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Non-specific binding and steric hindrance thresholds for penetration of particulate drug carriers within tumor tissue

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Abstract

Therapeutic nanoparticles (NPs) approved for clinical use in solid tumor therapy provide only modest improvements in patient survival, in part due to physiological barriers that limit delivery of the particles throughout the entire tumor. Here, we explore the thresholds for NP size and surface poly(ethylene glycol) (PEG) density for penetration within tumor tissue extracellular matrix (ECM). We found that NPs as large as 62 nm, but less than 110 nm in diameter, diffused rapidly within a tumor ECM preparation (Matrigel) and breast tumor xenograft slices *ex vivo*. Studies of PEG-density revealed that increasing PEG density enhanced NP diffusion and that PEG density below a critical value led to adhesion of NP to ECM. Non-specific binding of NPs to tumor ECM components was assessed by surface plasmon resonance (SPR), which revealed excellent correlation with the particle diffusion results. Intravital microscopy of NP spread in breast tumor tissue confirmed a significant difference in tumor tissue penetration between the 62 and 110 nm PEG-PS NPs, as well as between PEG-coated and uncoated NPs. SPR assays also revealed that

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AUTHOR CONTRIBUTIONS

JGD, CSS, GFW, JAW, and AJK conceived and designed the experiments. JGD, ASW, CSS, and JRHM performed the experiments. JGD, ASW, and CSS analyzed the data. JGD, ASW, CSS, GFW, JAW, and AJK wrote the paper. All authors discussed the results and commented on the manuscript.

Abraxane, an FDA-approved non-PEGylated NP formulation used for cancer therapy, binds to tumor ECM. Our results establish limitations on the size and surface PEG density parameters required to achieve uniform and broad dispersion within tumor tissue and highlight the utility of SPR as a high throughput method to screen NPs for tumor penetration.

Keywords

nanoparticles; PEG density; tumor tissue penetration; surface plasmon resonance (SPR); multiple particle tracking (MPT); intravital microscopy

INTRODUCTION

PEGylated liposomal doxorubicin (Doxil, size ~100 nm) and albumin-bound paclitaxel (Abraxane, size ~130 nm) are two examples of nanoparticle (NP) formulations currently approved for cancer therapy [1, 2]. The major advantage of these clinically approved NPs compared to conventional free drugs is significantly reduced adverse side effects, including lower cardiotoxicity for Doxil, and limited hypersensitivity reactions and peripheral neuropathy for Abraxane [3, 4]. While the enhanced permeability and retention (EPR) effect has been shown to improve preferential tumor accumulation of various agents [5–7], improvements in overall patient survival still remain modest [8]. This is likely due to tumor-related barriers to effective therapeutic delivery, including a relatively more dense and complex extracellular matrix (ECM) with narrower extracellular spaces and elevated interstitial fluid pressure. These tumor features hinder the penetration of drugs and NPs into and within the tumor interstitium [5, 9–13]. Indeed, Doxil and other clinically approved NPs have shown limited penetration within solid tumor tissue [14–20].

Recent studies have demonstrated that therapeutic NPs with diameters less than 50 nm exhibit improved tumor penetration compared to larger particles and effective tumor growth inhibition *in vivo*. Cabral and colleagues demonstrated that in a poorly permeable human tumor xenograft model characterized by low vascularity and dense fibrosis, only small micelles <50 nm in diameter were able to accumulate in tumors [21]. Similarly, Tang *et al.* evaluated three NP formulations to identify the optimal size for the most effective anticancer drug delivery [22]. They found that, ~50 nm particles had the highest tumor tissue retention due to deep tissue penetration, cancer cell internalization, and slow tumor clearance, ultimately leading to the highest efficacy against primary and metastatic tumors *in vivo*. In another study, Wang *et al.* found that increased tumor accumulation of 100 nm micelles with encapsulated SN38 prodrug did not result in significantly improved therapeutic efficacy because these large micelles had poorer tumor penetration than their smaller 30 nm counterparts [23]. While these studies provide valuable information about potential NP size limits for effective tumor penetration, the influence of surface properties on the optimal size and dispersion of NPs in tumors remains unclear. Previous studies have demonstrated that eliminating the non-specific binding of NPs by adding a dense low molecular weight polyethylene glycol (PEG) coating enables larger than expected particles to rapidly penetrate various biological environments [24–27]. Therefore, we speculated that (1) relatively large NPs (>50 nm) may penetrate in tumor tissues if they are densely coated with PEG and (2)

non-PEGylated clinically approved NPs such as Abraxane may exhibit non-specific binding towards tumor ECM components, and this could play a role in the limited clinical benefit observed with these formulations to date.

In this study, we examined the effect of size and surface PEG density on NP penetration within tumor tissue, both *ex vivo* and *in vivo*. Using polymeric polystyrene (PS) particles over the size range of 20–100 nm, we first tested how these parameters impact NP diffusion in a tumor ECM preparation (Matrigel) by multiple particle tracking (MPT) and MDA-MB-231 breast cancer xenograft tissue by MPT and intravital microscopy. Next, we investigated the effect of PEG surface density on the diffusion of ~60 nm PS NPs in the same models. Nonspecific binding interactions of the NPs to Matrigel were determined by a surface plasmon resonance (SPR) assay in an attempt to correlate *in vitro* interactions with *ex vivo* and *in vivo* diffusion behavior. Finally, biodegradable poly(lactic-co-glycolic acid) (PLGA) NPs with various surface PEG densities, as well as two clinical-grade NPs, Doxil and Abraxane, were evaluated by SPR to evaluate their non-specific binding to Matrigel as a surrogate for their ability to penetrate solid tumors. Through these studies, we determined valuable criteria for particle size and surface characteristics that mediate penetration of NPs within tumor tissue and identified key parameters of clinically-used NPs that may contribute to therapeutic efficacy.

