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Surface Engineering of Quantum Dots for *In Vivo* Vascular Imaging

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Abstract

Quantum dot–antibody bioconjugates (QD-mAb) were synthesized incorporating PEG cross-linkers and Fc-shielding mAb fragments to increase *in vivo* circulation times and targeting efficiency. Microscopy of endothelial cell cultures incubated with QD-mAb directed against cell adhesion molecules (CAMs), when shielded to reduce Fc-mediated interactions, were more specific for their molecular targets. *In vitro* flow cytometry indicated that surface engineered QD-mAb labeled leukocyte subsets with minimal Fc-mediated binding. Nontargeted QD-mAb nanoparticles with Fc-blockade featured 64% (endothelial cells) and 53% (leukocytes) lower nonspecific binding than non-Fc-blocked nanoparticles. Spectrally distinct QD-mAb targeted to the cell adhesion molecules (CAMs) PECAM-1, ICAM-1, and VCAM-1 on the retinal endothelium in a rat model of diabetes were imaged *in vivo* using fluorescence angiography. Endogenously labeled circulating and adherent leukocyte subsets were imaged in rat models of diabetes and uveitis using QD-mAb targeted to RP-1 and CD45. Diabetic rats exhibited increased fluorescence in the retinal vasculature from QD bioconjugates to ICAM-1 and VCAM-1 but not PECAM-1. Both animal models exhibited leukocyte rolling and leukostasis in capillaries. Examination of retinal whole mounts prepared after *in vivo* imaging confirmed the fluorescence patterns seen *in vivo*. Comparison of the timecourse of retinal fluorescence from Fc-shielded and non-Fc-shielded bioconjugates indicated nonspecific uptake and increased clearance of the non-Fc-shielded QD-mAb. This combination of QD surface design elements offers a promising new *in vivo* approach to specifically label vascular cells and biomolecules of interest.

INTRODUCTION

Inflammation is a complex process involving numerous cell types and surface proteins. It is characterized by leukocyte rolling and tethering along endothelial cells followed by transmigration into tissue, where their immunodefensive functions, such as phagocytosis, are elicited (1,2). Undesirable provocation of the inflammatory response is thought to be a detrimental feature of numerous diseases such as diabetes, atherosclerosis, and asthma (3–6). Treatment of inflammatory disease is challenging due to uncertainties associated with the roles of many of the cellular and biomolecular mediators. However, one developing strategy hinders inflammation by blockade of cell surface receptors either on the endothelium or on circulating leukocytes (7–9).

Detailed information about molecular mediators of inflammation might be acquired through *in vivo* imaging methods, since they can provide real-time data concerning the spatial and temporal dynamics of cellular activities and molecular expression throughout the time course

of the disease. However, disadvantages of current imaging techniques include limited optical accessibility to tissue, invasiveness (10,11), low or unstable signal intensity due to the use of organic fluorophores (12–14), or low spatial and temporal resolution achieved by the use of radiolabeled antibodies (15). No available technique provides a framework for the simultaneous imaging of multiple molecular participants on moving leukocytes and stationary endothelium and leukocytes in real time.

Imaging inflammation is a difficult task, as the cell types of interest have one or more of the Fc γ receptor family (CD16, CD32, and CD64) which bind to Fc fragments of Immunoglobulin G (IgG) antibodies with variable affinity (16). Binding of bioconjugates to these receptors can yield false positive results when attempting to detect vascular cell surface targets. In addition, it is well-known that nanoparticulate probes are subject to rapid uptake by the tissues of the reticuloendothelial system, such as liver and spleen (17). These immunodefensive mechanisms serve to either rapidly clear the probe from the circulation or nonspecifically bind the probe. However, the many advantages afforded by nanoparticles as bioconjugates, particularly quantum dots (QD), which feature size-tunable visible-IR emission spectra, the need for only one excitation source, and high quantum efficiency, warrant new methods to facilitate their continued application (18,19). Recent work has indicated that the surface functionalization of PEG chains on the quantum dot surface can substantially reduce nonspecificity and clearance problems (20,21). In addition, many studies have established QD amenability to bioconjugation and ease of encapsulation in water-soluble coatings (22), and its incorporation within targeted *in vivo* imaging applications (20,23,24).

