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Dynamic Visualization of RGD-Quantum Dot Binding to Tumor Neovasculature and Extravasation in Multiple Living Mouse Models using Intravital Microscopy

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We adopted quantum dots (Qdots) as a model spherical nanoparticle system to probe their binding and extravasation properties in small living animals. Qdots have exceptional fluorescent properties, exploiting the exquisite precision in their manufacture to yield highly size-controlled, single nanocrystals with tunable band gaps.[1,2] Therefore, they are highly conducive to biomedicine as *in vitro/ex vivo* fluorescent labels and in pre-clinical optical imaging.[1,3-9] While their use in *ex vivo* biomedical strategies such as those in cellular biology have been relatively well-characterized,[1,9] Qdots in living subjects remain promising yet comparatively uncharacterized. Nevertheless, they have recently been employed to target tissue-specific vascular biomarkers[5], cancer cells and blood vessels, [3,4,6,7,9,10] and to identify sentinel lymph nodes in cancer[11] with the potential for human translation.[1,2,9] While toxicity and clearance concerns remain major obstacles on the path toward clinical utility in humans,[12] Qdots' excellent intrinsic optical reporter properties make them a vital tool in pre-clinical biology. In the present application of Qdots as bright, intrinsically fluorescent model nanoparticles (for nanoparticles of comparable size and shape), they are indeed indispensable to analyze the binding parameters of targeted nanoparticles in the tumor neovasculature of various murine models and tumor types with high photostability.

Molecular imaging with nanoparticles is a rapidly growing discipline[13] which comprises the application of many different types of nanoparticles to deliver contrast that is specific for various types of imaging modalities, including optical, Raman, magnetic, photoacoustic, radionuclide, and other methods.[14,15] These are generally whole animal imaging methods which can provide dynamic macroscopic information, yet lack the ability to answer questions about microscale interactions. Common to these numerous imaging modalities, as far as exogenously-administered contrast, are the general methods used to target nanoparticles site-specifically. Cancer-targeted nanoparticles are directed to tumor by conjugating to the nanoparticles molecules that recognize over-expressed cell surface proteins either within the tumor blood vessels, on the tumor (or tumor-associated) cells, or both. The aforementioned macroscopic imaging modalities are excellent in reporting back

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the approximate quantity and general location of relevant reporter nanoparticles in the tumor. Nevertheless, there is a fundamental dearth of knowledge about how the nanoparticles reach the tumor, where and when they bind, and the general microscale dynamics and mechanisms by which these conjugated nanoparticles produce contrast in the whole animal images that are ultimately generated. We thus previously demonstrated the ability to image Qdots traversing and binding to tumor neovasculature.[10] However, it was unclear whether the mechanisms we described were specific to the tumor type we chose, and if not, whether the targeted binding was similar in quantity and pattern across tumor types. Critically, we also sought clarification on whether Qdots would extravasate in some tumor models since they did not in our original SKOV-3 model. Further, if they did, we asked whether this would affect vascular binding. These binding and extravasation issues are important because, among other objectives, they allow us to understand the generalizability of nanoparticle usage across tumor types – this has implications in clinical medicine. Thus, we devised a series of experiments across multiple tumor types (human ovarian cancer, glioblastoma, and adenocarcinoma) which examine, in real-time at the microscale level: (1) the passage of Qdots through the tumor blood vessels (2) binding of targeted Qdots to tumor neovasculature and (3) extravasation of Qdots from tumor blood vessels. The different tumor cell lines help to represent, in part, the heterogeneity of solid human tumors, both in terms of the molecular expression of the integrins to which our ligand binds and in the differences in vessel density, size, and permeability. This may be illustrative of the heterogeneity faced when imaging and treating human cancer. Contrast agents are thus needed which are capable of imaging cancer without regard to this inevitable heterogeneity – targeting of vasculature may therefore be optimal to minimize heterogeneous effects in imaging solid tumors. At the same time, therapy often requires that nanoparticles extravasate and reach tumor cells. Nanoparticles are a prudent choice as contrast agents because of the control engineers have over their size, shape, and surface charge. It is natural that intravital microscopy, a stalwart in tumor biology and immunology,[16] has recently begun to emerge as a tool for studying the *in vivo* behavior of nanomaterials.[4,10,17,18] Here we show the utility of intravital microscopy to elucidate the differences in the microscale binding and extravasation of targeted Qdots in living subjects using three different tumor cell lines. Our purpose is to explore the heterogeneity between tumor types in terms of binding and extravasation in order to better understand how nanoparticles behave when they are injected and to suggest improvements in nanoparticle formulation for tumor uptake.

