Published in final edited form as:

Nano Lett. 2008 September ; 8(9): 2599–2606. doi:10.1021/nl080141f.

Real-Time Intravital Imaging of RGD–Quantum Dot Binding to Luminal Endothelium in Mouse Tumor Neovasculature

Bryan Ronain Smith[†], Zhen Cheng[†], Abhijit De[†], Ai Leen Koh[‡], Robert Sinclair[‡], and Sanjiv Sam Gambhir^{*,†,§}

[†]The Molecular Imaging Program at Stanford (MIPS), Department of Radiology and Bio-X Program

[‡]Department of Materials Science and Engineering, Stanford University, Stanford, California 94305

[§]Department of Bioengineering, Stanford University School of Medicine, Stanford, California 94305

Abstract

Nanoscale materials have increasingly become subject to intense investigation for use in cancer diagnosis and therapy. However, there is a fundamental dearth in cellular-level understanding of how nanoparticles interact within the tumor environment in living subjects. Adopting quantum dots (qdots) for their excellent brightness, photostability, monodispersity, and fluorescent yield, we link arginine–glycine–aspartic acid (RGD) peptides to target qdots specifically to newly formed/forming blood vessels expressing $\alpha_v\beta_3$ integrins. Using this model nanoparticle system, we exploit intravital microscopy with subcellular ($\sim 0.5 \mu\text{m}$) resolution to directly observe and record, for the first time, the binding of nanoparticle conjugates to tumor blood vessels in living subjects. This generalizable method enabled us to show that in this model qdots do not extravasate and, unexpectedly, that they only bind as aggregates rather than individually. This level of understanding is critical on the path toward ensuring regulatory approval of nanoparticles in humans for disease diagnostics and therapeutics. Equally vital, the work provides a platform by which to design and optimize molecularly targeted nanoparticles including quantum dots for applications in living subjects.

Quantum dots (qdots) are single nanocrystal colloidal semiconductors with exceptional fluorescent properties. This makes them highly conducive to preclinical optical imaging.^{1–8} Qdots have been used in living subjects to target tissue-specific vascular biomarkers³ and cancer cells^{1,2,4,5,8} and to identify sentinel lymph nodes in cancer^{9–12} with the potential for

© Copyright 2008 by the American Chemical Society

*Corresponding author: mail, Sanjiv S. Gambhir, MD, PhD, Director, Molecular Imaging Program at Stanford (MIPS), Head, Division of Nuclear Medicine, Professor, Department of Radiology and Bio-X Program, The James H. Clark Center, 318 Campus Dr., East Wing, first Floor, Stanford, CA 94305-5427; phone, 650-725-2309; fax, 650-724-4948; sgambhir@stanford.edu.

Supporting Information Available: Videos showing RGD–qdot injection and binding, RGD–qdots in normal mouse vasculature and tumor vasculature, Cy5.5–RGD block of RGD–qdots in tumor, unconjugated qdots entering the tumor vasculature, and Cy5.5 and Cy5.5–RGD leaking out of the tumor neovasculature and descriptions of nanoparticle conjugates, the tumor model, intravital microscopy, statistics, electron microscopy, and U87MG cells incubated with RGD–qdots. This material is available free of charge via the Internet at <http://pubs.acs.org>.

human translation, in addition to the impact they continue to make as fluorescent tags in cell biology.^{6,8} While ongoing toxicity and clearance studies remain to establish their clinical utility in humans,^{13,14} their excellent intrinsic optical reporter properties have made them an indispensable tool in preclinical biology. This is true particularly for our application of the qdot as a model nanoparticle (for nanoparticles of comparable size and shape) to study the binding and extravasation parameters of targeted nanoparticles in tumor neovasculature.

Numerous types of nanoparticles have been shown to enhance contrast in tumors with optical, nuclear, magnetic, and other methods^{15–17} via whole animal imaging modalities. Nanoparticles are modified to detect cancer by diverse means, such as targeting molecules on the surface of tumor vasculature and cancer cells in living subjects. Though these nanoparticles typically are specifically designed to accumulate in tumors, little is understood about the mechanisms by which this occurs at the vascular, cellular, and subcellular levels. Nanoparticles often are either molecularly targeted to tumors by linking tumor-specific targeting biomolecules to the nanoparticle surface or designed to allow passive extravasation in the tumor via the enhanced permeability and retention (EPR) effect.¹⁵ Use of the EPR effect is simpler, but because different types of tumors located at different sites have varying levels of vascular leakiness,^{18,19} targeting (or a multimechanism approach including both targeting and EPR¹⁵) is preferred. We choose a strategy to target tumor neovasculature in this work in order to avoid the problem of differential extravasation and binding. Tumor neovasculature/angiogenesis has become a prevalent target for both therapeutics and diagnostics.²⁰ Intravital microscopy has been critical in beginning to unravel the architecture and physiology of tumor neovasculature and immunology,^{9,21,22} yet little information is available on the behavior of nanomaterials in such vasculatures at the cellular level. Only recently have intravital techniques begun to emerge as a vital tool for studying the behavior of nanomaterials in vivo.^{23–26} Still, no studies have explored nanoparticle binding in the tumor neovasculature. This critical step has regulatory and design implications for imaging and therapeutic nanoparticle targeting in tumor neovasculature.