In this study, we selected spectrally distinct quantum dot (QD) nanocrystals to enable high-resolution, multispecies imaging using a previously developed, noninvasive *in vivo* retinal vascular imaging system (25). In this application, which pursues the detection of vascular targets, mitigation of nonspecific uptake and clearance mechanisms are essential. To address this, monoclonal antibodies (mAb) targeting leukocytes, neutrophils (26), or the cell adhesion molecules PECAM-1, ICAM-1, and VCAM-1 were site-specifically conjugated to PEG-maleimide-activated QD surfaces via 2-MEA to preserve mAb orientation and binding affinity (27). QD-mAb were then adsorbed with Fc-blocking F(ab)₂ fragments to reduce nonspecific immunorecognition. When incubated with endothelial cells or leukocytes *in vitro*, the shielded probes were found to be more specific for their targets relative to controls. *In vivo* retinal imaging of streptozotocin (STZ)-treated diabetic rats using QD-mAb revealed upregulation of ICAM-1 and VCAM-1 but not PECAM-1. Imaging of a rat model of endotoxin-induced uveitis (EIU) showed the expected increase in stagnant leukocytes in the microcirculation. The high photostability of QD permitted post-experimental histological observations which confirmed the *in vivo* results. Real-time imaging of QD-IgG₁ conjugates indicated rapid clearance of conjugates lacking Fc-blocking F(ab)₂ fragments from the circulation. The distinct spectral emission characteristics of the QD enabled the simultaneous imaging of up to four biomarkers or cell types within the same animal with high specificity.

EXPERIMENTAL PROCEDURES

Synthesis and Characterization of Shielded Quantum Dot-Antibody Conjugates

QD nanocrystals with approximately 80 NH₂ groups/nanoparticle (ITK-NH₂, Invitrogen Corp.) were surface-functionalized with a heterobifunctional PEG-based cross-linker (NHS-PEG-MAL, Nektar Therapeutics) to couple reduced antibodies to the surface, followed by Fc-shielding F(ab)₂ surface adsorption as shown in Scheme 1. (1) 1 μ M of QD-ITK-NH₂ (1.2×10^{14} nanoparticles in 200 μ L solution) were maleimide-activated by incubation with a 20-fold molar excess of NHS-PEG-MAL (MW: 5218) for 2 h in PBS with 10 mM EDTA, pH = 7.4 (PBS-EDTA) at room temperature. Excess NHS-PEG-MAL was removed using two exchanges of PBS-EDTA on a 100K MWCO Amicon Ultra-4 column. The solution was

analyzed qualitatively for the presence of large aggregates (> 200 nm) using fluorescence microscopy, and was filtered through a 100 nm syringe filter if aggregates were observed. (2) mAb (Table 1) were buffer-exchanged in 500 μ g amounts into PBS-EDTA, with two exchanges on a 100K MWCO spin column device (Amicon Ultra-4, Millipore) according to manufacturer-supplied instructions. The retentate was resuspended to 10 mg/mL concentration in PBS-EDTA. The antibodies were then reduced specifically in the hinge region to create two monovalent IgG (r IgG) bearing 1–2 free sulfhydryl groups using 2-mercaptoethylamine (2-MEA, Pierce) (28) according to manufacturer's instructions. Briefly, 6 mg of 2-MEA was dissolved in 100 μ L of PBS-EDTA, and 1 μ L of this solution was added to each 10 μ L of mAb solution. The mixture was reacted for 120 min at 37 °C on a slowly rocking platform. Excess 2-MEA was removed from mAb using a NAP-5 desalting column (GE Healthcare) pre-equilibrated with degassed PBS-EDTA buffer according to manufacturer's instructions. The presence of free sulfhydryl groups generated from 2-MEA reduction was confirmed using Ellman's reagent (Pierce) on a Nanodrop ND-1000 spectrophotometer according to manufacturer's instructions. (3) The QD retentate from **1** was then added to the 2-MEA reduced and purified r IgG mixtures and incubated overnight at 4 °C. The following day, the reaction was terminated with 1 mg/mL L-cysteine (Sigma) to quench remaining maleimide groups. The QD-r IgG conjugates were purified by gel filtration chromatography according to manufacturer's instructions (Superdex 200, GE Healthcare). Fractions were eluted with PBS into 96-well microplates and evaluated by a UV-vis and fluorescence spectrophotometry (Nanodrop ND-1000 and ND-3300) or a fluorescence microplate spectrophotometer (Biotek Synergy HT) in order to identify appropriate QD-mAb fractions and to evaluate conjugation efficiency. In six separate conjugations of r IgG_{2a} to QD655, the average conjugation efficiency measured by UV-vis absorbance spectrophotometry on pooled QD-bound and unbound r IgG fractions was $59.33 \pm 11.34\%$. This corresponds to 18 r IgG/QD. (4) The bioconjugate fraction was incubated with goat anti-mouse IgG (Fc-specific) blocking F(ab)₂ (Sigma) at molar concentrations equal to primary antibody concentrations for 1 h at room temperature, and the resulting mixture was filtered through a 0.22 μ m syringe filter and stored at 4 °C until use. On the day of animal imaging, bioconjugates were briefly spun in an Eppendorf 5415R microcentrifuge for 10 min at 10 000 g to remove QD-mAb aggregates, reserving the supernatant for use. The supernatant was examined by fluorescence microscopy to ensure the absence of QD aggregates (> 200 nm).

