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Quantum Dots As A Visual Aid For Screening Bead-Bound Combinatorial Libraries

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A commonly used method for identifying protein ligands is to screen combinatorial libraries or diverse compound collections. A particularly convenient way to do so is to create a library of compounds on beads by using the classical split-and-pool technique,[1] then incubate a labeled protein with the library. Ideally, beads that display ligands able to bind the target protein 'light up' above whatever background might be observed, which allows their identification.[2] This approach is particularly attractive if one wishes to employ the ligands thus identified as immobilized protein capture agents, which is the main focus of our research.[3] However, the technique places stringent demands on the resin to be utilized for both synthesis and screening, since it must exhibit good swellability in both aqueous and organic solvents, low nonspecific protein binding, and good mechanical stability. After considerable experimentation, we found that TentaGel (Rapp Polymere) resin, which consists of a cross-linked polystyrene core overlaid with long, amine-terminated polyethylene glycol chains, has by far the best combination of these attributes. Unfortunately, TentaGel, like many polystyrene-based resins, exhibits high-level, broad-wavelength autofluorescence (see Figure 1). This is unfortunate

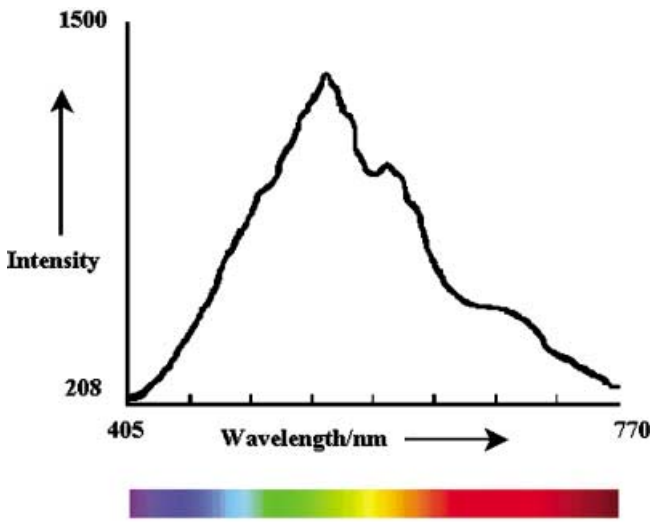

Figure 1. *Fluorescence emission spectrum of a TentaGel bead (excitation: 330 – 380 nm).*

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because, while there are many ways available to label a protein for a screening experiment, fluorescent tags have many advantages, including high sensitivity. Furthermore, fluorescence microscopes commonly available in many departments can be used to visually screen large libraries without the need for other specialized, expensive equipment. We have found that the signal from many such tags, for example fluorescein, is obscured by the bead fluorescence (data not shown). As is reported below and in detail elsewhere, this problem can be partially overcome by using Texas-Red-labeled proteins, since the bead fluorescence is lowest in the red region of the visible spectrum (Figure 1). Nonetheless, the significant background exhibited by TentaGel and related beads even in this region of the specturm makes microscope-based visual screening a tedious and time-consuming process, and also results in occasional false positives. We show herein that all of these problems can be avoided by using the unique fluorescent properties of a quantum dot to visualize captured proteins. The result is a simple and convenient two-color assay that easily distinguishes true 'hits' from beads lacking appreciable levels of captured protein with extremely high sensitivity.

Quantum dots are nanomaterials that provide a much higher quantum yield of fluorescence and exhibit better photochemical stability than organic fluorophores.[4] A critical feature of quantum dots is that they are semiconductor materials and therefore absorb energy in a broad and continuous region of the spectrum but have narrow and symmetrical emission peaks.[5] Recently, streptavidin (SA)-coated quantum dots that emit in the red region of the spectrum ($\lambda_{max} = 608$ nm) have become commercially available that should allow the detection of biotinylated proteins captured on beads. We therefore decided to compare the performance of a quantum dot with that of an organic fluorophore, Texas Red, in a protein binding experiment with peptides displayed on TentaGel beads.