In this work, we exploited intravital microscopy (IV-100, Olympus, Center Valley, PA) to examine the binding of neovascularly targeted fluorescent nanoparticles to tumor neovasculature via direct cellular-level visualization in living mice (Figure 1a). Arginine–glycine–aspartic acid (RGD) and control peptide qdot conjugates were prepared as previously described with some modifications⁵ (see Figure 1b and Supporting Information for protocols). Nanocrystals were 6–8 nm in diameter by transmission electron microscopy (TEM) (see Figure 2) and ~20 nm hydrodynamically.⁵ Approximately 30–50 covalently bound peptides enveloped each qdot, which advantageously emits in the near-infrared (NIR) region for in vivo imaging, and RGD-qdots were shown to selectively bind integrin $\alpha_v\beta_3$ in cell culture on various cell lines and ex vivo on excised tumor tissues.⁵ The distribution and properties of RGD-qdots were examined by TEM (Philips CM200 operating at 200 kV, FEI Co., Eindhoven, Netherlands) and light microscopy as well as spectrophotometry (DU 640 from Beckman Coulter, Fullerton, CA) and dynamic light scattering (DLS, using a ZetaPlus Analyzer from Brookhaven Instruments Corp., Holtsville, NY) (see Figure 2 and Table 1). RGD was employed because of the very significant role that RGD's target, integrin $\alpha_v\beta_3$, plays in tumor angiogenesis, proliferation, and metastasis.²⁷ Enhanced green fluorescent

protein (EGFP)-expressing SKOV3 cells were inoculated in the ears of mice and were imaged with intravital microscopy (Figure 1, see Supporting Information for methods). Angiosense 680 (VisEn Medical, Woburn, MA) was used to define blood vessel walls due to its ability to remain within the vasculature (Figure S1 in Supporting Information). We observed and recorded in real time the binding of RGD–qdot aggregates to tumor luminal endothelium soon after intravenous administration via tail vein (movie S1, in Supporting Information). Quantification of RGD–qdot binding events was performed by examining many fields-of-view (FOV) in the tumor, evaluating each FOV throughout the accessible range of optically sectioned focal planes. Compared to RGD–qdots in tumor, controls displayed minimal binding (Figures 3 and 4 and Table 1). Thus, for the first time we have demonstrated the ability to directly follow the specific binding of nanoparticles to biomolecules expressed on tumor neovascular endothelium.

We examined the vessel walls for indications of individual qdot binding, because binding of many single RGD–qdots on luminal endothelial cells would presumably fluorescently outline vessel walls. Instead, interestingly and unexpectedly, binding events appeared to be restricted to aggregates of qdots tethering to multiple, discrete sites. This pattern was unanticipated because the literature does not indicate particularly patchy or focal integrin $\alpha_v\beta_3$ expression on angiogenic vessels.²⁸ Excluding aggregate binding, no fluorescence was visible on the neovascular endothelial lining once qdots cleared the vasculature, implying minimal binding to luminal endothelial cells by individual qdots. To explain this, we hypothesized that too few individual qdots bound along the vessel wall per voxel for our instrument's (which employs photomultiplier tubes) detection sensitivity. To test this, $\alpha_v\beta_3$ -positive U87MG cells were labeled with RGD–qdots in culture and imaged using our intravital microscope—single RGD–qdots evidently outlined most cells (Figure S2 in Supporting Information). The labeled U87MG cells were subsequently injected into a mouse's ear and reimaged in the living mouse. Single U87MG cells were still visibly bound by RGD–qdots, despite their residence in the ear (Figure S2 in Supporting Information). This suggests our instrument is capable of detecting the binding patterns of individual RGD–qdots decorating single (and multiple) endothelial cells along the tumor neovasculature. We therefore concluded that very few single qdots bound (potentially due to laminar vascular flow or to the polyvalency effect, as aggregates have many more RGDs available to bind than single qdots). To further explore the aggregate phenomenon, fluorescence microscopy was performed directly on wet RGD–qdot samples prior to injection. Aggregates were clearly visible, with appearance analogous to that of the aggregates observed in the blood vessels of mice (Figure 2). To investigate the nature of the qdot distribution on the scale of individual qdots, TEM was performed on RGD–qdots in PBS and incubated in serum. Scanning through both samples surprisingly revealed no perceptible aggregation (Figure 2). The discrepancy between the two modalities is unclear, though TEM sample preparation (i.e., use of a dry sample in vacuum) may be responsible in part. To further shed light on the issue, dynamic light scattering was used to analyze aggregate dimensions. While the vast majority of the solution consisted of individual qdots (hydrodynamic diameter ~ 20 – 25 nm), aggregates were detected in three ranges: ~ 150 nm, ~ 500 nm, and ~ 1200 nm. Aggregates falling below the resolution of the instrument remain visible on the micrographs because sufficient fluorescence is emitted (an aggregate as small as ~ 150 nm contains nearly 500