METHODS

Materials

5 kDa MW polyethylene glycol (PEG), methoxy-PEG5k-amine was purchased from Creative PEGWorks (Winston Salem, NC). Red (20 nm, 40 nm, and 100 nm, 580/605 excitation/emission) and Green (40 and 100 nm, 505/515 excitation/emission) carboxyl (COOH)-modified PS FluoSpheres (PS-COOH NPs) were purchased from Invitrogen (Carlsbad, CA). Poly(lactic-co-glycolic acid) (PLGA, LA/GA molar ratio 50:50, MW 7,000–17,000 Da) and PEG-PLGA (MW 5,000:10,000 Da) were purchased from PolySciTech (West Lafayette, IN). Matrigel™ Basement Membrane Matrix was purchased from BD Biosciences (San Jose, CA). Lab-Tek glass-bottom tissue culture plates were purchased from ThermoFisher Scientific (Rochester, NY). Clinical-grade Abraxane and Doxil were obtained from the University of Maryland Greenebaum Cancer Center Katz Pharmacy. Cell culture materials were purchased from Invitrogen Corp. (Carlsbad, CA) unless specified. 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (sulfo-NHS), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification.

Nanoparticle preparation

To formulate PEG-coated nanoparticles (CNPs), 20, 40, and 100 nm PS-COOH NPs were covalently modified with methoxy-PEG5k-amine by carbodiimide chemistry, following a modified protocol previously described [24, 25, 28]. Briefly, 20 and 100 nm PS-COOH nanoparticles (1 mg) were mixed with methoxy-PEG5k-amine (4–5× equivalent to total COOH groups on surface of PS-COOH particles) in 100 mM borate buffer (pH 8.2), followed by addition of excess sulfo-NHS (2–3 mg) and EDC (0.5–1 mg) to a volume of 500

μ L. For 40 nm CNPs, a different proportion of methoxy-PEG5k-amine (0.1x, 0.25x, and 1x equivalent to total COOH groups on surface of PS-COOH particles) was used for PS-COOH particle PEGylation. After the reaction, particles were purified by centrifugation (Amicon Ultra-15 mL 100 kDa MW cut-off) with ultrapure water for a total of 3 washes. CNPs were resuspended in ultrapure water and stored at 4 °C until use.

Biodegradable PLGA and PEG-PLGA nanoparticles were prepared by the nanoprecipitation method. PLGA and PEG-PLGA at determined ratios were dissolved in tetrahydrofuran (THF) at a concentration of 28 mg/mL and added dropwise into 10 mL of Tween-20 (0.25% w/v in water) solution under magnetic stirring (700 rpm). After 4 h of stirring and the complete removal of THF by evaporation, nanoparticles were first purified by centrifugation (Amicon Ultra-15 mL 100 kDa MW cut-off) with ultrapure water for a total of 2 washes. The nanoparticles were further purified by microcentrifugation at $21.1 \times g$ for 10 min with ultrapure water (2 washes total). Following resuspension, particles were allowed to settle for 1 h and the top layer containing the well suspended NPs was collected, discarding the settled NPs.

Physicochemical characterization of nanoparticles

The physicochemical characteristics of COOH- and PEG-coated fluorescent nanoparticles of all sizes and biodegradable formulations were measured in 15× diluted PBS (~10 mM NaCl, pH 7.4). Hydrodynamic diameter and ζ -potential (surface charge) were determined by dynamic light scattering and laser Doppler anemometry using a Zetasizer NanoZS (Malvern Instruments, South Borough, MA). Particle size measurement was performed at 25 °C at a scattering angel of 173° and is reported as the number-average mean. The surface charge on the particles was calculated using the Smoluchowski equation and is reported as the mean ζ -potential. Data for diameter (nm) and ζ -potential (mV) represents the average of 3 independent experiments with 10 runs each $\pm$ SD.

Surface PEG density calculation

The surface concentration of PEG (# of PEG chains/100 nm²) and Γ/Γ^* , where Γ is the PEG surface coverage over the total surface area (Γ^*), was calculated from the ¹H integrals of the ethylene oxide peak of PEG (3.6 ppm) using a previously described method [25]. Briefly, nanoparticles were lyophilized, weighed and dissolved in chloroform-d (CDCl₃) containing 0.1% (v/v) trimethylsilane (TMS) as an internal standard. NMR spectra were obtained at 500 MHz using Agilent DD2 500 MHz Spectrometer. A calibration curve was obtained by plotting the ¹H NMR integrals of various concentrations of 5 kDa PEG (~3.6 ppm) in the same CDCl₃ solvent containing 0.1% (v/v) TMS. The average PEG surface density (# of PEG chains/100 nm²) on the surface of the nanoparticles was calculated by taking the total quantity of PEG detected by NMR and the total particle surface area. The surface area of nanoparticles was calculated assuming that the particles are made of individual particles of diameter equal to that measured by the Zetasizer using a density of 1.055 g/cm³ and 1.34 g/cm³ for PS and PLGA nanoparticles, respectively.