Flow Cytometry

Whole blood was collected in 2 $\times$ 3 mL aliquots from male Long-Evans rats into BD Vacutainer tubes spray-lined with K₃EDTA. Erythrocytes were lysed by incubation with BD PharMLyse at a 20:1 ratio of lysis buffer to whole blood for 15 min in the dark at room temperature to obtain a diffuse red suspension of white blood cells. The solution was then centrifuged at 400 g in an Allegra X-22R unit with swinging bucket rotor (Beckman) at room temperature. The leukocyte pellet was rinsed in 500 μ L PBS (pH = 7.2) containing 0.5% BSA, and 0.1% sodium azide (staining buffer) to reduce shedding of membrane antigens. Each pellet was rinsed twice with staining buffer. The cells were resuspended to a 1×10^6 leukocytes/mL concentration, and were incubated for 45 min with one of the following in staining buffer: 50 nM each of QD585-anti-RP-1 conjugate, QD585-anti-CD45 conjugate, Fc-blocked QD585-isotype control IgG₁, maleimide-activated and L-cysteine quenched QD585, Fc-blocked QD585-isotype control IgG_{2a}, non-Fc-blocked QD585-IgG_{2a} isotype control, or 1 μ g phycoerythrin (PE)-anti-RP-1 conjugate (BD Pharmingen). A separate unlabeled fraction was also retained for analysis. All samples were immediately analyzed ($n = 20,000$ gated events) using a BD LSR II multicolor flow cytometer equipped with a 488 nm Ar laser. Rat leukocyte subtypes could be readily resolved by forward and side scatter profiles as previously described (29). Band-pass emission filters were set at 585/42 nm to analyze QD or PE-labeled cells. Analysis was conducted using *Flowjo 7.0* (Treestar Software).

Cell Culture and Immunofluorescence Microscopy

YPEN-1 (CRL-2222, ATCC) rat prostate endothelial cells were cultured to confluency on Lab-Tek II 8-well chambered coverslips with media containing minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM sodium pyruvate, supplemented with 0.03 mg/mL heparin; fetal bovine serum, 5%. YPEN-1 were stimulated with 30 ng/mL TNF- α in media overnight as previously described (30) to upregulate cell adhesion molecules, or incubated with media alone as a control. The next day, YPEN-1 were rinsed with one wash of media and incubated with 10 nM amounts of QD bioconjugates for 1 h at 37 °C as follows: QD655-anti-PECAM, QD655-anti-ICAM, QD655-anti-VCAM, QD655 (reactive maleimide group quenched with 1 mg/mL L-cysteine), and QD-isotype control IgG₁ in Fc-blocked forms as described above or as synthesized without the Fc-blocking step. Cells were then rinsed six times in Dulbecco's PBS containing Ca and Mg, pH = 7.4, and incubated with 4% paraformaldehyde in PBS for 15 min at 25 °C, followed by rinsing three times, 5 min each, with PBS with 50 mM glycine. Fixed cells were imaged using a Nikon TE2000U inverted fluorescence microscope with filter settings as shown in Table 1. These experiments were performed in triplicate. Images were acquired using a Hamamatsu C7780 12-bit color CCD in conjunction with *Image Pro Plus 5.1* (Media Cybernetics).

Preparation of Animal Subjects

All experimental procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee. Diabetes was induced in Long-Evans rats by intraperitoneal injection of 65 mg/kg streptozotocin (STZ, Sigma) in 0.1 mM sodium citrate, pH = 4.5, with the same number of rats remaining untreated as experimental controls ($n = 6$ per group). Elevated blood sugar (> 250 mg/dL) was confirmed in STZ treated animals. Endotoxin-induced uveitis (EIU) was initiated in Long-Evans rats by intraperitoneal injection of 200 μ g lipopolysaccharide. Controls were age-matched untreated rats ($n = 6$ per group). Animals were anesthetized with intraperitoneally administered 15/85% ketazine/xylazine prior to imaging. Tail vein catheterization was performed for injection of QD-mAb. Both eyes were dilated with 1 drop each of 2.5% phenylephrine hydrochloride and 1% tropicamide ophthalmic solutions (Alcon). The right eye was placed on the plano-concave lens previously filled with 2% methyl cellulose (Aqua Poly-Mount, Polysciences).

In Vivo Retinal Imaging

Our imaging design is based on a previously published technique (25), in which an inverted fluorescence microscope (TE2000U Eclipse, Nikon) with 4 $\times$ and 10 $\times$ objectives and a plano-concave -6 D lens (Edmund Scientific) on the microscope stage were utilized to image the rat retinal circulation. In this study, this technique was modified to include a high sensitivity Andor Bioimaging iXon 885 EMCCD camera (Andor Bioimaging), Hamamatsu C7780 color camera, Exfo X-cite 120 metal halide lamp (Exfo Life Sciences), and a pulsed xenon arc flashlamp excitation source (FX 4400, Perkin-Elmer) triggered by a function generator configured for square wave output at 20 Hz (Tektronix). The metal halide lamp and flashlamp were utilized for the imaging of stationary endothelial targets and circulating leukocytes, respectively. Following measurements of pre-injection tissue autofluorescence levels in each QD-specific emission channel, QD-mAb were injected via the tail vein catheter. Injectate consisted of 200 μ L of a 500 nM solution in PBS-EDTA. Initial digital sequences were acquired using exposures ranging from 20 to 200 ms (depending on the QD and the filter set, Table 1) at 110 ms intervals. Subsequent sequences were acquired at approximately 30 min intervals for 2.5 h. In four animals, a QD-IgG₁ isotype control was used as a negative control. Animals were sacrificed with 150 mg/kg sodium pentobarbital administered via tail vein catheter.

