion was evaporated overnight and processed as above.

The mean hydrodynamic diameter and zeta potential of NPs were determined by dynamic light scattering with a NICOMP 380 ZLS (Particle Sizing Systems, CA, USA). For this step, 3 ml (100 µg/ml) NP suspension in water was sonicated for 30 s, and a 50 µl aliquot was

added to a borosilicate glass disposable culture tube (Kimble Chase, NJ, USA) for particle sizing. The same NP suspensions were used to measure zeta potential.

The surface morphology of NPs was characterized using atomic force microscopy (AFM). For this step, silicon wafers (Ted Pella, Inc., CA, USA) were first cleaned by dipping in a mixture of H₂O/H₂O₂/NH₄OH (4:1:1 volume) at 80°C for 5 min and then rinsed with water. Wafers were dried under an N₂ stream to prevent oxidation. A 100 µl aliquot of the freshly prepared NP suspension prepared as above in water was added onto the silicon wafer and dried in a dust-free environment. AFM images were taken with a BioScope II Atomic Force Microscope (Veeco Metrology, Inc., CA, USA) in tapping mode using a 125 µm-long silicon probe with a resonance frequency of approximately 300 Hz and a tip radius of <10 nm (Ted Pella, Inc.). Scanning was performed at a scan speed of 0.5 Hz and a set-point ratio of 1.0 with a resolution of 512 × 512 pixels. The acquired images were flattened using a second-order flattening routine in Nanoscope software version 7.30.

Characterization of NIR dye-loaded NPs for imaging—First we determined the correlation between the amount of NPs and signal intensity. A stock suspension of NPs (2 mg/ml in water) was prepared from which different dilutions of NPs in water were prepared. A 100-µl aliquot of each concentration of NP suspension was added to agar-coated white 96 well plates, as described above. The plate with SDA5177-loaded large NPs was imaged first after auto exposure to determine the exposure time required for image acquisition. The same exposure time was used to image the plate with SDB5700-loaded small NPs. To quantify the signal intensity for each concentration of NPs, ROIs were created and cloned to have the same area selected for each concentration of NPs. Average signal intensities from three replicates were then plotted against the amount of NPs for each formulation.

Dye encapsulation efficiency & dye release from NPs—Encapsulation efficiency of the dyes in NPs was determined by extracting the dye from the NPs. Each formulation of NPs (2 mg/ml) was suspended in methanol, left at 4 °C for 96 h, then centrifuged at 14,000 rpm (19,000 g), and the supernatant was analyzed for dye content using the Optical Imaging protocol as described above. Dye release from NPs was carried out in 1% bovine serum albumin solution in phosphate buffered saline (PBS). For this step, 2 mg NPs suspended in 1 ml of release buffer was added to a 1 ml Float-A-Lyzer (Spectrum Laboratories) with molecular weight cutoff of 500 D and dialyzed against 5 ml release buffer at 37°C with continuous shaking. At a predetermined time point, the dialysate in 5 ml release buffer was collected and replaced with fresh release buffer for analysis. The amount of dye released was determined by comparing the signal intensity of each dialysate to a standard plot for each dye (1–200 ng) prepared in 1% bovine serum albumin solution in PBS.

Animal studies—Cleveland Clinic's Institutional Animal Care and Use Committee (OH, USA) approved all animal procedures. All studies were performed with male athymic nude mice aged 5–6 weeks old (nu/nu athymic, Charles River, MA, USA).

Protocol for *in vivo* imaging

All images were acquired under the following setting. The blue filter set with a wavelength range 500–720 nm with exposure time set at 500 ms was used to capture autofluorescence; the NIR filter set with a wavelength range of 740–950 nm with exposure time set at 1200 ms was used for capturing the NIR dye-loaded NPs. For effective separation of signals from autofluorescence versus from NIR dyes, the filter/wavelength selection was set at narrow in the 'acquire fluorescence' tab in the Maestro Imaging software. All *in vivo* image acquisitions were performed with 2% isoflurane in oxygen as anesthesia. The images were processed using the instrument's imaging software (version 2.10.0). Large NPs (with

SDA5177) were coded green and small NPs (with SDB5700) as red, thus the regions of colocalization were represented as yellow. Autofluorescence acquired with the blue filter set was coded as white. Signal intensities for both large and small NPs were obtained by creating ROIs at various anatomical regions.