qdots) to enable visualization of the aggregate's point-spread function. The size of aggregates seen on the images therefore does not correlate well with the actual size of the aggregate.

At baseline dose of ~ 30 pmol, RGD–qdots bound on average more than once in every two FOVs; at 6 times this dose, RGD–qdots bound greater than once per FOV (see Figures 3 and 4 and Table 1). Qualitative differences appear between the two dose conditions, but there is not a statistical difference ($p < 0.13$ for significance) based on Poisson regression analysis using a negative binomial model with $p < 0.05$ taken to be significant. Despite the lack of a clear dose–response relationship, which might be due to saturation of available binding sites or insufficient FOVs for statistical purposes, RGD–qdots unambiguously bound in tumor vessels. To test if this phenomenon was specific to tumors, RGD–qdots were assessed in normal ear vessels (i.e., mice which had never been exposed to cancer cells). Though sporadic binding events were found in this condition, RGD–qdots at both doses tested bound significantly more frequently in tumor than in normal tissues ($p < 0.001$, see Figure 4 and Table 1). The binding events which occurred in normal vasculature may have been due to nonspecific binding of RGD–qdots, to expression of integrin $\alpha_v\beta_3$ in normal tissues (which has been reported, albeit diminished in quantity compared to angiogenic regions),²⁸ or to areas undergoing angiogenesis for reasons other than cancer (e.g., wound healing, which is unlikely because no evidence of wounds was observed, or young animals, 6–8 weeks of age). Lending credence to the hypothesis of nonspecific or nonrobust binding due to low levels of normal tissue integrin expression, RGD–qdots that were seemingly bound to the normal vessels were observed to escape from their binding sites back into circulation and to move around the vessel walls (see movie S2 in Supporting Information). Such departures from the vessel wall, which were also observed in other controls, were *never* observed in the experimental conditions of both doses of RGD–qdots in tumor neovasculature.

RGD peptide is a strong and fairly specific binder of integrin $\alpha_v\beta_3$ (~ 400 nM affinity²⁹) in vitro cell experiments and indirectly (without cellular-level evidence) in living subjects.^{4,5,29–31} Nonspecific binding in tumors could nevertheless be caused either by nonspecific adsorption of the RGD peptide or by the qdot surface to tumor neovasculature. To control for this, similar peptide RAD was employed on qdots. RAD–qdots are nearly equivalent in size and surface chemistry to RGD–qdots, yet lack RGD-specific interaction. RAD–qdots rarely (~ 0.02 events/FOV, 50 pmol) bound tumor neovasculature (see Figures 3 and 4 and Table 1). While the difference of RAD–qdots compared with RGD–qdot conditions is clearly significant (Figure 4), a significant difference was seen even compared to the RGD–qdots-in-normal-vessel condition ($p < 0.019$). In some instances, RAD–qdot aggregates were observed slowing down or stopping, yet they rapidly returned to the circulation (movie S3 in Supporting Information). Because the only difference in the RAD–qdot condition was a single amino acid substitution in the binding moiety, RGD peptide on RGD–qdots is highly likely mediating qdots' interaction with integrin $\alpha_v\beta_3$. To confirm this finding, in our subsequent control we blocked integrin $\alpha_v\beta_3$ binding sites by presaturating with an RGD–Cy5.5 conjugate which was previously validated to bind to $\alpha_v\beta_3$ in cell culture and in living mice.³⁰ Due to extravasation and consequent blurring of the vessel