Nanoparticle diffusion in Matrigel

The diffusion of individual fluorescent nanoparticles in Matrigel was quantified using multiple particle tracking (MPT) as previously described [25]. Fluorescent nanoparticles (2 $\mu\text{g/mL}$) were diluted in cold Matrigel (4 °C) and then added to Lab-Tek glass-bottom chamber. The chamber was placed in at 37 °C incubator for a minimum of 15 min before imaging to allow Matrigel to form into a gel matrix. The movement of individual nanoparticles in Matrigel was imaged at a frame rate of 20 frames/s for a total of 400 frames (20 s) for 40 and 100 nm nanoparticles and at a frame rate of 10 frames/s for a total of 400 frames (40 s) for 20 nm nanoparticles. Images were captured using a Zeiss LSM5 Duo slit scanning confocal microscope (Zeiss, Thornwood, NY) with a 63 $\times$ Plan-Apo/1.4 NA oil-immersion objective. Movies were analyzed using a custom written MATLAB automated tracking code to extract x , y -coordinates of nanoparticles over time, as previously described [24, 25]. Each particle type was imaged at least three separate times with at least 100 particles tracked per sample every time. The geometric mean of the mean squared displacement (MSD) was calculated per particle type and the average was calculated as a function of time scale.

Nanoparticle binding to Matrigel

Nanoparticle binding affinities to Matrigel were evaluated by SPR using a Biacore 3000 instrument at 25 °C. Matrigel was diluted to 100 $\mu\text{g/mL}$ in acetate buffer pH 4.0 and was conjugated to a CM5 Biacore chip with RU value of ~ 2000 . The first flow path (Fc1) was activated and blocked with ethanolamine to serve as a reference for each binding run, as suggested per manufacturer's protocol. The running buffer, 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl, 0.05% surfactant Tween 20 with 50 μM EDTA (HBS-P+), was degassed prior to use. For SPR experiments, samples were run at a flow rate of 20 $\mu\text{L/min}$ with an injection time of 3 min followed by a 2 min wait time for dissociation, before chip regeneration with 10 mM glycine, pH 1.75 (GE Healthcare). Nanoparticle binding was assayed with particle concentrations of 1 mg/mL diluted in running buffer. Data were analyzed using Biacore 300 Evaluation Software, where data from Fc1 was subtracted from the Fc2, Fc3, and Fc4 data to give the final sensorgrams.

Cell culture

MDA-MD-231 breast cancer cells were provided by Dr. Stuart Martin (University of Maryland School of Medicine (UMSOM)). Cells were cultured at 37 °C in a humidified incubator (95% air, 5% CO_2) in DMEM supplemented with 10% Fetal Bovine Serum (FBS) 1% of penicillin-streptomycin (1000 units/L), 0.25 mg/ml G418 sulfate (Corning, Manassas, VA), and 0.5 mg/ml hygromycin B (Corning, Manassas, VA).

Implantation of MDA-MB-231 breast cancer cells into immunodeficient mice

All animal procedures were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC) and the Office of Animal Welfare Assurance (OAWA). Athymic nude female mice (age, 6–8 weeks) were purchased from the UMSOM Veterinary Resources. For the tumor implantation procedure, animals were anesthetized via continuous flow of 2–3% isoflurane through a nose cone. MDA-MB-231 cells (2×10^6 cells) in 100 μL

of DMEM were mixed with 100 μ L of Matrigel and subcutaneously inoculated in both flanks of the mice.

Nanoparticle diffusion in breast tumor slices

The diffusion of individual fluorescent nanoparticles in tumor tissue slices was quantified via MPT as described above. Mice bearing MDA-MB-231 xenografts (~ 500 mm³ in volume) were euthanized and the tumor was harvested and sliced into 2 mm sections using a Zivic matrix slicer (Zivic Instruments, Pittsburgh, PA). Slices were added to custom microscope slide chambers and red fluorescent nanoparticles were injected (0.5 μ L of 20–50 μ g/mL stocks) into tumor slices using a Hamilton syringe aided by a stereotactic frame. Slides were sealed with super glue and allowed to incubate at 37 °C for a minimum of 15 min before imaging to allow tissue recovery and convection dissipation. The movement of individual nanoparticles in tumor tissue slices was analyzed by MPT as described above.

Nanoparticle penetration in breast tumor xenografts

Mice bearing MDA-MB-231 subcutaneous tumors were secured and anesthetized on a custom-designed, heated microscope stage from the UMSOM Confocal Microscopy Core. Animals were anesthetized with 2–4% isoflurane vaporizer and nose cone mixed with 1 L/min oxygen administration throughout the surgical procedure. The custom microscope stage has a heating source to maintain body temperature of 37 °C. A single midline incision was made in the skin and the skin-flap attached to the tumor was gently separated from the underlying musculature and then draped over parallel to the mouse using needle retractors. A Hamilton syringe was inserted gently into the exposed tumor at a depth of 100–200 μ m and a 0.5 μ L volume injection was made at this depth to deliver nanoparticle formulations (500 μ g/mL). The particle distribution within the tumor was assessed by a Zeiss LSM 710 2-photon confocal microscope (Zeiss, Thornwood, NY) using a 10x/0.45 NA air objective. Images were acquired every 10 min for a total of 1 h.