Separation of NIR signals & photostability of NIR dye-loaded NPs *in vivo*

Prior to the *in vivo* biodistribution study, we evaluated the ability to distinguish signals from the two types of NPs. For this, 10 μ l of small and 10 μ l large NPs were injected subcutaneously on the lateral side of the same mouse. In addition, a mixture of the two NPs (1:1 w/w) was also injected subcutaneously on the same lateral side. Each mouse was imaged as per the protocol described above. We also studied the photostability of the injected NPs. For this part, 10 μ l of NPs (3 mg/100 μ l) was injected into the lateral side of a mouse and ten successive images were taken and unmixed. Signal intensities of each image were analyzed as performed for the *in vitro* photostability analysis.

Tumor induction

Mice were anesthetized by intraperitoneal injection of a ketamine/xylazine cocktail at a dose of 150 mg/kg body weight for ketamine and 10 mg/kg body weight for xylazine. One million PC-3 prostate cancer cells in 100 μ l of PBS and 100 μ l Matrigel (BD Biosciences, CA, USA) for a total volume of 200 μ l were injected subcutaneously on the left flank. Tumor growth was monitored every other day for 2 weeks postinoculation by measuring the site with a digital caliper. Tumor volume was calculated as $(\text{length} \times \text{breadth}^2)/2$. Animals with tumors were used for biodistribution and pharmacokinetic studies when tumor size reached approximately 300 mm³.

Biodistribution of dye-loaded PLGA-NPs

Mice were injected via tail vein with a mixture of large and small NPs (2 mg each) in a total volume of 100 μ l saline and imaged at predetermined time points. The anatomical locations corresponding to liver, tumor and skin were selected and the signal counts determined for each area. Mice were euthanized at 48 h by intraperitoneal injection of 100 μ l pentobarbital and perfused with 10 ml heparinized saline by cutting the inferior vena cava and injecting the heparinized saline into the right ventricle. The liver, lungs, heart, spleen, kidneys and tumor were harvested and imaged using the above imaging protocol. To determine NP levels in the blood, mice were injected with a mixture of large and small NPs via tail vein as above. At predetermined time points, animals were euthanized by intraperitoneal injection of pentobarbital (150 mg/kg) followed by cardiac puncture to collect blood. A 200 μ l of blood was added to 200 μ l of PBS and centrifuged for 10 min at 1300 rpm. A 100 μ l aliquot of supernatant (plasma) was collected and imaged with the optical imaging system using the above imaging protocol.

Statistical analysis—Data are expressed as mean $\pm$ standard error of the mean. Statistical analyses were performed using Student's t-test. Differences were considered significant at p 0.05.

Results

Selection of NIR dyes

The maximum emission wavelengths (λ max) of the four NIR dyes selected ranged from 770 to 840 nm. However, the dyes SDB6825 (λ max = 780 nm) and SDB5491 (λ max = 830 nm) both exhibited broad emission spectra; SDB6825 also showed a secondary emission peak at 820 nm (Figure 1A). Because of their broad emission wavelengths, the signal from

these two dyes could not be unmixed, and therefore these two were not considered for further evaluation. The other two dyes, SDA5177 ($\lambda_{\text{max}} = 840 \text{ nm}$) and SDB5700 ($\lambda_{\text{max}} = 770 \text{ nm}$), exhibited narrow emission spectra, with their λ_{max} separated by 70 nm (Figure 1B). These dyes also exhibited significantly better photostability following laser excitation for ten successive images with total exposure time of 10 min; SDA5177 retained 81% and SDB5700 72% of yield as compared with 68% for quantum dots and 61% for Cy5.5, a commonly used NIR cyanine dye (Figure 1C).

Characterization of NIR dye loaded NPs

Following the initial screening of these latter two dyes, they were incorporated into small (SDB5700, color coded as red) and large (SDA5177, color coded as green) NPs. The mean hydrodynamic diameter of large NPs was 315 nm (polydispersity index [PI] = 0.1), whereas that of small NPs was 154 nm (PI = 0.07). A 1:1 w/w mixture of small and large NPs demonstrated the mean hydrodynamic diameter of 246 nm (PI = 0.18; Figure 2A). Images obtained by AFM show the spherical nature of these NPs (Figure 2B). Although both small and large NPs were prepared using the same composition of polymer and emulsifier, they acquired a different zeta potential. Small NPs were less negative, almost neutral ($\zeta = -0.81 \text{ mV}$), than large NPs ($\zeta = -16.15 \text{ mV}$); the 1:1 w/w mixture of small and large NPs exhibited a ζ of -10.23 mV (Table 1). Because of significant differences in their surface charge, small NPs henceforth are designated as S-neutral-NPs whereas large NPs as L-anionic-NPs. AFM phase images of the NPs show the difference in the surface texture; S-neutral-NPs showing a general distribution of light shaded crystalline patches whereas L-anionic-NPs showing these light shades as crystalline stripes radiating from a central region on the NP's surface (Figure 2B).