In this work, we exploited intravital microscopy to develop our *in vivo* nanoparticle binding and extravasation assays, comparing the experimental RGD-qdot condition with controls in various tumor models in living mice (for set-up, see Figure 1). Qdots were chosen to emit in the near-infrared due to improved optical tissue properties in that range. RGD-qdots displayed 30-50 covalently-bound peptides each, and were previously shown to specifically bind integrin $\alpha_v\beta_3$ in cell culture on a variety of cell lines and *ex vivo* on excised tumor tissues.[7] RGD was selected as the targeting molecule because of its convenient small size, specificity, and the very significant role that RGD's chief target, $\alpha_v\beta_3$ integrin, plays in tumor angiogenesis, proliferation, and metastasis.[19] While integrin $\alpha_v\beta_3$ is overexpressed both on neovasculature and on some tumor cells, we focused here on binding to neovasculature. Blood vessel walls were outlined via a long-circulating dye that remained within the vasculature for hours to days (data not shown). Compared to RGD-qdots, controls displayed minimal binding in tumor blood vessels (Figures 2-3 and Table 1).

In all tumor models we found that binding events were restricted to aggregates of Qdots tethering to multiple, discrete sites in the ear tumor (Figures 2-3, Supporting Data), similar to our previous SKOV-3 ear model.[10] Aggregate binding was not initially expected because the literature does not suggest the presence of patchy or focal integrin $\alpha_v\beta_3$ expression on neovascular vessels.[20] Instead, we would expect individual Qdot binding to

accumulate along the vascular endothelial lining and thus to be visualizable as disperse fluorescence along the blood vessels. Yet we found no detectable fluorescence on the vessel endothelial lining after Qdots cleared the vasculature in any tumor type other than bound aggregates. We previously labeled $\alpha_v\beta_3$ -positive U87MG cells with RGD-qdots *in vitro* as a control and injected them into the ear to test our instrument's sensitivity to individual Qdot binding; the instrument proved capable of visualizing individual RGD-qdot binding patterns on neovascular endothelial cells.[10] Yet since we observed no such binding in the neovasculature we concluded that individual RGD-qdots at most bound minimally to luminal endothelial cells. This observation may in part result from differences in polyvalent binding under shear flow: that is, the minimal number of bonds that a single 20-25 nm RGD-decorated qdot is capable of forming to its cognate integrins (each protein is 5-10 nm) compared with the many bonds potentially formed by a larger aggregate may lead to significantly decreased probability of binding. We further characterized our Qdot formulations: using fluorescence microscopy with Qdots on a slide we detected Qdot aggregates, while TEM showed no aggregates. Using dynamic light scattering, we found that, though the solution was almost completely individual Qdots, (hydrodynamic diameter ~20-25 nm) by volume, aggregates of ~150 nm, ~500 nm, and ~1200 nm were present. These aggregates appeared of similar size and shape using fluorescence *ex vivo* as they were in living subjects.

In our previous SKOV-3 work, using the baseline intravenously administered dose of ~30 pmol, RGD-qdot aggregates bound on average more than once in every 2 FOVs (Figure 2 and Table 1), which was not significantly different than the binding observed using a six-fold greater dose.[10] U87MG and LS174T tumors generated using their respective cell lines in the nude mouse ear appeared different than SKOV-3 in terms of blood vessel morphology (see Supporting Data). Yet when RGD-qdots were intravenously injected into mice with these tumors, binding events were observed very similar to the SKOV-3 condition in terms of aggregates of RGD-qdots binding with little-to-no apparent individual RGD-qdot binding (see Figure 3, Table 1, and Supporting Data). The ratios of binding events to FOV in LS174T and U87MG were similar to the ratio observed in SKOV-3 tumor; the differences between them were statistically insignificant ($P=0.053$, see Table 1).