Statistical analysis

A nested mixed-effects model was used to estimate and compare the average MSD for Matrigel and tumor tissue slice groups over time [29]. MSD values were log transformed (base 10) for statistical analyses to assure approximate normality and to decrease variability. The data have a hierarchical structure, or represent a nested design. There is more than one size of experimental unit and a smaller experimental unit is nested within a larger one, i.e. a particle is nested within a group (uncoated or coated). A nested mixed model was used to estimate and compare outcome (log MSD) between two groups. The model included the fixed effects of nanoparticle size, coating density, time, and their interaction. Common factor for random effects was specified using unique particle number. The reported *p*-values correspond to the tests for overall difference between the average MSD for Matrigel and tumor tissue slice groups at 1 second for each nanoparticle type. All tests were performed at the 0.05 level of significance. Statistical analyses were performed using JMP Pro 10.0.2 (SAS Institute Inc., Cary, NC) and SAS 9.4 (SAS Institute Inc., Cary, NC).

RESULTS

Surface modification and characterization of PS nanoparticles of different sizes

We first formulated 20, 40, and 100 nm PEG-coated PS nanoparticles (CNP) and compared these formulations to 20, 40, and 100 nm uncoated carboxyl-modified PS (PS-COOH) nanoparticles (UNP) (Table 1). The CNPs exhibited a ~10 to 20 nm increase in hydrodynamic diameters and more near-neutral ζ -potentials compared to their negatively-charged UNP counterparts of the same size, as expected for nanoparticles with dense PEG coatings.

Diffusion of nanoparticles of different sizes in Matrigel

We have previously shown that CNPs up to ~100 nm in diameter are able to diffuse rapidly through brain tissue ECM, provided they have sufficiently dense PEG coatings [24, 25]. Here, we applied similar dense PEG coatings to 20, 40 and 100 nm PS particles (CNPs) and examined their diffusion in Matrigel, an ECM preparation derived from a mouse EHS sarcoma that is thought to resemble the extracellular environment found in most solid tumors [30], to establish size criteria for CNPs in penetrating tumor tissue. Fluorescent 20, 40, and 100 nm CNPs or UNPs (Table 1) were mixed with Matrigel and MPT was used to test the diffusion rates of individual particles. All three sets of UNPs (20, 40, and 100 nm), regardless of size, were immobilized in a Matrigel matrix (Fig. 1A). Additionally, the 100 nm CNPs were also immobilized in the Matrigel matrix (Fig. 1A), despite a dense PEG coating, suggesting that 100 nm CNPs were too large to fit through the pores in the tumor tissue ECM. In contrast, 20 and 40 nm CNPs exhibited more diffusive trajectories, which were quantitatively observed by the upward shift in the mean square displacement (MSD) vs time scale (τ) curve compared to 20, 40, 100 nm UNP and 100 nm CNP (Fig. 1A). The calculated MSD at a time scale (τ) of 1 s for 20 and 40 nm CNP formulations were statistically greater than 20 and 40 nm UNP formulations (Fig. 1B). There was also a statistically significant difference in MSD between 20 nm CNP and 40 nm CNP and between 40 nm CNP and 100 nm CNP at $\tau = 1$ (Fig. 1B). To ensure that the observed rapid diffusion for 20 and 40 nm CNPs was not biased by a small fraction of fast-moving outliers, we examined the distribution of individual particles. We found that a substantial fraction of 20 and 40 nm CNPs exhibited a more diffusive transport compared to 100 nm CNPs and the other UNP formulations (Fig. 1C).

Diffusion of nanoparticles of different sizes in breast tumor slices

Fluorescent 20, 40, and 100 nm CNPs or UNPs (Table 1) were injected directly into freshly dissected MDA-MB-231 breast tumor slices and MPT was used to test the diffusion rates of individual particles. Consistent with the Matrigel experiment, 100 nm CNPs and all three UNP formulations were immobilized or strongly hindered within the breast tumor ECM. In contrast, 20 and 40 nm CNPs exhibited rapid diffusion in breast tumor tissue. This can be quantitatively observed by the upward shift in the MSD vs time scale (τ) curve for 20 and 40 nm CNPs compared to 20, 40, 100 nm UNPs and 100 nm CNPs (Fig. 2A). The calculated MSD at a time scale (τ) = 1 for the 20 nm CNP formulation was statistically greater than 20 nm UNP (Fig. 2B). There was also a statistically significant difference in MSD between 20 nm CNP and 100 nm CNP and between 40 nm CNP and 100 nm CNP at $\tau = 1$ (Fig. 2B).

There was no statistically significant difference in the MSD between 100 nm UNP and 100 nm CNP at $\tau = 1$. By examining the distribution of individual particle diffusivities, a substantial fraction of 20 and 40 nm CNPs exhibited a more rapidly diffusive fraction compared to the other formulations (Fig. 2C).

Surface modification and characterization of PS nanoparticles with different PEG densities

We selected 40 nm CNPs (actual hydrodynamic size ~63 nm) for surface PEG density variation studies based on their ability to rapidly diffuse in both Matrigel and MDA-MB-231 breast tumor slices. The 40 nm CNPs with three different surface PEG densities were formulated by varying the amount of methoxy-PEG_{5k}-NH₂ that was added to the reaction mixture. As expected, there was an inverse relationship between PEG density and ζ -potential; specifically, as the amount of PEG on the surface increases, the ζ -potential becomes more neutral (Table 2). We also saw a correlation between NP size and PEG density with 40 nm CNP (high) exhibiting the largest diameter of ~63 nm and highest surface PEG coating with Γ/Γ^* of 3.6 (Table 2), where Γ is the PEG surface coverage over the total surface area (Γ^*). The 40 nm CNP (med) and 40 nm CNP (low) formulations displayed particle diameters of 57 and 54 nm with Γ/Γ^* of 1.4 and 0.8, respectively.