Dye SDA5177, selected for incorporation into L-anionic-NPs, demonstrated almost 100% entrapment efficiency, whereas dye SDB5700 used for incorporation in S-neutral-NPs demonstrated 93% entrapment efficiency. Dye loading did not significantly change the mean particle size or surface charge of NPs (Table 1). Furthermore, an insignificant amount of the incorporated dye is released from NPs under *in vitro* conditions (3.1% from S-neutral-NPs and 2.4% from L-anionic-NPs in 48 h). The imaging data show that the signal from S-neutral-NPs and L-anionic-NPs can easily be differentiated from each other; thus making these dye-loaded NPs suitable for simultaneous imaging (Figure 2C). Interestingly, dye-loaded NPs demonstrated better photostability than the free dye under the same exposure conditions (Figure 1C vs Figure 2D); with SDA5177-dye-loaded L-anionic-NPs retaining 97.9% and SDB5700-dye-loaded S-neutral-NPs retaining 99.9% of the initial signal after ten successive images with a total exposure time of 10 min. The dye loaded NPs exhibited a linear increase in signal intensity correlating with the amount of NPs (Figure 2E). More importantly, the S-neutral-NPs and L-anionic-NPs demonstrated almost the same signal yield per μg of NPs (slope for small NPs = 13.14 and for large NPs = 14.12; Figure 2F).

Biodistribution & pharmacokinetics of dye-loaded NPs

Prior to this biodistribution study, we confirmed that the two types of NPs can be detected independently *in vivo* and that their signal intensity can be quantified without interference from each other following their subcutaneous injection into mice (Figure 3A). Optical imaging of the animals following intravenous injection of equal doses of S-neutral-NPs and L-anionic-NPs shows their presence throughout the body, including in the tumor (Figure 3B). These images also show that S-neutral-NPs and L-anionic-NPs have a different biodistribution, as evident from their respective color codes. There are areas in the animal where S-neutral-NPs are predominantly present (red) and other areas where mostly L-anionic-NPs are present (green). In addition, some areas overlap, with both S-neutral-NPs and L-anionic-NPs seen together (yellow). The images also show the change in

biodistribution of the injected NPs over time. Immediately following injection, both S-neutral-NPs and L-anionic-NPs are seen throughout the body, but as time passes, the two types are seen to slowly accumulate, primarily in the liver, until approximately 2 h postinjection. Thereafter, S-neutral-NPs are seen to slowly redistribute into other body compartments, including into the tumor and gut, whereas L-anionic-NPs mostly remain confined within the liver. Merged images show the presence of both S-neutral-NPs and L-anionic-NPs in the liver. Imaging of the tumor by mapping the ROIs over time showed a rapid decay in signal for the L-anionic-NPs; by contrast, the signal for the S-neutral-NPs, after an initial rapid drop, persisted until the end of the study at 48 h (Figure 4B).

In a separate set of experiments, blood samples were collected at different time points following intravenous injection of both S-neutral-NPs and L-anionic-NPs and analyzed for the presence of both types of NPs. The results show that the signal from L-anionic-NPs dropped immediately following their injection, whereas that from S-neutral-NPs remained in the circulation up to 6 h postinjection (Figure 4C). The overall $t_{1/2}$ of clearance of S-neutral-NPs was 96 min whereas that for L-anionic-NPs was 13 min. The decay in the signal in the tumor tissue for S-neutral-NPs was slower than the decay in signal in the blood samples; however, there was no significant difference in the signal decay in the blood and tumor for L-anionic-NPs (Figure 4B vs 4C).