edges, binding of RGD–Cy5.5 to tumor vessel walls was not observable (see red channel in movie 4 in Supporting Information). Administration of RGD–qdots (50 pmol) shortly after blocking peptide led to only rare binding of the conjugate to vessels (see Figures 3 and 4 and Table 1). More common were instances in which RGD–qdots appeared to gently rock, roll, and temporarily stop as they moved across the neovasculature endothelial lining (movie S4 in Supporting Information). We calculated that our blocking dose of RGD–Cy5.5 was insufficient to saturate all RGD binding sites (approximately half should have been blocked). Supported by recent work on the potency of RGD polyvalency,^{31,32} we hypothesize that the rocking motions may be due to partial binding (i.e., fewer RGDs bind because many integrins are blocked) which slows and sometimes even halts the conjugates' movements, but is insufficient to retain binding.

The red color in movie S4 in Supporting Information is caused by leakage of RGD–Cy5.5 from tumor neovasculature into the tumor interstitium—due to the extravasation and consequent blurring of the vessel edges, we did not observe binding of RGD–Cy5.5 to vessel walls. Indeed, we observed that RGD–Cy5.5 rapidly extravasated from tumor neovasculature (movie S5 in Supporting Information). This is not unexpected due to the EPR effect.¹⁵ On the other hand, we observed that neither RGD–qdots nor control qdot conditions extravasated from tumor neovasculature in our SKOV-3 ear tumor model (nor in our SKOV-3 flank tumor model, in which SKOV-3 cells were implanted in the mouse hind leg, unpublished observations) despite their relatively small 20–25 nm hydrodynamic diameter (see Figure 3, near-infrared channel, and movies S1–S4 and S7 in Supporting Information). Because the EPR effect and many studies suggest that nanostructures extravasate in tumors,^{1,2,5,15,18} we performed a positive control to verify the feasibility of extravasation in SKOV-3 cell tumors. Cy5.5 dye was administered intravenously, and within 20 min extravasated fluorophore (dye) had diffused to infiltrate the majority of tumor (see movie S6 in Supporting Information and Figure 3f), while normal vasculature permitted considerably less dye to leak (unpublished observations). Furthermore, during our blocking experiment we observed that RGD–Cy5.5 conjugate rapidly leaked from tumor neovasculature (movie S5 in Supporting Information), which caused the fairly pervasive red hue in the red channel of movie S4 in Supporting Information. Of note, in contrast with the dye and dye–conjugate which extravasated rapidly, little-to-no extravasation was detected even when very small qdots (~5 nm in hydrodynamic diameter) were administered to an SKOV-3 tumor mouse (unpublished observations). In general, commercially available Angiosense remained in the vasculature at approximately constant levels for at least 2–3 h postinjection, while qdots cleared from the vasculature (via reticuloendothelial system, or RES, uptake³³) in a linear fashion within 1–1.5 h (Figure S1 in Supporting Information). Many studies report extravasation of nanostructures (and larger particles^{18,23,34}) from tumor vessels in animal models,^{1,2,4,5,23} but the molecular size cut-offs in such vessels have been reported to vary significantly between different xenograft models reported¹⁸ and even between different regions of the same vessel of a given tumor.²³ Our results suggest that this cutoff size variability occurs even in the low nanoscale range. We found that although qdots do not extravasate in the SKOV-3 ear or flank xenografts, they clearly and rapidly extravasate when exposed to the ears of mice inoculated with certain other tumor cell lines (e.g., LS174T). Furthermore, we have shown that although 5 nm qdots do not extravasate in

the SKOV-3 model, ~1 nm RGD–Cy5.5 easily does. Thus, the intravital method we employ can be used for further study of differential extravasation parameters, as this variability is likely also to be a key hurdle for nanoparticle delivery in human oncology.