Biacore screening of nanoparticles for non-specific binding to Matrigel

Our group has previously used SPR assay to evaluate non-specific binding of PS-based CNPs to mouse brain ECM proteins immobilized on a Biacore chip, and these results correlated with *in vivo* imaging of CNP spread in the brain [24]. Here, we sought to use a similar SPR assay to examine non-specific binding of UNPs and CNPs to a Matrigel-coated chip. We found that 40 nm UNPs bound strongly to the Matrigel-coated Biacore chip (Fig. 3A). The CNP formulation with the lowest PEG density on the surface, 40 nm CNP (low), also showed relatively strong binding to the Matrigel-coated chip, although not as strongly as UNP (Fig. 3B). In contrast, the other 40 nm CNP formulations with medium and high PEG density, 40 nm CNP (med) and 40 nm CNP (high), did not bind appreciably to the Matrigel-coated chip, suggesting minimal non-specific interactions between the particles and Matrigel components (Fig. 3B).

Diffusion of nanoparticles with different PEG density in Matrigel and breast tumor slices

Fluorescent 40 nm CNP (low), 40 nm CNP (med), and 40 nm CNP (high) were mixed with Matrigel and MPT was used to test the diffusion rates of individual particles. The 40 nm CNP (high) formulation was equivalent to 40 nm CNPs used in Figure 1. We found that uncoated 40 nm UNPs were largely immobilized in the Matrigel matrix (Fig. 4A). In contrast, 40 nm CNP (low), 40 nm CNP (med), and 40 nm CNP (high) exhibited enhanced transport rates. The calculated MSDs at a time scale (τ) = 1 for 40 nm CNP (low), 40 nm CNP (med), and 40 nm CNP (high) were significantly greater than 40 nm UNP and 40 nm CNP (low) (Fig. 4B).

We also tested the diffusivity of the same 40 nm UNP and 40 nm CNPs in MDA-MB-231 breast tumor slices *ex vivo*. Similar to their transport behavior in Matrigel, 40 nm UNP and 40 nm CNP (low) particles were immobilized in the tumor tissue slice (Fig. 5A). In contrast, the calculated MSDs at a time scale (τ) = 1 for 40 nm CNP (med) and 40 nm CNP (high)

formulations were statistically greater than 40 nm UNP formulation. There was also a statistically significant difference in the calculated MSDs between 40 nm CNP (low) and 40 nm CNP (med) formulations at $\tau = 1$ (Fig. 5B).

Nanoparticle penetration in live animals bearing breast tumor xenografts

We directly examined *in vivo* penetration of NPs in breast tumor xenografts using live-animal microscopy to determine if our findings obtained using Matrigel (SPR and MPT assays) and tumor tissue slices (MPT assay) could be extended to the *in vivo* setting. Red fluorescent 40 nm UNPs and green fluorescent 40 nm CNPs (high) were co-injected into the tumor at a depth of ~ 100 μm below the tumor surface and NPs were visualized by intravital fluorescence microscopy. We found that 40 nm UNPs were immobilized in the tissue at the injection site, whereas 40 nm CNPs penetrated over 500 μm into the tumor (Fig. 6A). To further study the differences between size and particle coating, we performed a co-injection of red fluorescent 40 nm UNPs and green fluorescent 100 nm CNPs. Similar to the results observed in the SPR and MPT assays, 40 nm UNPs and 100 nm CNPs did not penetrate into the tumor, as evidenced by an overlay between red and green fluorescence in the merged image (Fig. 6B).

Synthesis and physicochemical characterization of biodegradable nanoparticles

Biodegradable NPs composed of block copolymers of poly(lactic-*co*-glycolic acid) (PLGA) and PEG were formulated to have different surface PEG densities by varying the amount of PEG-PLGA that was added to the reaction mixture. We formulated PLGA and PEG-PLGA NPs with 1, 2.5, and 5% PEG by weight. As expected, uncoated PLGA NPs exhibited the most negative ζ -potential compared to the other formulations (Table 3). We found an inverse relationship between PEG density and ζ -potential; specifically, as the amount of PEG on the surface increased, the ζ -potential becomes more neutral. In addition, there was an inverse relationship between NP size and PEG density with uncoated PLGA NPs exhibiting the largest diameter of 187 nm and 5% PEG-PLGA NPs having the smallest diameter of 109 nm. Using a nuclear magnetic resonance (NMR)-based method to quantify the PEG density, we estimated that 1, 2.5, and 5% PEG-PLGA NPs had PEG conformation (Γ/Γ^*) of 2.1, 2.7, and 2.8, respectively.

Biacore screening of PLGA nanoparticles, Doxil, and Abraxane for non-specific binding to Matrigel

We used the same SPR assay as described earlier to screen biodegradable PLGA-based and two clinically approved NPs for non-specific binding to tumor ECM. The uncoated PLGA NP formulation and 1% PEG-PLGA particles bound to the surface of the Matrigel-coated chip (Fig. 7A). In contrast, the other PEG-PLGA formulations with 2.5% and 5% PEG did not bind to the Matrigel-coated chip, suggesting minimal non-specific interactions between the particles and tumor ECM components (Fig. 7B). Using another Matrigel-coated chip that was prepared in the same way (same RU ~ 2000), we detected relatively strong non-specific binding of Abraxane to tumor ECM components (Fig. 7C). On the other hand, the clinical-grade Doxil did not bind appreciably to the Matrigel-coated chip, suggesting minimal non-specific binding of this therapeutic NP formulation to tumor ECM components (Fig. 7C).