Immunofluorescence Microscopy of Retinal Tissue

Following euthanization of animals, both eyes were enucleated. Retinal flat mounts were prepared of the right eye, which was the eye imaged by our *in vivo* retinal imaging system. Eyes were fixed in 4% paraformaldehyde in PBS overnight and flat-mounted on a microscope slide mounted with fluorescence mounting media. The left eye was fixed in 4% paraformaldehyde and paraffin embedded for sectioning. Sections and flat mounts were analyzed by fluorescence microscopy (Nikon TE 2000U) using the same filters as those used in *in vivo* imaging (Table 1).

Image Analysis

For analysis of QD-mAb binding to YPEN-1, fluorescence micrographs acquired under identical settings and conditions (matched cell densities in field of view) were measured in the Red CCD channel (the chip with which QD655 emission is captured) for fluorescence intensity using *Image Pro Plus 5.1*. From these data, the mean Red channel fluorescence intensity was obtained and standard deviation determined. Background correction was performed on image intensities by subtracting the mean Red CCD channel intensity obtained from the same density of unlabeled YPEN-1. Intensities were analyzed for statistical significance using an unpaired two-tailed *t*-test (*SigmaStat 3.0*, SYSTAT). Background-corrected data was then plotted using *SigmaPlot 9.0* (SYSTAT). Statistical significance was interpreted by $P < 0.05$.

For *in vivo* image quantification of leukostasis, the area of image analysis of rat fundi was a circle of two optic disk diameters from the center, allowing for counts of stagnant leukocytes and observations of leukocyte trafficking and cell adhesion molecule expression within major arteries and veins as well as the microcirculation. A leukocyte was assumed to be stagnant if no displacement larger than one cell diameter was observed within 60 s of continuous imaging. The number of stagnant QD-labeled cells was quantified for STZ, EIU, and wild-type retinas postacquisition from digital video using *Andor iQ 1.6*, and compared using an unpaired two-tailed *t*-test in *SigmaStat 3.0*.

Quantitative analysis of Fc-blocked and non-Fc-blocked QD-IgG₁ bioconjugates was performed by analyzing 60 consecutive digital frames of wild-type rat retina before and after systemic injection of 500 nM (200 μ L) of each probe. An observation area of 2 optic disc diameters from center was utilized for the analysis. Using *Andor iQ 1.6* image analysis software, the mean fluorescence intensity of 60 consecutive frames was plotted as a function of time. Data were plotted using *SigmaPlot 9.0*.

RESULTS

In Vitro Studies

In cell culture studies, the QD655-mAb Fc-shielded conjugates specifically labeled CAMs on TNF- α -stimulated YPEN-1 rat endothelium (TNF+) (Figure 1A–D; Figure 2B,E). In order to simulate the *in vivo* environment, no traditional immunofluorescence blocking steps (e.g., serum, Fc receptor blocking mAbs) were utilized. Under TNF+ conditions, a statistically significant increase in Fc-blocked QD655-anti-VCAM binding to YPEN-1 (Figure 1B,D) was observed, whereas TNF+ conditions did not significantly affect QD655-anti-PECAM binding (Figure 1A). These observations were consistent with previous reports (31, 32). QD655-anti-ICAM bioconjugates bound TNF+ YPEN-1 by over 10-fold as compared to untreated cells (Figure 2E). A strong reduction in nonspecific binding was observed when comparing binding of Fc-blocked and non-Fc-blocked QD655-anti-ICAM bioconjugates (Figure 2A,B,E). A reduction in nonspecific binding was also observed when comparing measured intensities of YPEN-1 labeled with Fc-blocked or non-Fc-blocked QD655-isotype control IgG₁ nanoparticles (Figure 2C–E), suggesting that the Fc fragment is responsible for a substantial

percentage of QD-mAb nonspecific binding. Immunofluorescence analysis of YPEN-1 indicated that non-Fc-blocked QD655-mAb constructs were aggregated in focal locations along cell membranes, shown by arrowheads (Figure 2A–C). This observation may be due to two features: the upregulation of Fc γ receptors on endothelial cells following TNF- α stimulation, and the patching and capping effect observed previously for Fc receptor–ligand combinations (33). Specifically, patching and capping refers to the accumulation of surface-bound IgG-Fc receptor pairs at distinct, punctate locations at the membrane to form clumps of Fc receptor-IgG pairs. This incidence of cap formation was less evident in TNF+ YPEN-1 cells incubated with Fc-blocked bioconjugates (Figure 2B). Maleimide-quenched QD which were not conjugated to Mab (Figure 1C) did not bind appreciably to cell surfaces. These data collectively suggest that nonspecific binding of unshielded probe was likely due to the binding of mAb Fc fragment to endothelial Fc receptors.