To account for nonspecific binding by the qdots themselves, unconjugated qdots were administered (255 pmol). Unconjugated qdots were observed entering and flowing through tumor neovasculature (see movie S7 in Supporting Information); however, these bare qdots rarely (~0.02 events/ FOV) were associated with the tumor neovasculature (see Figures 3c and 4 and Table 1). In all six experimental conditions (Table 1), many aggregates were observed flowing through tumor and normal vasculatures (movies S1–S4 and S7 in Supporting Information and unpublished data), yet only when RGD–qdots were exposed to neovascular tumor environments did significant binding occur. We have thus directly observed, at the cellular-to-subcellular level, the specific binding of RGD–qdots to $\alpha_v\beta_3$ integrins in tumor neovasculature. The in vivo assay employed here is generalizable to assess the binding of any nanoparticle type, targeting ligand, and in any vasculature (diseased or normal). Using macroscopic optical imaging, Cai et al. showed that near-infrared RGD–qdots target tumors in living mice.⁵ However, whether these RGD–qdots extravasated and bound to tumor cells or bound to tumor vasculature was unknown. On the other hand, Tada et al. recently demonstrated binding of single Her-2/neu antibody-conjugated qdots to tumor cells in living mice using a high-speed camera at high magnification.² Neither study, however, explains the process by which nanoparticle accumulation actually occurs in tumors. Further, previous approaches would not provide access to the “near-binding” characteristics seen in controls (see movies S2–S4 in Supporting Information) that our method facilitated. This information is likely to lead toward a better model of the mechanism of binding and thus improved nanoparticle construction. The present study bridges the gap between bulk phenomena/macrosopic optical imaging and single qdot studies by demonstrating significant binding in the FOVs of experimental conditions. This is a critical step on the path to understanding the parameters that will enable the design of next-generation nanoparticles with superior tumor targeting properties and will ultimately help accelerate the approval of nanoparticles by regulatory agencies for clinical use.

We are trying to understand on a deeper level why only RGD–qdot aggregates, rather than individual qdots, bind to the neovasculature and to determine if this is a more general phenomenon that could be exploited in the pursuit of high efficiency delivery of nanostructures to cancer. For instance, we confirmed that the binding pattern observed with RGD–qdots was not restricted to the SKOV-3 model—LS174T tumor neovasculature displayed a similar binding pattern (unpublished data). Our approach can be further generalized by assessing the binding properties of other nanoparticles and targeting ligands, as well as other sites of tumor implantation (e.g., orthotopic models). Clearly other sites of implantation or other species may produce different results. We are now addressing how mathematical models of these in vivo processes can help us explain the phenomena we observed by reference to the strength of shear forces and stresses on nano-to-microsized structures bound to vessel walls compared with the strengths of varying polyvalent affinities and nonspecific interactions keeping the structures tethered. Because of the significance of

these affinity interactions to the effect we observed, we intend to quantify the precise differences in binding affinity to $\alpha_v\beta_3$ integrins between single qdots and aggregates to validate and guide our mathematical models. It is known that fluid shear modulates targeted nanoparticle adhesion to vessel walls³⁵ and that multiple copies of targeting ligand facilitate polyvalent binding to the cell surface, which significantly strengthens the interaction.³² However, polyvalent binding to cell-surface biomolecules can only occur if the density of surface biomolecules is sufficiently high. Considering that the diameter of each cell-surface biomolecule may be 5–10 nm, binding of targeting ligands to multiple biomolecules may be compromised for a qdot that is 20–25 nm in diameter. Indeed, the ligand-to-receptor surface density ratio is known to be a key feature in targeted nanoparticle design.³⁶ Comparison of the force of monovalent and polyvalent binding to the shear forces experienced by the nanoparticles will thus yield insight into this issue. However, a mathematical model conforming more closely to the environment existing within living subjects must be employed to generate credible data for comparison and to draw reliable conclusions. In our study, though larger aggregates are subject to larger shear (the force is proportional to the square of the radius),³⁶ the larger surface area and increased number of surface-bound RGDs may enable aggregates to remain bound compared to individual qdots. This suggests a hypothesis that a bimodal or polydisperse^{37,38} size distribution of injectable nanoparticles may be superior for optimal uptake of nanoparticles in heterogeneous tumors rather than conventional monodisperse formulations (e.g., use smaller nanoparticles for potential EPR uptake and larger nanoparticles for binding to vessel walls). Note, however, that such a polydisperse distribution would be simultaneously difficult to reproducibly generate from the manufacturing standpoint and to obtain approval from the regulatory perspective. We must also consider the contribution of laminar flow in the vessels to the access that differently sized nanoparticles/aggregates have to vessel walls. We do not yet understand the reasons for aggregate binding and the evident lack of single RGD–qdot binding. Our data nevertheless clearly and consistently indicate this phenomenon in our model's tumor neovasculature. We have shown that our instrument is capable of visualizing disperse (individual) RGD–qdots binding on single cells in vivo (Figure S2 in Supporting Information) and effectively excluded the possibility that disperse binding is occurring in our tumor model.