DISCUSSION

Particle size and surface characteristics are fundamental, modifiable features of therapeutic NPs that mediate *in vivo* behavior and thus the eventual therapeutic ratio of encapsulated drugs [10, 11, 13]. Here, we examined the size and surface characteristics that affect particle adhesivity to tumor ECM (Matrigel) and particle movement within breast tumor xenograft tissue, *ex vivo* and *in vivo*. Using densely PEGylated, polystyrene (PS) NPs, we determined the diffusivity-size threshold and found a steric limit between 63 and 116 nm diameter. Intravital microscopy of NP spread in living tissue confirmed a significant difference in tumor tissue penetration between 63 and 116 nm PEG-PS NPs, as well as between PEG-coated and uncoated NPs. Further examination of the particle surface PEG density-diffusivity relationship using SPR and MPT techniques revealed a PEG density threshold related to tumor ECM binding that was consistent across multiple particle types, including PS NPs, biodegradable PLGA NPs, and two clinically-approved NPs, Abraxane and Doxil.

Penetration of therapeutic agents within tumor tissue has been found to be a critical mediator of treatment duration, toxicity, and efficacy following delivery of free drug, protein-drug conjugates and drug-loaded particles [10, 13, 31, 32]. This is particularly true in tumors where high intratumoral pressure limits movements of therapeutic agents into the tissue and a dense ECM network hinders diffusive transport throughout the tumor [33–35]. The importance of the tumor ECM barrier has been acknowledged in previous studies that have focused on reducing the tumor ECM matrix barrier by either directly degrading tumor ECM components or using a multistage delivery approach where NP size decreases in response to matrix metalloproteinases (MMPs) present in the tumor microenvironment [8, 36, 37]. Previous findings from other groups revealed that sub-50 nm NPs are required for deep tissue penetration and retention in tumors [8, 21–23]. Our current study provides *in vivo* evidence that larger NPs can penetrate tumor ECM if densely coated with PEG. This result is significant in the context that most clinically tested NP formulations, including liposomes (Doxil, ~100 nm), albumin-coated NPs (Abraxane, ~130 nm), and PLGA NPs (Accurins, ~100 nm) typically have a formulation size limit of >50 nm [35].

Surface chemistry is another parameter that plays an important role in NP interactions with tumor ECM components and movement within tumor tissues [38, 39]. It has been shown that NPs coated with PEG accumulate more efficiently in tumor compared to similar uncoated NPs, due in part to improved blood circulation time and their ability to avoid clearance mechanisms [40–42]. With regard to particle penetration within tumor tissue, it is thought that dense PEG coatings promote dispersion of NP within tumors by reducing binding to tumor tissue components [8, 35, 43]. High surface PEG density has been shown to assume a brush like conformation, as opposed to a mushroom configuration, at low PEG density [43, 44], limiting exposure of electrostatic and hydrophobic surfaces that facilitate non-specific adhesion. In a prior study by Nance and colleagues that focused on brain tissue penetration, a nuclear magnetic resonance (NMR)-based method was used to quantify the PEG coating density that correlated with brain penetration. The authors found that in order for NPs to diffuse in human and rodent brain tissue, 100 nm PS NPs must have ~9 PEG molecules per 100 nm² of particle surface and a PEG layer in a brush regime with $\Gamma/SA \geq 2$, where Γ is the PEG surface coverage over the total surface area (SA) [25]. Similarly, we

found that negatively charged PS and PLGA NPs with exposed hydrophobic regions exhibit hindered diffusion in tumor tissues regardless of the NP size. We observed diffusive behavior in tumor tissues for 40 nm CNP (med) and 40 nm CNP (high) with $\Gamma/\Gamma^* = 1.4$, whereas the coating density of $\Gamma/\Gamma^* = 0.8$ for 40 nm CNP (low) was not sufficient for tumor penetration. Interestingly, the PEG-PLGA NP formulations exhibited a slightly higher threshold for minimum PEG density to prevent non-specific binding to the Matrigel-coated chip. We found that PLGA and PEG-PLGA NPs need to be formulated with at least 2.5% PEG on the surface with $\Gamma/\Gamma^* = 2.7$ and $\Gamma/\Gamma^* = 2.1$ for 1% PEG-PLGA NPs was not sufficient to prevent the non-specific binding to tumor ECM components. This discrepancy between the PS and PLGA NPs is likely due to incomplete partitioning of the hydrophilic PEG segments to the surface of the PEG-PLGA nanoparticles during the formulation process by the nanoprecipitation method [45]. Thus, our NMR-based quantification analysis of total PEG content in NPs may overestimate the minimum surface PEG density of PEG-PLGA NPs required to reduce non-specific binding to the Matrigel-coated chip. In addition, relatively porous and rough surface structures of the PEG-PLGA NPs (compared to PS NPs) may further reduce the “effective” PEG chain surface coverage around the NPs. Therefore, the estimated PEG surface density required for tumor penetration will likely depend on the material being coated as well as PEG molecular weight. We recognize that PEG surface coating is not a requirement for tumor penetration, as long as the exposed surfaces do not bind non-specifically to tumor ECM components [46]. However, based on our work, most drug carriers that we tested including PS NPs, PLGA NPs, and Abraxane, suffered from strong non-specific binding to tumor ECM components when the particle surfaces were not shielded by PEG coatings.