Ubiquitin was labeled with biotin and used as the target protein. The labeled ubiquitin (1 μM) was incubated with beads displaying either a seven-residue ubiquitin-binding peptide[6] or a negative control peptide that does not bind ubiquitin. As a positive control, a third set of beads was created in which biotin was linked covalently to the bead surface. After washing, the beads were then exposed to either Texas-Red-labeled streptavidin (250 nM) or SA-coated quantum dots (50 nM).[7]

The top panel in Figure 2 shows the results obtained by using Texas-Red-labeled streptavidin as the 'sandwich agent' and employing the excitation filter commonly used for this dye (555 – 590 nm band pass). Both the beads displaying the covalently linked biotin and ubiquitin-binding peptide exhibit significant fluorescence. The fluorescence intensity from beads displaying the ubiquitin-binding peptide is clearly greater than that observed for the negative control peptide, but this modest difference illustrates the problem with using Texas-Red-labeled proteins for library screening experiments. The 'hits' are brighter than the background, but the level of contrast is not huge. This problem necessitates careful and painstaking visual analysis of beads under a microscope field, which is a tedious process.

In contrast, the lower panel in Figure 2 shows the results obtained by using SA-coated quantum dots as the sandwich

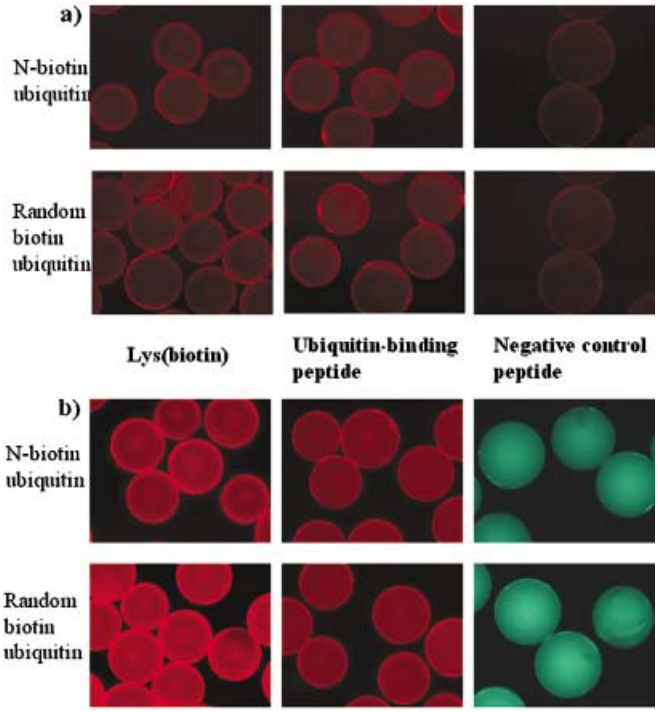

Figure 2. 'Sandwich assays' detecting the capture of biotinylated ubiquitin (Ub). TentaGel beads displaying a Ub-binding peptide or negative control peptide were incubated with biotinylated Ub, then with a) Texas Red-conjugated SA (excitation filter: 555 – 590 nm; exposure: 300 ms; magnification 10 ×) or b) SA-coated quantum dots (excitation filter: 390 – 410 nm; exposure: 150 ms; magnification 10 ×). Beads displaying biotinylated lysine were employed as a positive control for binding of the labeled SA. Results from identical experiments utilizing Ub biotinylated at a lysine side chain (random biotin ubiquitin) or labeled uniquely at the N terminus (N-biotin ubiquitin) are shown.

agent and employing the excitation filter commonly used for 4′,6-diamidino-2-phenylindole (DAPI) dye (390 – 410 nm band pass filter). Both the biotinylated beads and the beads displaying the ubiquitin-binding peptide exhibit a bright red fluorescence. However, the negative control beads are bright green, which is expected from the fluorescence spectrum of TentaGel beads shown in Figure 1. The same result was obtained with ubiquitin that had been biotinylated at lysine side chains as with ubiquitin labeled specifically at the N terminus (Figure 2). These results show that the much greater emission intensity of the quantum dot and its extraordinary Stokes shift provide a signal that is not obscured by the autofluorescence of the beads. The striking color difference between the beads that display the ubiquitin-binding peptide and those that display the negative control peptides provides a visually convenient, high-contrast 'two-color assay' that would make library screening experiments much easier.