Controls were performed in each tumor model, including non-peptide conjugated (bare, amino-group functionalized) Qdots and control peptide-conjugated Qdots. Though sporadic binding occurs in normal vasculature,[10] RGD was clearly specific to tumor vasculature ($P<0.001$). Normal vasculature may engender binding events for a number of reasons, including (1) non-specific binding of RGD-qdots, (2) expression of integrin $\alpha_v\beta_3$ in normal tissues, which is known to occur in reduced concentration in comparison to the vessels located in angiogenic regions,[20] and (3) areas which produce angiogenesis due to non-cancer related physiology. We explored (1) further through control Qdots. The literature shows that RGD peptide binds fairly specifically with relatively high affinity to integrin $\alpha_v\beta_3$ (~400 nM affinity[21]) both in *in vitro* cell experiments and indirectly using histology in living animals.[6,7,21,22] However, because non-specific binding could occur due to charge-based or other interactions between the Qdot surface and the endothelial cell surface, we used unconjugated Qdots as a control. When injected, even at an approximately tenfold higher dose than RGD-qdots, control Qdots were observed to be rarely associated with the tumor neovasculature in all three tumor models as hypothesized (see Figures 2-3 and Table 1) – RGD-qdots bound significantly more than controls across all tumor conditions, with $P<0.0005$. The RGD-qdot condition also bound significantly more than the control condition in all three of the SKOV-3, U87MG, and LS174T tumors, with p-values equal to 0.000002, 0.000945, and 0.000004, respectively. In another control to interrogate the same vasculature, we injected RAD-conjugated qdots (RAD is a peptide similar to RGD, but without the specificity to integrin $\alpha_v\beta_3$, see Methods section) prior to RGD-qdots. RAD-qdots were

allowed to clear from the circulation and revealed minimal binding; subsequently, RGD-qdots were injected and binding events increased 3-fold. Based on these controls, the RGD peptide is likely to be mediating the specific interaction between RGD-qdots and integrin $\alpha_v\beta_3$ in all three tumor models.

Taken together, this binding data indicates that the binding rates and binding patterns between tumor types (SKOV-3, U87MG, and LS174T cell lines) do not vary significantly, independent of the molecular and vascular heterogeneity between tumor types (Supporting Data). This is important for potential translation of nanoparticles into the clinic since human cancer is also extremely heterogeneous. This heterogeneity demands the ability to robustly preserve targeting no matter the variances between individual, site, and type of tumor. It is further notable that the present work partially simulates the heterogeneity of human cancer in the sense that diverse molecular pathways/cancer cell types are observed in humans just as displayed in these cancer cell lines (from brain cancer to ovarian cancer). The present work suggests that even when challenged by different types of cancer in mouse models, neovascularly-directed Qdots comparably target (and can thus be used to image) tumor vasculature despite the differences in extravasation, vascularity, and other physical properties (see Supporting Data).

The Chen group initially demonstrated RGD-qdot targeting to tumor using optical imaging at the macroscopic scale in mouse shoulder.[7] They showed that RGD-qdots are selectively localized within U87MG tumors compared with controls (bare Qdots). However, preferential uptake of RGD-qdots was demonstrated without reference to the mechanism by which they accumulated in tumor. Whether the binding was specific to targeting of the tumor neovasculature (including whether single Qdots or aggregates bound), specific to tumor cells themselves, or some heretofore unidentified mechanism was unknown. Our previous work showed how Qdots behave in SKOV-3 tumors,[10] but uncertainty remained if the ovarian tumor used in that work (SKOV-3) could be generalized to glioblastoma (U87MG). Our current study indicates that uptake in U87MG tumors such as those employed by the Chen group likely occurred through binding to blood vessels and to a much lesser degree from Qdot extravasation and subsequent tumor cell binding, though the difference in tumor site potentially could change its characteristics.[18]