More than 20 therapeutic NPs have been approved for clinical use by the FDA and many others are currently being evaluated in clinical trials [8]. There have been very few studies using SPR to test non-specific interactions of nanomedicines such as Doxil and Abraxane with biologically-relevant molecules. Our work highlights the value of SPR as a method to assess the NP adhesivity and thereby estimate tumor penetration capability of many clinically relevant NPs, which cannot be analyzed via conventional microscopy without additional surface modifications or fluorescent tags [47]. Using this SPR methodology, we determined that Abraxane, but not Doxil, displayed strong non-specific interaction with tumor ECM components. This has important clinical implications. For example, the PEG coating density on Doxil, 5 wt % of 2 kDa PEG, is sufficient to prevent the non-specific binding to tumor ECM components, which suggests that the inability of Doxil to penetrate in tumors [15] is likely due to steric size and/or particle stability limitations. This new finding motivates further development of Doxil-based PEGylated liposomal formulations with smaller sizes (<100 nm). Also, while there is some preclinical evidence that Abraxane disintegrates into smaller size NPs (~10 nm) after it enters the bloodstream [8], the majority of the particles are found along the tumor blood vessels, with very few diffusing into the tumor tissue [19, 20]. Our SPR results revealed relatively strong nonspecific binding of Abraxane to tumor ECM components, which may explain the lack of tumor penetration *in vivo* in addition to the potential steric obstruction of the intact Abraxane particles (~130 nm). Therefore, we expect that effective shielding of the exposed surfaces of Abraxane, such

as with PEG coating, may enhance its tumor penetration and further improve therapeutic efficacy.

In conclusion, the findings in this study provide new insights into the NP size and surface PEG density thresholds related to tumor tissue penetration and adhesivity to extracellular tumor components. In particular, this work may help explain *in vivo* differences between clinically-relevant particulate delivery systems including albumin-based nanoformulations, PEGylated liposomes, and biodegradable polymeric NPs. Finally, the results from this study are likely to help guide the design of new particulate drug carriers for enhanced tumor tissue penetration and greater therapeutic efficacy.

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Figure 1. Diffusion of nanoparticles of different sizes in Matrigel

The diffusion of individual fluorescent polystyrene (PS) NPs in Matrigel was quantified using multiple particle tracking (MPT). (A) Ensemble-averaged mean square displacements (MSD) as a function of time scale for 20, 40, and 100 nm uncoated (UNP) and PEG coated (CNP) PS NPs. (B) The ensemble-averaged MSD of nanoparticles at a time scale of 1 s. (C) Distributions of the logarithms of individual MSDs for UNPs and CNPs of different sizes at a time scale of 1 s. Larger MSD values indicate faster transport rates of nanoparticles. Data represents at least three experiments, with $n = 100$ particles per experiment. $**P < 0.01$.

Figure 2. Diffusion of nanoparticles of different sizes in MDA-MB-231 tumor tissue *ex vivo* MDA-MB-231 breast cancer cells were injected into the flank of mice and tumors were allowed to grow to $\sim 500 \text{ mm}^3$. Tumor slices were prepared, fluorescent NPs were injected into the tumor, and the diffusion of individual NPs was quantified using multiple particle tracking (MPT). For these experiments, the transport rates of all three particle sizes, with and without PEG coatings, were measured in the same tumor tissue. (A) Ensemble-averaged mean square displacements (MSD) as a function of time scale. (B) The ensemble-averaged MSD of nanoparticles at a time scale of 1 s. (C) Distributions of the logarithms of individual MSDs for UNPs and CNPs of different sizes at a time scale of 1 s. Data represents at least three experiments, with $n = 100$ particles per experiment. * $P < 0.05$, ** $P < 0.01$.

Figure 3. Surface plasmon resonance (SPR) analysis of 40 nm nanoparticles with varying surface PEG densities

SPR experiments were performed by flowing various NP formulations into flow cells and across the surface of a Matrigel-coated sensor chip. Data is displayed as sensograms showing resonance units (RU) as a function of time. (A) SPR analysis measuring the binding of 40 nm uncoated (UNP) and coated (CNP) nanoparticles ranging in PEG-coating densities (low, medium, high) to a Matrigel chip. (B) Expanded view of boxed region in A.

Figure 4. Diffusion of nanoparticles with varying PEG densities in Matrigel

The diffusion of individual fluorescent NPs that were either uncoated or coated with a low, medium, or high PEG density in Matrigel was quantified using multiple particle tracking (MPT). (A) Ensemble-averaged mean square displacements (MSD) as a function of time scale. (B) The ensemble-averaged MSD of nanoparticles at a time scale of 1 s. Data represents at least three experiments, with $n = 100$ particles per experiment. $**P < 0.01$.

Figure 5. Diffusion of nanoparticles with varying PEG densities in MDA-MB-231 tumor tissue *ex vivo*

MDA-MB-231 breast cancer cells were injected into the flank of mice and tumors were allowed to grow to $\sim 500 \text{ mm}^3$. Tumor slices were prepared and the diffusion of individual fluorescent NPs was quantified using multiple particle tracking (MPT). (A) Ensemble-averaged mean square displacements (MSD) as a function of time scale. (B) The ensemble-averaged MSD of nanoparticles at a time scale of 1 s. Data represents at least three experiments, with $n = 100$ particles per experiment. * $P < 0.05$, ** $P < 0.01$.