To simulate a screening experiment, a few beads displaying the ubiquitin-binding peptide were added to an excess of beads that display the negative control peptide. This mixture was incubated with biotinylated ubiquitin (1 μM) and then, after washing, the beads were incubated with SA-coated quantum dots (50 nM). Photomicrographs of the same group of beads were then taken with the Texas Red (Figure 3 a) or DAPI (Figure 3 b) excitation filters. A green and a red bead were

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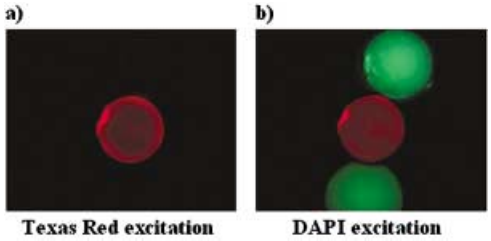

Figure 3. Fluorescence micrograph of a mixture of beads displaying the ubiquitin-binding peptide and a negative control peptide after incubation with biotinylated ubiquitin followed by SA-coated quantum dots. a) Texas Red excitation filter. Exposure: 2000 ms. b) DAPI excitation filter. Exposure: 150 ms. Magnification: 10 × in each case.

picked from the field shown and their sequences were determined by Edman sequencing. As expected, the bright red bead displayed the ubiquitin-binding peptide while the green beads (dark in the left-hand panel) displayed the negative control peptide. This result indicates that quantum-dot-based visualization allows one to choose true hits in the midst of many other beads reliably.

To test the sensitivity of quantum-dot-based detection in this type of assay, we used TentaGel beads displaying a peptide (YAPPLPPRNRPRA) that binds the Src homology 3 (SH3) domain of human c-Src protein with a dissociation constant, K_D, of 300 nM.[8] Again, beads displaying a different peptide were employed as a negative control (Figure 4). A dilute solution of

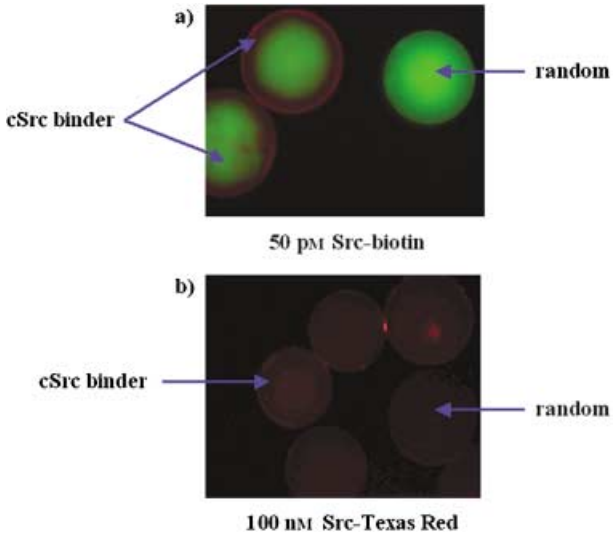

Figure 4. Photomicrograph of beads that display an Src-binding peptide or a negative control peptide taken through the DAPI excitation filter after incubation of the beads with dilute Src (50 pM) followed by SA-coated quantum dots. The Src binder exhibits a red halo on the surface of the bead. The negative control shows the green fluorescence bead background. Exposure: 150 ms. Magnification: 10 ×.

biotin-labeled c-Src (50 pM) was incubated with each type of bead. After washing, two beads displaying the c-Src-binding peptide and one of the control-peptide beads were placed in the same field of view under a microscope and a photograph was taken through the DAPI band pass filter (390 – 410 nm) for excitation. As shown in Figure 4 a, two of the beads have readily observable red halos around the green core, while one is solid green. The input level of protein was well below the K_D value of the complex, therefore only a small amount of biotinylated c-Src should be retained by the beads displaying the Src-binding peptide, especially after washing. Since the photograph is not a confocal image, the red halo pattern is expected as in this region of the micrograph the signal due to multiple layers of quantum dots is summed. When this experiment was repeated with the streptavidin – Texas Red conjugate in place of the quantum dots, no bound protein could be observed. When the DAPI filter was used, all of the beads appeared green with no red halo visible. When we repeated the experiment with Texas-Red-labeled Src, 100 nM was the lowest concentration of protein at which binding could be detected visually above the bead background (Figure 4 b). This lack of contrast is a serious limitation for performing on-bead assays at low concentrations of a Texas-Red-labeled protein. This experiment shows that quantum-dot-based detection is superior for the detection of modest-affinity ligands under demanding conditions.