Ex vivo images of the organs, including tumor samples collected at 48 h postinjection of NPs, confirmed the general biodistribution observed from *in vivo* imaging (Figure 4D). The organs were collected following immediate perfusion of euthanized animals to ensure that the signal seen is due to the NPs retained in the tissue. L-anionic-NPs showed greater accumulation in the organs of the reticuloendothelial system (RES), such as the liver and spleen, whereas S-neutral-NPs showed greater accumulation in highly vascularized organs, such as the lungs, kidneys and heart (Figure 4E). The S-neutral-NPs accounted for over 75% of the total NPs accumulated in the tumor. The relative tumor-to-liver signal intensity ratio for S-neutral-NPs was 0.41 and that for L-anionic-NPs was 0.03 – that is, over 13-fold greater for S-neutral-NPs than for L-anionic-NPs.

Discussion

The goal of our studies was to highlight the significance of heterogeneity in NP-based drug delivery systems on biodistribution and targeting of NPs. As we have demonstrated in this study, S-neutral-NPs and L-anionic-NPs, when injected together at the same doses, show significantly different patterns of biodistribution (Figures 3B & 4E), blood clearance (Figure 4C) and tumor uptake and retention (Figure 4B & E). The data obtained from such studies that analyze the *in vivo* characteristics of the different NP fractions could be very useful to refine parameters to enrich the formulation with a fraction of NPs that is indeed effective in accumulating in the target tissue. Such an approach can potentially reduce toxicity due to nonspecific distribution of ‘other species’ present in the formulation, improve specificity of drug delivery and reduce the dose of nanocarriers required to achieve the desired therapeutic outcome.

The other important aspect of our study was the ability to simultaneously track the biodistribution of NPs of different physical properties. Current strategies for studying the biodistribution and pharmacokinetics of NPs involve formulating NPs of different properties and evaluating them separately in different groups of animals [18]. However, these studies do not address the issue of the heterogeneity of NPs present in the same formulation. Furthermore, evaluation of formulations in different groups of animals may not take into account the competition for blood proteins to different fractions of NPs present in the formulation. In addition, when tested in a separate group of animals, it may not take into

account the competition for space within a particular tissue that such different NP fractions may exhibit.

The NIR dyes incorporated into PLGA-NPs emit very stable signals, and the signals from two formulations of NPs can easily be distinguished, allowing their simultaneous detection. These dyes have not been investigated for biomedical applications, but are commonly used in credit card and security card technology. The NIR emission wavelengths of these dyes make them suitable for *in vivo* imaging, and because of their hydrophobic nature, they can easily and efficiently be incorporated into PLGA-NPs without requiring chemical conjugation, and high-fluorescence yield and photostability make them suitable for biodistribution and pharmacokinetic studies of NPs [19,20]. The conventional technique, by which fluorophores are conjugated to the surface of NPs, can alter the characteristics of NPs and thus their biodistribution [21]. Furthermore, the conjugated dye might quickly dissociate from the surface of NPs, limiting their use for a long-term biodistribution study. Most importantly, the conjugated dye might photo-bleach with each exposure, thus limiting the sensitivity of detection [22,23]. Since the dyes used in our studies are incorporated into the polymer matrix of NPs, they are retained within the NPs, allowing us to monitor their biodistribution for prolonged periods (Figure 2D). In addition, the incorporated dyes do not influence the NP properties, hence the observed biodistribution and pharmacokinetics seen in our studies are representative of NPs without the dye.

Several studies have demonstrated that size and shape, as well as surface characteristics, exert significant effects on the interaction of NPs with their biological environment [5,24]. Although we did not determine the protein binding onto our NPs, it is known that physical characteristics of NPs significantly influence the amount and identity of proteins adsorbed onto their surface, and therefore play a significant role in their interactions with cells and tissue, and hence biodistribution [25,26]. For example, Tenzer *et al.* have shown that protein corona is significantly different in composition for silica NPs that vary in size even by 10 nm [27]. Similarly, chemical functional groups [28] and presence of hydrophilic polymers at the NP interface have been shown to influence protein binding and biodistribution of NPs [29]. Recently, the effect of particle shape and size on vascular transport and adhesion of NPs has been studied [30], and it has been suggested that thin disk-like particles could more effectively target the diseased microvasculature compared with spheres and slender rods [31]. These studies thus emphasized the role of particle geometry to enhance the specificity of delivery [32].