This study demonstrates, to our knowledge, the first direct visualization of nanoparticle conjugate binding to tumor neovasculature. We effectively performed a nanoparticle binding assay in living subjects while directly observing the process. Using this method, we concluded that RGD–qdots do not extravasate in an SKOV-3 mouse ear tumor model, specifically bind their target in tumor neovasculature as aggregates, and can be recorded doing so. The knowledge thus gained is invaluable in helping to design and obtain approval for future nanoparticle formulations. The study thus portends the promise of studying nanoscale structures interacting with microscale entities in living subjects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported in part by the following grants: NCI U54 CA119367 (S.S.G.) and NCI ICMIC P50 CA114747 (S.S.G.). B. R. Smith is supported by an NIH R25T postdoctoral training grant and Stanford Dean's Fellowship. We gratefully acknowledge the assistance of Jarrett Rosenberg with statistics and to Carsten Nielsen for help with animal studies. Please address requests for reprints to Sanjiv S. Gambhir, at Molecular Imaging Program at Stanford, E 150 Clark Center, 318 Campus Drive, Stanford, CA 94305-5427. Phone: 001 650 725 2309. Fax: 001 650 724 4849. E-mail: sgambhir@stanford.edu.

References

1. Gao X, Cui Y, Levenson RM, Chung LW, Nie S. In vivo cancer targeting and imaging with semiconductor quantum dots. *Nat Biotechnol.* 2004; 22:969–976. [PubMed: 15258594]
2. Tada H, Higuchi H, Wanatabe TM, Ohuchi N. In vivo Real time Tracking of Single Quantum Dots Conjugated with Monoclonal Anti-HER2 Antibody in Tumors of Mice. *Cancer Res.* 2007; 67:1138–1144. [PubMed: 17283148]
3. Akerman ME, Chan WC, Laakkonen P, Bhatia SN, Ruoslahti E. Nanocrystal targeting in vivo. *Proc Natl Acad Sci USA.* 2002; 99:12617–12621. [PubMed: 12235356]
4. Montet X, Montet-Abou K, Reynolds F, Weissleder R, Josephson L. Nanoparticle imaging of integrins on tumor cells. *Neoplasia (Ann Arbor MI US).* 2006; 8:214–222.
5. Cai W, Shin DW, Chen K, Gheysens O, Cao Q, Wang SX, Gambhir SS, Chen X. Peptide-labeled near-infrared quantum dots for imaging tumor vasculature in living subjects. *Nano Lett.* 2006; 6:669–676. [PubMed: 16608262]
6. Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S. Quantum dots for live cells, in vivo imaging, and diagnostics. *Science.* 2005; 307:538–544. [PubMed: 15681376]
7. Li ZB, Cai W, Chen X. Semiconductor quantum dots for in vivo imaging. *J Nanosci Nanotechnol.* 2007; 7:2567–2581. [PubMed: 17685272]
8. Medintz IL, Uyeda HT, Goldman ER, Mattoussi H. Quantum dot bioconjugates for imaging, labelling and sensing. *Nat Mater.* 2005; 4:435–446. [PubMed: 15928695]
9. Soltész EG, Kim S, Laurence RG, DeGrand AM, Parungo CP, Dor DM, Cohn LH, Bawendi MG, Frangioni JV, Mihaljevic T. Intraoperative sentinel lymph node mapping of the lung using near-infrared fluorescent quantum dots. *Ann Thoracic Surg.* 2005; 79:269–277.
10. Ballou B, Ernst LA, Andreko S, Harper T, Fitzpatrick JAJ, Waggoner AS, Bruchez MP. Sentinel lymph node imaging using quantum dots in mouse tumor models. *Bioconjugate Chem.* 2007; 18:389–396.
11. Knapp DW, et al. Sentinel Lymph Node Mapping of Invasive Urinary Bladder Cancer in Animal Models Using Invisible Light. *Eur Urol.* in press.
12. Frangioni JV, Kim SW, Ohnishi S, Kim S, Bawendi MG. Sentinel Lymph Node Mapping With Type-II Quantum Dots. *Methods Mol Biol.* 2007; 374:147–160. [PubMed: 17237537]
13. Gopee NV, Roberts DW, Webb P, Cozart CR, Siitonen PH, Warbritton AR, Yu WW, Colvin VL, Walker NJ, Howard PC. Migration of intradermally injected quantum dots to sentinel organs in mice. *Toxicol Sci.* 2007; 98:249–257. [PubMed: 17404394]
14. Choi HS, Liu W, Misra P, Tanaka E, Zimmer JP, Ipe BI, Bawendi MG, Frangioni JV. Renal clearance of quantum dots. *Nat Biotechnol.* 2007; 25:1165–1170. [PubMed: 17891134]
15. Ferrari M. Cancer nanotechnology: opportunities and challenges. *Nat Rev.* 2005; 5:161–171.
16. Panchapakesan B, Wickstrom E. Nanotechnology for sensing, imaging, and treating cancer. *Surg Oncol Clinics North America.* 2007; 16:293–305.
17. Veisheh O, Sun C, Gunn J, Kohler N, Gabikian P, Lee D, Bhattarai N, Ellenbogen R, Sze R, Hallahan A, Olson J, Zhang M. Optical and MRI multifunctional nanoprobe for targeting gliomas. *Nano Lett.* 2005; 5:1003–1008. [PubMed: 15943433]
18. Hobbs SK. Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment. *Proc Natl Acad Sci USA.* 1998; 95:4607–4612.