In contrast to macroscopic imaging, Tada et. al. demonstrated the *in vivo* binding and internalization of a single Her-2/neu antibody-conjugated Qdot to an individual tumor cell at very high magnification.[4] Their work showed the level of detail possible in studying targeting in tumor interstitium; conversely, our work demonstrates bulk targeting (at the microscale) of blood vessels as well as properties of tumor extravasation. This is necessary to understand whole populations of nanoparticles rather than individual units. The current work fills the gap between the previous approaches by helping to expose the mechanisms involved in nanoparticle collection and retention in tumors in comparison to controls by searching through many FOVs for evidence of binding (and extravasation).

The *in vivo* binding assay and visualization described above is generally applicable to various nanoparticles, targeting moieties, and blood vessel types. We have built upon our previous work to generalize our discussion of the vascular targeting properties of Qdots given the reality of high tumor heterogeneity. We have moreover extended our work beyond vascular targeting by studying the differences in extravasation of these nanoparticles across multiple tumor models. Indeed, extravasation studies have typically been performed at the level of small molecules and drugs[23,24] or larger nanoparticles/microparticles (100-1000 nm),[17,24] but not at the “meso”-nanoscale of commercial Qdots (20-25 nm in diameter).

While neovascular binding was similar across the different tumors, extravasation proved starkly different. We observed that neither RGD-qdots nor controls extravasated from the tumor neovasculature in SKOV-3 tumor in the ear (see Figure 2 and Figure 4, near-infrared channel). Yet the Enhanced Permeation and Retention (EPR) effect and many studies suggest that nanostructures do extravasate in tumors.[3,4,7,14,25] We thus became interested in why the relatively small (20-25 nm in hydrodynamic diameter) Qdots did not extravasate in the SKOV-3 model (Figure 2 and Supporting Data). Furthermore, little-to-no extravasation was detected when very small Qdots (~5 nm diameter) were injected intravenously to a mouse with an SKOV-3 ear tumor (unpublished observations). Similarly, when we tested U87MG tumors, we noticed little extravasation of Qdots from the tumor vasculature. However, even ~25 nm commercially-available Qdots unequivocally and rapidly extravasated from blood vessels when we challenged LS174T tumors with the same Qdot doses (Figures 4-5). We found that the circulating Angiosense dye (a high molecular weight macromolecule) did not extravasate at all, remaining in the vasculature at nearly constant levels for at least ~3 hours post-injection in all tumor types. Qdots cleared (presumably via reticuloendothelial system, or RES, uptake[26]) within 1-2 hours in all models, whether or not they extravasated from blood vessels.

It is well-known that nano-sized compositions and even larger particles can extravasate in animal tumor models.[1,2,4,5,17,25,27] Further, due to tumor heterogeneity, even intra-tumoral heterogeneity, the molecular size cut-off for vessel permeability varies broadly between different models and even different sites of the same tumor for larger particles (>100 nm).[17,25] Yet nanoparticles are typically defined in size between 1-100 nm. Extravasation in this critical range, which has enormous implications for cancer nanomedicine, has thus far gone unstudied apart from histologic examination. We observed a huge diversity in extravasation within a small sample of only three tumor types. Qdots did not extravasate at all from SKOV-3 and escaped from U87MG tumor blood vessels only minimally (see Supporting Data). These results are in agreement with the literature based on histological examination.[28] While nanoparticles are typically expected to extravasate in tumors, the fact that no extravasation was observed in the SKOV-3 model is likely a feature of the heterogeneity in tumor neovasculature across tumor types. In particular, since extravasation is due to pores in the vasculature, the unique SKOV-3 microenvironment likely supports the growth of tumor blood vessels with very small pores. This is supported by the work of Kong et. al., which also showed that nanoparticles do not extravasate in SKOV-3 tumor vasculature (in normothermic conditions) likely due to the very small pore size exhibited.[27] Moreover, as might be anticipated given the minimal but nonzero 25 nm Qdot extravasation we observed in U87MG models, further studies demonstrated that small nanoparticles (<10 nm in hydrodynamic diameter) extravasate relatively well in U87MG. [29] The typical pore size for blood vessels in U87MG tumors therefore appears to be in the 10-25 nm range, though a dependence on tumor site likely exists. However, while Qdot extravasation was minimal to nonexistent in U87MG/SKOV-3, Qdots escaped fairly rapidly and intensely from vessels in LS174T tumor. We furthermore observed that the pattern of extravasation displayed in LS174T was heterogeneous even along single blood vessels (e.g., Figure 4).