In our study, although small and large NPs were prepared using the same PVA concentration, they demonstrated different zeta potentials and surface textures (Figure 2B & Table 1). This difference could be due to the amount of PVA associated with NPs. The amount of surface-associated PVA is dependent on the particle size and this surface-associated PVA influences the interfacial properties of NPs as well as their interactions with cells [33]. Thus the apparent difference in the biodistribution of the two NP formulations could be the effect of a combination of factors, not just NP size. These observations underline the complexity of the changes that may occur in NP characteristics, even if the investigator's intention is to only change one parameter. Further characterization of NP surface, particularly for the presence of functional groups at the interface via x-ray photon spectroscopy, hydrophilicity/hydrophobicity, surface roughness and so on may further explain the differences in the behavior of two formulations of NPs used in our study.

The interesting observation was the slow redistribution of S-neutral-NPs, mainly from the liver to other body compartments approximately 2 h postinjection (Figure 3B). This change in biodistribution with time may be related to the changes in the protein corona around NPs. It has been shown that NP-protein interaction is a dynamic process; proteins that are in

excess in the circulation bind first to NPs, and then these bound proteins equilibrate with high-affinity binding proteins over time [26,34]. This exchange of proteins is a function of size, surface charge and morphology with S-neutral-NPs perhaps taking on a more 'stealthy' character over time because of changes in their protein corona, and hence they redistribute to the non-RES organs.

The decay in the signal from the tumor tissue for the S-neutral-NPs was slower than the decay in the signal from the blood samples, suggesting their localization in the tumor itself. However, there was no significant difference in the signal decay in blood and tumor for L-anionic-NPs, suggesting that these NPs remain mainly in the blood circulation and are not retained in the tumor (Figure 4B vs C). S-neutral-NPs are seen in the gut, which could be due to their excretion via the liver biliary route [35,36]. By contrast, L-anionic-NPs, despite their localization in the liver, are not seen in the gut, suggesting that the hepatic clearance of NPs via the biliary route is size and/or charge dependent. The other possibility could be that S-neutral-NPs localize into hepatic cells, from which they are excreted into the biliary ducts, whereas L-anionic-NPs are taken up by Kupffer cells, a specialized form of immune cells that only exist within the liver. Further histological analysis of the liver tissue may provide further insight into the mechanism of clearance of NPs from the liver to the gut. Our data also show that L-anionic-NPs accumulate more in the spleen than S-neutral-NPs, whereas S-neutral-NPs accumulate more in the kidney than L-anionic-NPs (Figure 4D & E). Although other parameters, particularly NP surface charge may also be playing a role in biodistribution and clearance of NPs, it seems feasible that on the basis of size, one could develop NPs targeting preferentially to a particular organ.

It is worth noticing that all harvested organs, except the heart, show the signal for both S-neutral-NPs and L-anionic-NPs, although the signal ratio varies (Figure 4D). For example, tumor shows a 75% signal due to S-neutral-NPs, whereas liver shows an 83% signal due to L-anionic-NPs (Figure 4E). One possibility could be that different tissues/organs have the capacity to take up both L-anionic- and S-neutral-NPs, but that the efficacy of this uptake varies from organ to organ. The other possibility could be the overlapping size distribution between small and large NPs. Despite our best efforts to make NPs of two distinctly different size ranges, we found that 7% of the fraction present in large NPs is of an overlapping size with small NPs and that 8% of the fraction present in small NPs is of an overlapping size with large NPs. Hence, it is possible that the signal seen due to large NPs may be due in part to the smaller NP fraction present in large NPs and *vice versa*.

Recently, several studies have reported a significant variation in tumor response to nanocarrier systems [37]. This difference in response has been attributed primarily to the heterogeneous nature of the tumor's vasculature, which could significantly influence the uptake of a nanoparticulate systems via the enhanced permeability retention effect [37]. However, a contributing factor could also be the heterogeneous nature of nanocarrier systems. Lengyel *et al.*, using cryo-electron tomography, have shown that six different sized fractions were present in a liposomal formulation of doxorubicin (Doxil[®], Janssen Biotech Inc., PA, USA), with particle diameters ranging from <60 to >120 nm, and various fractions ranging from 2 to 9% [38]. Thus it is not clear which particular fraction(s) present in the formulation is indeed localizing into the tumor and what percentage of any particular fraction is in the formulation. Our imaging technique could potentially be used to tag liposomal particles of different sizes present in the formulation and determine which 'species' of the particles consistently and with high efficiency target the tumor. The data obtained from such studies could be used to refine and enrich the formulation containing those particular 'species' to maximize the efficacy of drug delivery. The size may influence the drug loading efficiency, which could particularly be an issue with macromolecular therapeutics, as small-size NPs generally have lower loading capacity than large-size NPs.