19. McDonald DM, Baluk P. Significance of blood vessel leakiness in cancer. *Cancer Res.* 2002; 62:5381–5385. [PubMed: 12235011]
20. Folkman J. Angiogenesis: an organizing principle for drug discovery. *Nat Rev Drug Discovery.* 2007; 6:273–286.
21. Brown EB, Campbell RB, Tsuzuki Y, Xu L, Carmeliet P, Fukumura D, Jain RK. In vivo measurement of gene expression, angiogenesis and physiological function in tumors using multiphoton laser scanning microscopy. *Nature Med.* 2001; 7:864–868. [PubMed: 11433354]
22. Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: present and future therapeutic targets. *Nat Immunol.* 2005; 6:1182–1190. [PubMed: 16369557]
23. Stroh M, Zimmer JP, Duda DG, Levchenko TS, Cohen KS, Brown EB, Scadden DT, Torchilin VP, Bawendi MG, Fukumura D, Jain RK. Quantum dots spectrally distinguish multiple species within the tumor milieu in vivo. *Nature Med.* 2005; 11:678–682. [PubMed: 15880117]
24. Yuan F, Leunig M, Huang SK, Berk DA, Papahadjopoulos D, Jain RK. Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Res.* 1994; 54:3352–3356. [PubMed: 8012948]
25. Tsourkas A, Shinde-Patil VR, Kelly KA, Patel P, Wolley A, Allport JR, Weissleder R. In vivo imaging of activated endothelium using an anti-VCAM-1 magnetooptical probe. *Bioconjugate Chem.* 2005; 16:576–581.
26. Voura EB, Jaiswal JK, Mattoussi H, Simon SM. Tracking metastatic tumor cell extravasation with quantum dot nanocrystals and fluorescence emission-scanning microscopy. *Nature Med.* 2004; 10:993–998. [PubMed: 15334072]
27. Mizejewski GJ. Role of integrins in cancer: survey of expression patterns. *Proc Soc Exp Biol Med.* 1999; 222:124–138. [PubMed: 10564536]
28. Max R, Gerritsen RRCM, Nooijen PTGA, Goodman SL, Sutter A, Keiholz U, Ruiter DJ, De Waal RMW. Immunohistochemical analysis of integrin $\alpha_v\beta_3$ expression on tumor-associated vessels of human carcinomas. *Int J Cancer.* 1997; 71:320–324. [PubMed: 9139861]
29. Montet X, Yuan H, Weissleder R, Josephson L. Enzyme-based visualization of receptor-ligand binding in tissues. *Lab Invest.* 2006; 86:517–525. [PubMed: 16568109]
30. Cheng Z, Wu Y, Xiong Z, Gambhir SS, Chen X. Near-infrared fluorescent RGD peptides for optical imaging of integrin $\alpha_v\beta_3$ expression in living mice. *Bioconjugate Chem.* 2005; 16:1433–1441.
31. Li ZB, Cai W, Cao Q, Chen K, Wu Z, He L, Chen X. ^{64}Cu -labeled tetrameric and octameric RGD peptides for small-animal PET of tumor $\alpha_v\beta_3$ integrin expression. *J Nucl Med.* 2007; 48:1162–1171. [PubMed: 17574975]
32. Montet X, Funovics M, Montet-Abou K, Weissleder R, Josephson L. Multivalent effects of RGD peptides obtained by nanoparticle display. *J Med Chem.* 2006; 49:6087–6093. [PubMed: 17004722]
33. Schipper ML, Cheng Z, Lee SW, Bentolila LA, Iyer G, Rao J, Chen X, Wu AM, Weiss S, Gambhir SS. microPET-based biodistribution of quantum dots in living mice. *J Nucl Med.* 2007; 48:1511–1518. [PubMed: 17704240]
34. Yuan F, Dellian M, Fukumura D, Leunig M, Berk DA, Torchilin VP, Jain RK. Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. *Cancer Res.* 1995; 55:3752–3756. [PubMed: 7641188]
35. Blackwell JE, Dagia NM, Dickerson JB, Berg EL, Goetz DJ. Ligand coated nanosphere adhesion to E- and P-selectin under static and flow conditions. *Ann Biomed Eng.* 2001; 29:523–533. [PubMed: 11459346]
36. Decuzzi P, Ferrari M. Design maps for nanoparticles targeting the diseased microvasculature. *Biomaterials.* 2007; 29:377–384. [PubMed: 17936897]
37. Decuzzi P, Causa F, Ferrari M, Netti PA. The effective dispersion of nanovectors within the tumor microvasculature. *Ann Biomed Eng.* 2006; 34:633–641. [PubMed: 16568349]
38. Decuzzi P, Ferrari M. The role of specific and non-specific interactions in receptor-mediated endocytosis of nanoparticles. *Biomaterials.* 2007; 28:2915–2922. [PubMed: 17363051]