Fc-blocked QD585-anti-RP-1 (neutrophils) and QD585-anti-CD45 (leukocyte common antigen) were shown to retain bioactivity and specificity *in vitro* (Figure 3). QD585-anti-RP-1 conjugates were shown to specifically bind to neutrophils and not other leukocyte subsets (Figure 3A), and QD585-anti-CD45 were shown to label the three main subclasses of leukocytes as shown by flow cytometric analysis of erythrocyte-lysed whole blood (Figure 3B,D). QD-anti-RP-1 was observed to have over 4-fold enhancement in fluorescence over PE-RP-1 (1003 vs 233), a positive control antibody coupled to phycoerythrin (Figure 3C). QD585-IgG2a (matching the isotype of QD585-anti-RP-1), when Fc-blocked with an anti-Fc F(ab)₂ fragment, featured 53% lower mean fluorescence levels than that due to the non-Fc-blocked conjugate (Figure 3C), indicative of reduced nonspecific binding of Fc-blocked bioconjugates. Fc-blocked QD585-anti-CD45 conjugates used to label leukocytes yielded a mean fluorescence of 1430, over 17-fold greater than cells labeled with Fc-blocked QD-IgG₁ isotype control (82) and 22-fold greater than unlabeled cells (65) (Figure 3D).

***In Vivo* Imaging**

Intravenous injection of QD bioconjugates targeted to ICAM-1 or VCAM-1 resulted in a maximum increase in vascular fluorescence in streptozotocin (STZ)-treated diabetic rats within 30 and 90 min, respectively (Figure 4). This increase in fluorescence was observed in major vessels as well as the microcirculation. Control rat retinas did not exhibit the same fluorescence intensity with either the ICAM (Figure 4A–C) or VCAM (Figure 4D–F) conjugates. Injection of QD anti-PECAM produced increases in fluorescence which were similar in both STZ-treated and untreated animals (Figure 4G–I). Injection of QD-IgG₁, a nonspecific control, did not result in fluorescence signal accumulation in the retinal vasculature to any appreciable degree throughout the duration of imaging up to 2.5 h (Figure 4J–L).

Intravenous injection of QD585-anti-RP-1 conjugates enabled the fluorescence detection and long-term tracking of neutrophils in STZ-treated diabetic animals as shown in Figure 5A–D. Using a pulsed xenon flashlamp, we visualized individual free-flowing leukocytes (Figure 5A,B), leukocyte rolling along endothelial walls (Figure 5C), and leukostasis (Figure 5D) in major vessels as well as capillaries. Individual neutrophils were resolved in all vessels including arteries, and an increase in QD-anti-RP-1 positive stagnant neutrophils in the microcirculation was observed in the STZ-treated group (5.83 ± 2.86 , $n = 6$) vs untreated animals (0.50 ± 0.55 , $n = 6$) ($P < 0.01$). Fluorescence micrographs of stagnant neutrophils in flat-mounted STZ-treated rat retinas confirmed *in vivo* observations (Figure 5G).

Injection of the QD655-anti-CD45 bioconjugate in LPS-treated EIU rat models revealed a significant number of stagnant leukocytes in the microcirculation relative to untreated animals (Figure 5E,F). The number of QD-anti-CD45 positive stagnant leukocytes in LPS-treated rat retinas (20.33 ± 8.73 , $n = 6$) was elevated over control retinas (2.17 ± 1.17 , $n = 6$) *in vivo* ($P < 0.01$).

Ten seconds after tail vein injection, comparison of the retinal fluorescence from Fc-shielded and non-Fc-shielded bioconjugates showed significantly less fluorescence from the non-shielded bioconjugate (Figure 6). Tracers injected intravenously into a tail vein travel through the hepatic circulation, heart, and lungs before the remainder reaches the eye. Our data indicate that non-Fc-blocked nonspecific QD-IgG₁ is cleared rapidly within 2 h of circulation, returning retinal fluorescence levels to pre-injection values (Figure 6E–H,J). For Fc-blocked nonspecific QD-IgG₁, an apparent bolus is observed within 12 s of injection (Figure 6B), and fluorescence levels by 2 h have not returned to background values (Figure 6A–D,I). These results suggest that Fc blockade substantially enhances the circulation lifetime of the QD-mAb due to reduced uptake and clearance.

Ex Vivo Imaging of Retinal Tissue

Immunofluorescence analysis of flat-mounted retinas showed correlation of *in vivo* features, such as enhanced CAM expression areas within the microcirculation, with *ex vivo* microscopy (Figure 7A,B). Analysis of postmortem retinal tissue also indicated low nonspecific binding of Fc-shielded bioconjugates (Figure 7C,D) within blood vessels. The microcirculation in a flat mount from an EIU rat featured leukocyte adhesions and extravasations similar to those observed *in vivo* (not shown), and similar to the result shown for the STZ-treated rat (Figure 5G).