In such cases, one may have to balance the effect of size of nanocarriers on targeting and the amount of therapeutics that they can carry. However, for hydrophobic drugs, the loading difference with size of nanocarriers may be insignificant. Overall, the effective fraction of nanocarriers would be the one that delivers more therapeutic agent to the target site. Another factor to consider is the biodistribution of nanocarriers. Certain drugs may have a specific toxic effect to a particular organ/tissue. In such cases, nanocarriers that minimize drug localization to that particular organ are important to reduce nonspecific toxicity. Doxorubicin is one such example where drug biodistribution is altered with a liposomal formulation, Doxil, to reduce cardiac drug uptake and hence its cardiac toxicity.

In addition to studying the effect of physical properties on biodistribution, our imaging technique could potentially be explored to simultaneously study the biodistribution of two formulations of NPs conjugated to different targeting ligands or of NPs conjugated to multiple targeting ligands versus a single targeting ligand or to study the effect of ligand density on targeting efficiency [39]. Simultaneous monitoring of the biodistribution of NPs conjugated to different ligands can potentially be useful to determine whether such an approach produces an additive targeting effect or interfere with each other, resulting in antagonistic effect. One could also study the biodistribution of nanostructures of different shapes and architecture to understand their *in vivo* behavior to explore the targeting approach based on the physical properties of NPs [40]. Thus the dyes investigated in our studies for simultaneous imaging of NPs can be explored in many other applications to optimize drug delivery.

Conclusion

In this study, we investigated new NIR dyes for incorporation into PLGA-NPs to simultaneously monitor biodistribution of NPs that vary in physical characteristics. Our data show that a change in particle size of PLGA NPs also influences their surface charge and texture, and heterogeneity in NP formulation significantly influences biodistribution of NPs, their clearance and efficiency of tumor localization. The data obtained from such studies could potentially be explored to design and develop nanocarriers of specific properties to enhance their efficacy for targeted drug therapy.

Future perspective

One of the objectives of nanomedicine is to improve drug therapy via targeted drug delivery using nanocarriers, such as NPs, liposomes and dendrimers, among others. Despite significant efforts, a large fraction of the administered dose of nanocarriers localizes into nontarget tissue, reducing efficacy and increasing the risk of toxicity. This lack of targeting specificity could be due to the heterogeneous population of nanocarriers present in the formulation, which have distinctly different physical properties and hence dissimilar *in vivo* behavior. The biodistribution of nanocarriers in target tissue versus in other body compartments can largely determine their efficacy as a targeted drug delivery system. In this study, we demonstrated how heterogeneity of NPs influences biodistribution and targeting, underscoring the importance of formulating nanoparticles of specific properties to enhance their targeting efficacy. This would require developing sophisticated manufacturing techniques to produce NPs of specific and uniform properties, purification techniques to remove unwanted species from the formulation and establishing new quality control parameters to ensure uniformity in the formulation.

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Executive Summary

- The biodistribution and targeting of nanoparticles (NPs) significantly depends on their physical characteristics. Hence, a formulation containing heterogeneous population of NPs with different physical characteristics could significantly influence the targeting efficacy of the formulation.
- Formulations containing NPs of uniform physical properties can improve targeting efficiency of drug therapy.
- The size of NPs can change characteristics of NPs such as surface charge and texture, among others. that could potentially alter their interactions with proteins, cells and tissue, and hence biodistribution. Hence, the altered biodistribution seen with size may also be the effect of other factors.
- Optical imaging could offer a convenient, quantitative and effective way to simultaneously track the biodistribution of NPs of different characteristics *in vivo*.
- One could potentially develop targeted NPs based on their physical characteristics; however, this would require careful understanding of the effect of various physical characteristics of NPs on their biodistribution and targeting.

Figure 1. Spectral analysis of near infrared dyes and photostability

(A) Emission spectra of SDB5700 (red), SDA5177 (green), SDB5491 (blue) and SDB6825 (cyan). (B) Emission spectra of SDB5700 (red) and SDB5177 (green) showing distinguishable maximum emission wavelengths. (C) Photostability of near infrared dyes SDA5177 and SDB5700 in comparison to Cy5.5, a conventionally used near infrared cyanine dye, and quantum dots.