Figure 6. Nanoparticle penetration of breast tumor tissue *in vivo*

MDA-MB-231 breast cancer cells were injected into the flank of mice and tumors were allowed to grow to $\sim 500 \text{ mm}^3$. Fluorescent nanoparticle formulations were injected at a depth of 100–200 μm and particle distribution within the tumor was assessed by 2-photon confocal microscopy. (A) Direct comparison of the distribution of fluorescent uncoated and PEG coated 40 nm PS NPs after direct tumor co-injection into mice. Images were acquired within 10 minutes after injection. (B) Direct comparison of the distribution of PEG-coated 100 nm PS NPs (100 nm CNP) and uncoated 40 nm PSCOOH NPs (40 nm UNP). Images were acquired within 10 minutes after injection. Arrow indicates approximate position of injection needle. Scale bar is 500 μm .

Figure 7. Surface plasmon resonance (SPR) analysis of biodegradable nanoparticles
 SPR experiments were performed by flowing various NP formulations into flow cells and across the surface of a Matrigel-coated sensor chip. Data is displayed as sensograms showing resonance units (RU) as a function of time. (A) SPR analysis measuring the binding of PLGA and PEG-PLGA nanoparticles ranging in PEG-coating percentages (1%, 2.5%, 5%) to a Matrigel chip. (B) Expanded view of boxed region in A. (C) Screening of the FDA-approved nanodrugs Abraxane and Doxil for non-specific binding to tumor ECM. Data was compiled from separate runs using the same Matrigel chip.

Table 1

Physicochemical properties of NPs of different sizes.

Nomenclature	Base NP type	Surface modification	Hydrodynamic diameter (nm) ^a	ζ-potential (mV) ^b
20nm UNP	20nm PSCOOH	None	25 ± 1	-41.6 ± 8.6
20nm CNP	20nm PSCOOH	mPEG _{5K} -NH ₂	37 ± 2	-2.9 ± 0.8
40nm UNP	40nm PSCOOH	None	50 ± 1	-47.7 ± 2.4
40nm CNP	40nm PSCOOH	mPEG _{5K} -NH ₂	63 ± 1	-4.8 ± 0.5
100nm UNP	100nm PSCOOH	None	96 ± 1	-59.4 ± 3.8
100nm CNP	100nm PSCOOH	mPEG _{5K} -NH ₂	116 ± 2	-4.4 ± 0.4

^aDiameter (number mean) measured by dynamic light scattering. Data represents the average of 3 independent experiments +/- SD.

^bMeasured at 25°C in 15× diluted PBS with ~9 mM NaCl, pH 7.4. Data represents the average of 3 independent experiments +/- SD.

Table 2

Physicochemical properties of low, medium, and high PEG-coated 40 nm NPs.

Nomenclature	Base NP type	Surface modification	Hydrodynamic diameter (nm) ^a	ζ -potential (mV) ^b	Surface PEG density (#/100nm ²) ^c	PEG conformation [Γ/Γ^*] ^d
UNP	40nm PSCOOH	None	50 ± 1	-47.7 ± 2.4	NA	NA
40nm CNP (low)	40nm PSCOOH	mPEG5K-NH ₂	54 ± 2	-16.7 ± 1.2	3.7 ± 1.6	0.8 ± 0.4
40nm CNP (med)	40nm PSCOOH	mPEG5K-NH ₂	57 ± 1	-8.0 ± 0.5	6.2 ± 0.7	1.4 ± 0.2
40nm CNP (high)	40nm PSCOOH	mPEG5K-NH ₂	63 ± 1	-4.8 ± 0.5	15.7 ± 8.6	3.6 ± 2

^aDiameter (number mean) measured by dynamic light scattering. Data represents the average of 3 independent experiments ± SD.

^bMeasured at 25°C in 15× diluted PBS with ~9 mM NaCl, pH 7.4. Data represents the average of 3 independent experiments ± SD.

^cPEG Surface density determined by NMR.

^dPEG surface coverage/total surface area (value < 1 indicates mushroom coverage (low density), whereas > 1 indicates brush regime (high density)).

Table 3

Physicochemical properties of biodegradable NPs.

Nomenclature	Hydrodynamic diameter (nm) ^a	PDI ^b	ζ-potential (mV) ^c	Surface PEG density (#/100nm ²) ^d	PEG conformation [Γ/Γ*] ^e
PLGA	187 ± 3	0.31 ± 0.04	-59.2 ± 0.5	NA	NA
PLGA-PEG 1%	135 ± 5	0.16 ± 0.02	-13.8 ± 0.6	9.1 ± 0.2	2.1 ± 0.1
PLGA-PEG 2.5%	128 ± 1	0.07 ± 0.01	-9.1 ± 0.7	11.9 ± 1.6	2.7 ± 0.4
PLGA-PEG 5%	109 ± 1	0.10 ± 0.01	-10.4 ± 0.9	12.3 ± 0.5	2.8 ± 0.1

^aDiameter (number mean) measured by dynamic light scattering. Data represents the average of 3 independent experiments +/- SD.

^bPDI (Polydispersity Index) indicates the distribution of individual molecular masses in a batch of nanoparticles. Measured by dynamic light scattering. Data represents the average of 3 independent experiments +/- SD.

^cMeasured at 25°C in 15× diluted PBS with ~9 mM NaCl, pH 7.4. Data represents the average of 3 independent experiments +/- SD.

^dPEG Surface density determined by NMR.

^ePEG surface coverage/total surface area (value <1 indicates mushroom coverage (low density), whereas >1 indicates brush regime (high density)).