The blue curve in Figure 5 demonstrates that extravasation as expected does not occur linearly, but it does appear to saturate at nearly an hour post-injection. It is likely that this LS174T Qdot extravasation curve (Figure 5) is predominantly a function of the blood vessel pore size, RES clearance of Qdots (which chiefly determines their blood concentration over time), and inevitable diffusion of Qdots through tumor extracellular matrix (Qdots do not necessarily remain confined to the site where they initially localize, which affects ROI-based measurement). Because nanoparticles must extravasate to interact with tumor cells, optimization of the Figure 5 curve will be vital to the future of each tumor cell-targeted

imaging or therapeutic nanoparticulate agent, i.e., maximum impact on tumor cells will only occur if extravasation is optimal. While the curve will presumably change for every tumor type and site, understanding how the nanoparticle's physical properties (including size, shape, density, surface charge, and surface material) affect crossing the endothelium from vessel to interstitium will result in superior targeting characteristics. Testing these nanoparticle properties will likely be best assessed using intravital microscopy akin to the experiments described herein across a range of nanoparticles.

Even if nanoparticles are “optimized” for extravasation, a major difficulty is that the Figure 5 curve will vary for every tumor type and nanoparticle/molecule: from vanishing entirely (SKOV-3 tumors) to Qdots in LS174T as in Figure 5 to ultra-fast extravasational kinetics in SKOV-3 engendered by small molecules.[10] One solution to generalize nanoparticle treatments to all tumor types is to specifically target the vasculature since tumors larger than ~1 mm require a blood supply via the angiogenic switch.[30] Unlike tumor interstitium, all intravenously delivered nanoparticles have the opportunity to “see” solid tumor blood vessels no matter the tumor type or site. Although there is certainly a broad range of vascular density, sizes, shapes, and endothelial cell structures among various tumors (Supporting Data and [31]), the present work preliminarily indicates that the targeting of blood vessels may be similar in binding pattern, quantity, and specificity across considerable tumor diversity. This is important because this similarity could facilitate superior reliability. Thus, although blood vessel targeting will not be the optimal method for excellent uptake in every tumor type, its consistency might be depended upon to deliver a certain amount of the injected dose no matter the tumor type or site. This is important for imaging, since detection itself or monitoring of therapy is often the goal;[32] yet vascular targeting is also vital for some forms of therapy, such as that used in vascular normalization.[33] Because extravasation is not guaranteed in every tumor type, a strong argument can be made that it is critical to optimize the shape and size of nanoparticles to target tumor blood vessels in addition to extravasating from them. Indeed, while regulatory approval would ostensibly be challenging, perhaps a combination of nanoparticles for blood vessel targeting and for extravasation will prove optimal.

RGD-qdot binding ratios for the three different tumors were intriguingly not significantly different despite variations in blood vessel density, size, etc. This cellular-level data, showing similarity in binding across the different types of tumors and models, could portend a promising future for neovascularly-targeted imaging and therapy by demonstrating the potential to help overcome tumor heterogeneity. Yet to clarify and confirm this initial result, a larger scale study of the dependence of tumor type on the binding of nanoparticles to blood vessels will need to eventually be performed. For instance, tumor site is an important parameter[25] in tumor growth and physical attributes because of the chemical and physical milieu in which it is located. Thus further study would need to include multiple sites (instead of the ear alone) and species to determine the consistency in nanoparticle binding one might expect across the spectrum of human disease. We also note that the surprising phenomenon of our initial finding that RGD-qdots bind to blood vessels only as aggregates in SKOV-3 tumor is not restricted to a single tumor type. While our understanding of the mechanism causing aggregates to bind and individual Qdots not to bind is currently lacking, it is nevertheless apparent that single targeted Qdots do not frequently bind tumor neovasculature across multiple tumor models.