Figure 2. Characterization of nanoparticles

(A) Hydrodynamic diameter of S-neutral- and L-anionic-NPs and a mixture of the two NPs, as determined by dynamic light scattering. (B) Morphology of S-neutral- and L-anionic-NPs as determined with atomic force microscopy. Small and large NPs demonstrate different surface characteristics, as evident from the phase images from atomic force microscopy. (C) Separation of signal of SDB5700 dye-loaded S-neutral-NPs and SDA5177 dye-loaded L-anionic-NPs. (D) Photostability of the dye-loaded NPs. SDB5700 in S-neutral-NPs results in no loss of signal, while SDA5177 in L-anionic-NPs results in <3% loss of signal after ten consecutive images. (E) Color coding for S-neutral- and L-anionic-NPs, showing increasing

color intensity with increasing nanoparticle amount, and (F) S-neutral- and L-anionic-NPs show increasing signal intensity with nanoparticle amount. This relationship is linear with adjusted R^2 of 0.99 for L-anionic-NPs and 0.98 for S-neutral-NPs. The two near infrared dyes used also show similar fluorescent yield per μg of NPs. L-anionic-NP: Large anionic nanoparticle; NP: Nanoparticle; S-neutral-NP: Small neutral nanoparticle.

Figure 3. Biodistribution of small and large nanoparticles in tumor-bearing mice

Equal doses of L-anionic-NPs loaded with SDA5177 and S-neutral-NPs loaded with SDB5700 were mixed and injected intravenously in prostate tumor-bearing mice. (A) Unmixing of the signal from S-neutral-NPs and L-anionic-NPs injected subcutaneously in mice. (B) Biodistribution over time of S-neutral-NPs and L-anionic-NPs following their intravenous injection in mice. Arrows indicate tumor. L-anionic-NP: Large anionic nanoparticle; S-neutral-NP: Small neutral nanoparticle.

Figure 4. Quantification of *in vivo* signal of small- and large-sized nanoparticles

(A) Region of interest created over the anatomic location of tumor. (B) Tumor accumulation of nanoparticles. Intravenous injection results in rapid uptake of NPs, which gradually drain out of tumor. $p = 0.03$ at 4 h postinjection in tumor. Data are shown as mean $\pm$ standard error of the mean, $n = 6$. (C) Clearance of L-anionic- and S-neutral-NPs from blood. (D) *Ex vivo* imaging of tissues excised from mouse 48 h postintravenous injection of L-anionic-NPs and S-neutral-NPs. Mice were perfused with heparinized saline to remove NPs remaining in blood vessels before *ex vivo* imaging. Red indicates S-neutral-NPs, green L-anionic-NPs and white indicates autofluorescence. L-anionic-NPs show a greater uptake into organs of the RES and little accumulation in other tissues. S-neutral-NPs show a greater uptake into tumor, kidneys, lungs and heart. This difference is probably due to their small size and neutral charge, which allows them to bypass the reticuloendothelial system, resulting in increased half-life, allowing them to accumulate in other tissues. (E) Average signals from excised tissue after imaging with Maestro™ Ex Optical Imaging System (Caliper Life Sciences [PerkinElmer], MA, USA) was determined by drawing regions of interests around each tissue. L-anionic-NPs show a fivefold accumulation in the liver compared with S-neutral-NPs. S-neutral-NPs show a threefold accumulation in tumor and also show increased uptake by kidney. Data are shown as mean $\pm$ standard error of the mean ($n = 6$). L-anionic-NP: Large anionic nanoparticle; S-neutral-NP: Small neutral nanoparticle. * $p < 0.03$; ** $p < 0.01$.

Table 1

Characterization of nanoparticles of different size

Formulations	Size (nm)	Polydispersity index	Zeta potential (ζ /mV)
Small NPs	154.5 $\pm$ 4.1	0.06	-3.3 $\pm$ 0.46
Dye-loaded small NPs	150.6 $\pm$ 1.1	0.07	-0.81 $\pm$ 0.86
Large NPs	312.9 $\pm$ 8.1	0.11	-18.46 $\pm$ 0.16
Dye-loaded large NPs	315.7 $\pm$ 5.7	0.12	-16.45 $\pm$ 1.32
Mixed small and large NPs (1:1 w/w)	246.3 $\pm$ 5.3	0.18	-10.23 $\pm$ 0.92

Data are represented as mean $\pm$ standard error of mean (n = 3).

NP: Nanoparticle; w/w: Weight by weight.