Figure 1.

Intravital microscopy used to investigate derivatized nanoparticles. (a) Our intravital microscope setup, with callout of a mouse being imaged under a 10× objective. Ears were affixed to double-sided tape for motion stabilization. (b) The chemistry used to conjugate cyclic RGD and RAD peptides to qdots for injection into mice.

Figure 2.

TEM and fluorescence of RGD-qdots. RGD-qdots dispersed in PBS (left) and in mouse serum (middle) as shown by TEM. Overall concentration of qdots was identical in both conditions. Random (typical) fields-of-view were chosen. No aggregates were observed. Fluorescence microscopy (right), by contrast, displays various aggregates.

Figure 3.

Direct visualization of binding of RGD-qdots to tumor vessel endothelium and controls. (a) Panel displays different output channels of the identical imaging plane along the row with scale bars. In the green channel, individual EGFP-expressing cancer cells are visible (marked by thick horizontal blue arrows; vertical blue arrow points to a hair follicle), while the red channel outlines the tumor's vasculature via injection of Angiosense dye. The NIR channel shows intravascularly administered qdots which remain in the vessels (i.e., they do not extravasate). Binding events are visible by reference to bright white signal. These are

demarcated by arrows in the rightmost merged image, in which all three channels have been overlaid. (b) Displays the same as (a) in a different mouse, except that 6 times the RGD–qdot dose has been injected. Individual cells are not generally visible. Six binding events are observed in this FOV, as marked by arrows in the merged image at right. White arrows in the bottom merged image designate areas of tissue autofluorescence. Typical images of no binding in each control condition are shown in (c–f). Tumor neovasculature containing unconjugated qdots (c), normal vasculature containing RGD–qdots (d), and tumor neovasculature containing RAD–qdots (e). (f) Tumor vasculature shortly after Cy5.5 injection (left) and ~20 min post-Cy5.5 injection (right). Individual cancer cells are visible before (left) and after dye extravasates (right, dyed red). Also see movie S6 in Supporting Information. Horizontal white arrows indicate tissue autofluorescence, vertical blue arrows denote hair follicles (which generally display autofluorescence in their center), and thick horizontal blue arrows indicate individual cancer cells.

Figure 4.
Comparison of binding rates between conditions tested. Displays the binding rate (calculated as events/FOV) for each experimental condition with 95% confidence intervals.

Table 1

Statistics for Experimental Conditions

condition ^a	binding rate	FOVs	95% conf int	rate ratio	95% conf int	P
normal	0.110	109	0.059–0.206			
RGD low dose	0.562	32	0.333–0.889	5.11	1.92–13.62	0.001
RGD high dose	1.162	37	0.841–1.565	10.31	4.65–22.85	0.001
RGD block	0.047	85	0.013–0.120	0.43	0.14–1.34	0.146
RAD	0.018	110	0.002–0.066	0.17	0.04–0.75	0.019
unconjugated	0.022	45	0.001–0.118	0.20	0.03–1.55	0.124

^a Statistics for experimental conditions. Poisson regression analysis was used to compare all qdot conditions to the normal mouse (no tumor) condition injected with RGD–qdots. Binding rate, number of FOVs examined, rate ratio, *p*-value, and 95% confidence were calculated. RGD low and high doses in tumor were significantly different from normal, as were RAD–qdots.