In conclusion, despite differences in tumor type and extravasation, nanoparticles bound similarly to the vasculature. Indeed, different tumor types display broad variations in extravasation. This is key since extravasation can play a major role in total tumor uptake and is necessary for binding of nanoparticles directly to tumor cells. Our findings demonstrate high heterogeneity across tumor types, individual tumors, and single blood vessels. We were

also able to quantify the average rate of extravasation from blood vessels to interstitium in highest level-of-extravasation LS174T tumors. As approximately spherical nanocrystals, Qdots can be considered a model spherical nanoparticle. This work may thus serve as a foundation for future investigations that explore other spherical and non-spherical particulates both experimentally and theoretically. Indeed, this level of understanding will lead to further studies and models on the mechanism of nanoparticle binding and tumor accumulation, which could engender more rapid regulatory approval of nanoconjugates administered to humans and should simultaneously enable researchers to better design optimized nanoparticle formulations *de novo* for targeted delivery to tumors. Nanoparticle delivery is a critical barrier for both imaging and therapeutic applications of nanotechnology in humans and the direct microscale study of these nanostructures interacting in living subjects should lead the biomedical nanotechnology field much closer to these important objectives in human medicine.

Experimental Section

Nanoparticle conjugates

Amino-functionalized PEGylated quantum dots with 800 nm emission spectra were obtained from Invitrogen (Amino Qdots 800, Carlsbad, CA). Experiments involved Qdots without modification, or conjugated to RGD. Peptide conjugation and characterization was performed as previously reported[7] with modifications as described.[10] Sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) was purchased from Pierce Biotechnology, Inc (Rockford, IL). All other chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO), unless otherwise noted. Briefly, peptides targeting integrin $\alpha_v\beta_3$ (c(RGDfC) (cyclo-(Arg-Gly-Asp-D-Phe-Lys)) and control peptide c(RADfC) (cyclo-(Arg-Ala-Asp-D-Phe-Lys)) (Peptides International, Inc. Louisville, KY) were conjugated to PEGylated Qdots using sulfo-SMCC and PEG-Qdots at a 1000:1 molar ratio. Peptide (c(RGDfC) or c(RADfC) was reacted with the sulfo-SMCC-PEG-Qdot conjugate at a 1000:1 molar ratio. After purification, conjugates were reduced in volume via use of a PD-10 column (GE Healthcare, Piscataway, NJ) and stored until use. When animal experiments commenced, the final nano-bioconjugate was reconstituted in PBS for intravenous injection.

Spectrophotometry was used to establish Qdot conjugate concentrations. A DU 640 instrument (Beckman Coulter, Fullerton, CA) was used with an extinction coefficient of 3.1×10^6 l/mol*cm at 488 nm. Qdots were also analyzed by dynamic light scattering (DLS, using a ZetaPlus Analyzer from Brookhaven Instruments Corporation, Holtsville, NY) and Transmission Electron Microscopy (TEM, Philips CM200 operating 200 kV, FEI Co., Eindhoven, Netherlands) for hydrodynamic radius and aggregates.

Tumor model

To generate a color-encoded tumor microenvironment for visualization, human ovarian carcinoma cells (SKOV-3), human colon carcinoma cells (LS174T), and U87MG human glioblastoma cells (all obtained from American Type Culture Collection and tested negative for murine virus in December, 2008) were transfected with enhanced green fluorescent protein (EGFP). The stable EGFP-expressing cell lines were established with a lentiviral vector (pRRLsin18.CMV-EGFP, a gift from Luigi Naldini, HSR-TIGET, Italy) carrying an EGFP transgene. The cells were incubated overnight in media containing high titer virus, after which cells with very high EGFP expression were sorted using fluorescence activated cell sorting (FACS). Nude mice (nu/nu, Charles River, Wilmington, MA) were inoculated with 0.5-1 million EGFP-transfected tumor cells in the ear in low volume by gently pulling the skin of the ear and carefully sliding a 30 gauge needle through the skin a few mm.