DISCUSSION

Fc-blocked QD-mAb were found to be specific for CAM and leukocyte targets, as shown by immunofluorescence (Figure 1, Figure 2), flow cytometry (Figure 3), and *in vivo* and *ex vivo* retinal imaging (Figure 4–Figure 7). QD-IgG that were nonspecific in reactivity did not bind to retinal vasculature (Figure 6), which suggests that Fc-blocked QD-antibody bioconjugates feature reduced nonspecific binding tendencies while retaining the native binding affinity conferred by the mAb. Our technique, employing PEGylation, 2-MEA reduction, and Fc-blockade by Fc-specific F(ab)₂, is a rapid, cost-effective method for the site-specific bioconjugation and immune-shielding of mAb on nanoparticle surfaces. The 2-MEA reduction process was found to work equally well on mouse IgG₁ and IgG_{2a} subclasses using the same protocol. As an alternative to removal of the Fc fragment from the antibody, we choose instead to adsorb it with an antibody fragment which itself has no Fc region. Nontargeted QD-IgG conjugates adsorbed with an Fc-blocking F(ab)₂ were less susceptible to nonspecific binding to leukocyte and endothelial cell surfaces compared to the same conjugates without Fc-blockade (Figure 1–Figure 3). This may be the result of steric inhibition conferred upon the bioconjugate by the F(ab)₂, which reduces Fc receptor access to its Fc fragment binding site on the IgG. Fc-blockade of CAM-targeted QD did not affect their binding affinities toward endothelial cell proteins *in vitro* or *in vivo* (Figure 1, Figure 2, Figure 4). Fc-blockade by itself also may potentially enhance the circulation half-life of QD-mAb when employed in conjunction with PEG. Fc-blocked, nontargeted QD-IgG were present in the retinal circulation in much higher fluorescence levels relative to the same dose of non-Fc-blocked constructs (Figure 6), suggesting that Fc-blockade of IgG may substantially reduce first-pass nonspecific clearance of probes due to liver and spleen immunorecognition. Therefore, our probe design features target selectivity with a reduced tendency for nonspecific binding.

Analogues of our strategy exist for reduction of nonspecific binding of bioconjugates for *in vitro* applications, and we have applied these *in vitro* techniques for the first time to *in vivo* imaging. For example, the commercial reagent FcBlock (anti-CD16, anti-CD32, BD Biosciences) has long been utilized to block Fc receptors on hematopoietic and endothelial cells prior to immunofluorescence (34) or flow cytometric (35) analysis for the reduction of background staining using cell surface protein antibodies. Direct blockade of the Fc

fragment of antibodies rather than Fc receptors occurs often in biology. Blockade of the antibody Fc domain with streptococcal proteins has been found to reduce Fc-mediated nonspecific binding (36). Herpes Simplex Virus type 1 (HSV-1), when bound by an IgG, uses its Fcγ receptors to occupy the Fc fragment of the same IgG, thus preventing Fc-mediated immunodefensive mechanisms (37). By adsorption of the QD-IgG conjugate with an Fc-targeted F(ab)₂, it is likely that the adsorbed fragment makes the Fc binding site of the targeting antibody sufficiently inaccessible to immune surveillance, thus enhancing specificity and circulation time.

Other strategies to reduce Fc-mediated immune recognition of antibodies or antibody-based bioconjugates are reported in the literature. Specifically, antibody fragmentation techniques using papain, pepsin, or ficin require several reaction steps and much reagent quantity and time optimization for each antibody species and subclass, and generally do not yield a biomolecule with a site-specific cross-linking site suitable for nanoparticle conjugation. Instead, primary amines which reside in the antigen-binding site of the antibody could be cross-linked to the nanoparticle, obliterating its antigen binding affinity following bioconjugation. Protein engineering techniques now provide for a means of producing antibody fragments with engineered hinge region cysteines (Fab') which allow for site-specific nanoparticle conjugation while preserving the antigen-binding region (38,39). However, this process is time-consuming and costly, and may not be suitable in applications where multiple IgGs are to be conjugated to nanoparticles for multiplexed imaging on a small scale, or where IgG targets are quickly examined and replaced for purposes of drug discovery or biology. Our process provides for the site-specific conjugation of antibodies to nanoparticles, without IgG subclass-specific optimization, with similar benefits of immune system evasion afforded by antibody fragmentation techniques.