Tumors and neovasculature were visible within 24-48 hours, and imaging was performed as soon as three days post-inoculation. All animal studies were approved by Stanford University's Institutional Animal Care and Use Committee.

Intravital Microscopy

Intravital studies consisted of imaging an ear tumor model in 3 or more living mice per group. Mice were anesthetized with isoflurane and positioned with the ear fixed beneath the IV-100 intravital microscope objective (Olympus, Center Valley, PA). Angiosense 680 (VisEn Medical, Woburn, MA), a long-circulating dye, was injected to outline the vasculature. Unconjugated Qdots (~250 pmol per mouse) or RGD-qdots (33 pmol) were subsequently injected intravenously and the mouse was imaged with Olympus objectives using 488 nm and 633 nm lasers with 3 output channels (green, red, and near infrared). The different fluorophores were scanned sequentially to avoid filter bleed-through. Qdot aggregate binding events in each field-of-view (FOV) were counted across the range of accessible optically-sectioned depths using a 20X objective (NA 0.75), which corresponds to $459.2 \mu\text{m} \times 459.2 \mu\text{m}$ FOV dimensions. Each binding event, occurring in the Qdot near-infrared channel, was verified by reference to (lack of) autofluorescence in the spectrally-separated green and red channels and by reference to a characteristic point-spread-function pattern (in comparison to the pattern of qdot aggregates observed in solution on a slide) as the bound aggregate was optically sectioned in the z-axis. Moreover, each FOV was carefully examined for disperse fluorescence along the length of blood vessels for evidence of individual Qdot binding. Data were acquired from approximately 10 minutes post-injection up to 3 hours post-injection and included between at least 15 FOVs per tumor group. Data were analyzed using Olympus IV-100 software as indicated in the Statistics section to generate a statistical basis for comparison across conditions in our binding assay. Controls in SKOV-3 tumor were performed as described.[10] In a different control, in order to investigate how the exact same vasculature and FOVs would react to sequentially administered control and experimental Qdots, RAD-qdots (a control peptide similar to RGD with a single amino acid substitution and thus decreased specificity to the integrin) were injected and binding events were counted prior to and after injection of RGD-qdots.

For quantification of extravasation, z-stacks were generated by optically sectioning through the region of interest (10-20 sections per region) at each time-point. To process images, depth stacks were acquired at each time point and appended to other depth stacks in the same location using the Olympus Fluoview software. Once the complete depth and time stack was completed, an image projection was created at each time point for the depth stack. Regions of interest (ROI) were then drawn on the image projection both within the blood vessels (as defined by long-circulating dye) and in tumor interstitium (non-vascular areas). The ROIs in each group were averaged to sample the average intensity values in the vessel and interstitium at each time-point. Using the Olympus software, the average extravasation curve was determined by applying this method across the entire time series to compute the influx of qdots into tumor interstitium.

Statistics

We tested statistical significance for the specific binding of RGD-qdots over unconjugated qdots and for the binding of RGD-qdots across various tumor types. Binding rates between RGD-qdots and control Qdots across the different tumor types were compared using a Wilcoxon test stratified by tumor type. We employed an exact Poisson regression to test the treatment condition (RGD) against the control condition in each tumor type. Binding rates of RGD-qdots between tumor types were compared using a Kruskal-Wallis test. Binding events in every FOV for each condition were organized and compared, and binding rates and

confidence intervals at 95% were calculated. We considered comparisons between conditions with p-values <0.05 to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Intravital microscope set-up used for mouse experiments. The call-out to the bottom demonstrates the imaging of our ear tumor model in nude mice. Blood vessels in the ear are visible to the eye and easily accessible to the microscope optics.

Figure 2.