Our *in vivo* imaging results are consistent with studies of ICAM and VCAM expression in diabetic and nondiabetic tissue, in which their upregulation is implicated in an inflammatory cascade that causes various complications of the disease (6,40,41). QD fluorescence due to ICAM and VCAM expression is apparent on major vessels as well as the microcirculation in disease models, which appears as an out of focus fluorescent background behind the major vessels (Figure 4A–F). QD targeted against PECAM, a panendothelial marker, produced increases in fluorescence which were similar in both STZ-treated and untreated animals (Figure 4G–I), thus suggesting its utility as an internal control or vascular counterstain in this imaging application. Our technique was found to be suitable for the long-term imaging of stagnant leukocytes commonly observed in both the STZ-treated rat model of diabetes (6,9) and the LPS-treated rat model of EIU (42).

Photobleaching is a problem with current techniques which probe leukocyte dynamics *in vivo*, such as acridine orange fluorography (43,44). Thus, long-term imaging of leukocytes is hindered. Using QD-based fluorography, we were able to continuously observe entrapped leukocytes in the circulation over an hour of continuous illumination without bleaching (Figure 5). Therefore, our QD-based imaging technique constitutes a potential alternative to current methods of imaging leukocytes due to the ability to simultaneously image multiple leukocyte subsets and stationary biomarkers with one excitation source with high specificity.

Our imaging technique provides noninvasive optical access to the *in vivo* retinal vasculature and continuous monitoring of vascular events. Spatial resolution on the order of a cell is achieved with this technique. QD have superior optical properties, such as high quantum efficiency and photostability, as well as distinct size-tunable emission wavelengths with the need for only one excitation source. By harnessing these QD properties while immune-shielding the nanoparticle surface, it was readily possible to image three CAMs and one leukocyte subset within the same animal, without spectral overlap, using one blue excitation

source to sufficiently illuminate QD-tagged species within the circulation. This feature enables detailed multiplexed studies of disease and biological processes. We used this approach to simultaneously observe the molecular expression of three different biomarkers, while also observing leukocyte-endothelial interactions. Other applications of this imaging strategy could readily be applied to studies in which multiple cellular and biomolecular vascular functions remain to be elucidated such as cancer, ocular angiogenesis, and atherosclerosis. While qualitative information was primarily presented in this initial application of our technique, we foresee the calculation of additional parameters including rolling velocities for various subtypes, relative fluorescence intensities of specific biomolecules along vascular walls, density of entrapped leukocytes, and important features of the inflammatory process *in vivo*.

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Figure 1. Evaluation of Fc-blocked QD-mAb conjugate specificity toward TNF- α stimulated (TNF+) or untreated (TNF-, image insets) rat endothelial cells (YPEN-1). (A–C) Fluorescence micrographs of QD655-anti-PECAM (A), QD655-anti-VCAM (B), and QD655 (C). Insets show incubation of TNF- YPEN-1 with matched bioconjugates. (D) Mean image intensities from immunofluorescence analysis of TNF $\pm$ YPEN-1 incubated with QD655-mAb bioconjugates (reported as mean + S.D., $n = 3$ images per sample). Intensities for each TNF+ and TNF- pair were compared by t -test for statistical significance ($P < 0.05$ indicated by asterisk).

Figure 2.

Comparison of Fc-blocked and non-Fc-blocked QD655-mAb conjugate specificity toward TNF- α -stimulated YPEN-1 (TNF+). (A–B) TNF+ YPEN-1 incubated with non-Fc-blocked (A) and Fc-blocked (B) QD655-anti-ICAM bioconjugates (magnification 100 $\times$). Arrowheads indicate possible Fc receptor capping, which was substantially reduced when using Fc-blocked conjugates. (C,D) TNF+ YPEN-1 incubated with non-Fc-blocked (C) and Fc-blocked (D) QD655-IgG₁ isotype control bioconjugates, with capping sites labeled with arrowheads (magnification 400 $\times$). (E) Mean image intensity analysis reveals lower nonspecific binding of bioconjugates featuring Fc blockade (reported as mean + S.D., $n = 3$ images per sample).

Intensities for each matched Fc-blocked and non-Fc-blocked bioconjugate were compared by *t*-test for statistical significance ($P < 0.05$ indicated by asterisks).

Figure 3.

FACS analysis of leukocytes labeled with QD-mAb bioconjugates *in vitro*. (A) QD585-anti-RP-1 (Fc-blocked) specific labeling of neutrophils in peripheral blood. (B) QD585-anti-CD45 (Fc-blocked) labeling of all leukocyte subsets, with arrow from B to D indicating fluorescence histogram of gated leukocytes (D). (C) Gated neutrophil populations from rat peripheral blood (arrow from A to C shows a gated neutrophil population used for analysis of 1 sample), (488 nm Ar laser excitation, phycoerythrin (PE) emission filter (585/42 nm)), with mean fluorescence intensity of each sample indicated in bold. Light blue: PE-anti-RP-1 dye-labeled positive control mAb. Purple: QD585-anti-RP-1 (Fc-blocked). Blue: QD585 quenched with ϵ -cysteine. Brown: QD-Ms-IgG2a isotype non-Fc blocked. Green: QD-Ms-IgG2a isotype Fc blocked. Red: Unlabeled rat peripheral blood. (D) Rat leukocytes labeled with QD585-anti-CD45 (red, gated leukocytes from (B)), QD585-Ms IgG1 Fc blocked isotype control (blue) or unlabeled (green).

Figure 4.

Frames from *in vivo* digital videos of STZ treated and untreated rat retinas. Pre-injection digital videos using the appropriate fluorescence filters for the spectrally distinct biomarkers of interest (left column; A, D, G, J) were acquired prior to bioconjugate injection. QD585-anti-ICAM, QD655-anti-VCAM, QD525-anti-PECAM, and QD565-Ms IgG₁ were systemically injected in STZ-treated rats. Untreated control rat fundi (middle column; B, E, H, K) and STZ-treated rat fundi (right column; C, F, I, L) were imaged either 30 min (ICAM) or 90 min (VCAM, PECAM, IgG₁) postinjection under identical acquisition settings used for background autofluorescence measurements for each biomarker channel. Fluorescence enhancement due to target binding in untreated controls (B, E, H, K) was primarily observed for PECAM (H).

The retinal vasculature of STZ-treated rats (C, F, I, L) showed a marked increase in fluorescence due to binding of QD-mAb conjugates to ICAM (C) and VCAM (F), with at least 5-fold increases in fluorescence intensity over background measured for labeled vasculature. Injection of conjugates to PECAM (I) produced similar levels of fluorescence under both conditions (H, I). Fluorescence signal from nonspecific IgG₁ mAb bioconjugates (J–L) was not detected in either control or STZ retinas. Images shown were acquired at imaging intervals resulting in optimal signal-to-noise ratio.

Figure 5.

In vivo imaging of endogenously QD-mAb-labeled leukocyte trafficking in two rat models of inflammation. The CCD acquired images with exposure times ranging from 50 to 200 ms with 110 ms between exposures. Flowing of QD585-RP-1 labeled cells in vessels (A,B) or leukocyte rolling (C) in STZ model as detected by four pulses of a xenon flashlamp at 50 ms intervals within 30 min of probe injection. Leukostasis was frequently observed in the STZ-treated rat model (D in right panel). Stagnant neutrophil appears as a hyperfluorescent dot in the microcirculation. Stagnant neutrophils were continuously visible in the retinal vasculature for over 1 h of *in vivo* imaging. Stagnant leukocytes labeled *in vivo* with QD655-anti-CD45 were present at high densities in EIU rat models (E) relative to untreated controls (F). Stagnant neutrophils labeled in STZ-treated diabetic animals were visible in postmortem retinal flat mounts (G).

Figure 6.

Comparison of Fc-blocked (A–D, I) and non-Fc-blocked (E–H, J) QD-IgG₁ mAb bioconjugates *in vivo*. (A) Pre-injection autofluorescence in QD655 emission channel. (B–D) Fc-blocked QD655-IgG₁ in retina observed 12 s postinjection (B) where the first pass of bolus through retinal circulation is observed, 1 h postinjection (C), and 2 h postinjection (D), by which time Fc-blocked QD655-induced fluorescence is still above background levels (I, dashed line). (E) Autofluorescence levels in QD565 channel. (F–H) non-Fc-blocked QD565-IgG₁ bolus was not qualitatively visible at 12 s postinjection (F), and subsequent image acquisitions at 1 h (G) and 2 h (H) revealed eventual return of image intensity to pre-injection levels (J, solid line), suggesting a relatively rapid clearance and/or uptake of non-Fc-blocked QD-mAb.

Figure 7.

In vivo microcirculatory VCAM-1 expression (A) can be confirmed using immunofluorescence analysis postmortem (B), as shown for QD655-anti-VCAM-1 labeled vasculature. While QD655-VCAM-1 (Fc-blocked) conjugates were shown to specifically stain vasculature in retinal histological sections (C), no background staining due to QD-IgG₁ (Fc-blocked) was observed above background autofluorescence levels (D).

Scheme 1 a.

Table 1

Quantum Dot-mAb Conjugates

study target (function)	targeting monoclonal antibody	QD excitation	QD emission
PECAM-1, endothelial cell (transmigration)	Ms anti-rat CD31 IgG1 (BD)	excitation filter E460SPUV v2 peak emission 405 nm	525 nm, detection filter 525WB20
ICAM-1, endothelial cell (arrest)	Ms anti-rat CD54 IgG1 (BD)		605 nm, detection filter 605WB20
VCAM-1, endothelial cell (arrest)	Ms anti-rat CD106 IgG1 (BD)		655 nm, detection filter 655WB20
RP-1, neutrophil (unknown)	Ms anti-rat RP-1 IgG2a (BD)		585 nm, detection filter 585WB40 or 620WB90
CD45, leukocytes (multiple essential functions)	Ms anti-rat CD45 IgG1 (BD)		655 nm, detection filter 655WB20
nonspecific	Ms IgG1 or IgG2a isotype control (BD)		565 nm, detection filter 565WB20