Displays the direct visualization of RGD-qdots and unconjugated Qdots in SKOV-3 tumor in the nude living mouse ear. RGD-qdot aggregates bind to tumor vasculature, while unconjugated Qdots do not. The subpanels across the top and bottom display the three simultaneous output channels and merged image of SKOV-3 tumor and blood vessels. This includes the EGFP channel (left), the angiosense channel (second from left), the near-infrared Qdot channel (second to right), and the merged image (right) as labeled. Binding events occurred predominantly in the RGD-qdot condition and are visible by reference to the bright white areas, which are indicated by black arrowheads in the merged image. The RGD-qdot image was acquired 1.5 hours post-injection. In the RGD-qdot subpanels, Qdots have cleared from the vasculature, leaving only bound aggregates. A typical image (acquired approximately 1 hour post-injection) in the unconjugated Qdots condition is displayed across the bottom panel. In this condition, circulating Qdots remain as visualized by the blood vessel outline, yet no bound aggregates are visible. Note that individual tumor cells are often visible using our technique as displayed in the bottom tumor EGFP image (circular green spots); hair follicles are visible in the unconjugated Qdot condition as round dark areas in the EGFP channel. Scale bars are 50 μm .

Figure 3.

Shows show the merged images of LS174T (top panel) and U87MG (bottom panel, **see also** Supporting Figure 2) tumors with tumor (green), angiosense to outline blood vessels (red), and RGD-qdots (white). On the left, in both tumor types RGD-qdots bind to tumor blood vessels as indicated in each field-of-view by black arrows. On the right, in typical images, unconjugated Qdots do not bind to tumor blood vessels. LS174T images were acquired at 20 minutes and 10 minutes post injection for RGD-qdot and unconjugated Qdots, respectively. Both U87MG images were taken at 40 minutes post-injection. Scale bars are 50 μm across the top panel, and the magnified images below have scale bars of 20 μm .

Figure 4.

Displays the differential extravasation of nanoparticles between tumor types. A particular field-of-view was chosen for each tumor type. The FOV was imaged within minutes of Qdot injection (left) and again 1.5 hours after injection (right). The top panel shows clear extravasation from blood vessels in LS174T tumor. Note the heterogeneous pattern of extravasation which qualitatively does not appear to depend entirely on vessel density or width. The bottom panel shows SKOV-3 tumor. In the early time point, Qdots are visible in the vasculature as the reddish-gray color (a combination of Qdots and angiosense); at the late time point, the vessels are purely red due to Qdot clearance and the continued residence of red angiosense. While Qdots have cleared from circulation due to reticuloendothelial system uptake, no Qdots extravasate from the blood vessels in the SKOV-3 tumor model. Scale bars are 50 μm .

Extravasation of Qdots from LS174T Tumor Vessels

Figure 5.

The graph demonstrates the extravasation of Qdots from LS174T blood vessels over 90 minutes using a region of interest analysis of the fluorescence intensity with Olympus IV-100 software. ROIs in a typical field-of-view were chosen and monitored regularly as z-stacks after Qdot injection. The blue curve represents averaged ROIs from outside the vasculature – this measures the rate of extravasation in the selected region. The pink curve is indicative of the blood vessel fluorescence intensity, which remains relatively constant.

Table 1

Displays the statistics for representative experimental and control conditions of SKOV-3, LS174T, and U87MG tumors in nude mouse ear. Wilcoxon, Kruskal-Wallis, and exact Poisson regression. tests were used to compare various Qdot conditions in tumor to control conditions and binding rate, 95% confidence intervals, and p-values were calculated.

Condition	Binding Rate	95% c.i.
No Tumor, RGD-qdots	0.175	0.070-0.361
SKOV-3, Unconj. Qdots	0.050	0.001-0.279
SKOV-3, RGD-qdots	0.583	0.235-1.202
LS174T, Unconj. Qdots	0.000	0.000-0.527
LS174T, RGD-qdots	0.857	0.315-1.866
U87MG, Unconj. Qdots	0.000	0.000-0.284
U87MG, RGD-qdots	0.500	0.156-1.463