With these model experiments complete, we then turned to employing the quantum dot technology in a genuine library-screening experiment, again comparing this technique to our standard protocol with Texas-Red-labeled protein. α1-Antitrypsin (AAT) was labeled with Texas Red or biotin. The labeled protein was purified to remove excess free label and was then used to screen a library of octamer peptides built from 10 amino acids (A, D, E, H, K, N, R, V, W, Y) by split-and-pool synthesis on TentaGel. Figure 5 shows photomicrographs from each screen-

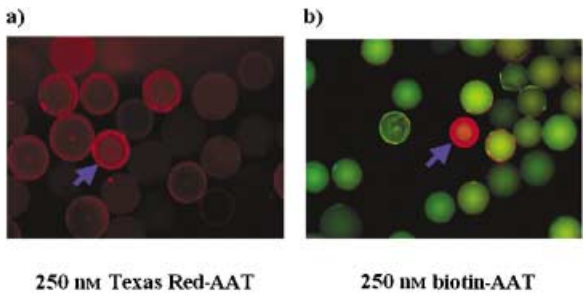

Figure 5. Screening of a peptide library with AAT. Two portions of the library were incubated with the corresponding labeled AAT. a) 250 nM Texas Red – AAT. Exposure: 2000 ms. b) 250 nM biotin – AAT and then 50 nM SA – quantum dot. Exposure: 300 ms. The arrows indicate the hits isolated for characterization. Magnification: 4 × in each case.

ing experiment. Each image shows a bead scored as a 'hit' (indicated by the blue arrow). Figure 5 a is typical of the results we obtained when screening against a number of Texas-Red-labeled proteins. A spectrum of beads, ranging in appearance from quite bright (the hit) to less bright to quite dark, is observed and considerable time is required to look carefully at each microscopic field and decide which are the best hits. In contrast, the micrograph from the experiment with the quantum dot technology (Figure 5 b) shows the stark contrast between a hit and a negative result. The hit is obvious since it is the only bead showing any red color. This contrast makes visual screening

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much faster and completely eliminates the possibility of picking false positives, which is a significant problem when using Texas-Red-labeled proteins. The 'hit molecule' from the quantum-dot-based screen is being incorporated into a protein-detecting microarray and its characterization, along with that of many other protein ligands, will be reported elsewhere.

All of the photomicrographs shown in this study were obtained with a standard monochromatic CCD camera. Images taken with different filter sets were then processed by using standard software to provide the false-color images shown. More expensive and sophisticated color cameras are not required to produce these images. More importantly, the red and green colors of the hit and negative beads, respectively, are readily visible when one views the beads through the eyepiece of the microscope and the type of picture shown in Figure 5 b is typical of what one sees, so only a standard microscope with the appropriate set of filters is required for screening. Indeed, if one employs large macrobeads (reported to generate high-loading compound-bead libraries)[9] that can be seen with the naked eye for library synthesis, the quantum dot method allows visualization of captured biotinylated protein with the naked eye by using a simple hand-held UV light source (365 nm irradiation) of the type employed to visualize fluorescent TLC plates (data not shown). This approach makes library screening accessible to essentially any laboratory, even those lacking access to a fluorescence microscope.

In summary, we have shown that visualization of protein binding to a ligand immobilized on polystyrene-based beads can be realized much more easily by using quantum dots than with traditional organic fluorophores. In particular, the visually striking two-color readout that is obtained when the beads are irradiated with 390 – 410-nm light provides a sensitive, high-contrast measure of the level of protein captured on the bead. This technique should prove to be an enormous aid in library screening experiments that involve libraries synthesized on beads or spotted onto other surfaces with significant intrinsic fluorescence.

Experimental Section

Proteins, peptides, and instrumentation: 9-fluorenylmethoxycarbonyl (Fmoc) amino acids were purchased from Novabiochem. Streptavidin Qdots 605 were purchased from Quantum Dot Corporation. Streptavidin Texas Red was purchased from Molecular Probes. Lysine- and N-terminally biotinlylated ubiquitin were purchased from Boston Biochemicals. Human Src was purchased from Upstate Biotechnology Inc. and was biotinylated with sulfo-NHS-LC-biotin (Pierce) according to the protocols that were provided by the manufacturer. The peptides were synthesized on TentaGel macrobeads (Rapp Polymere; 140 – 170 μm, 0.51 mmol g^{-1} capacity) by standard Fmoc chemistry on a Symphony peptide synthesizer (Protein Technology Incorporated). Biotin was attached to the TentaGel macrobeads by using Fmoc lysine (Advanced Chemtech) activated with *O*-benzotriazoyl-1-yl-*N*,*N*,*N*,*N*-tetramethyluronium hexafluorophosphate. On-bead sequencing was performed on a 476A ABI Protein sequencer. The fluorescence of the beads was recorded on a hyperspectral microscope constructed in the laboratory of Prof. Harold Garner (University of Texas Southwestern).[10] The on-bead fluorescence assays were visualized with a Nikon Eclipse TE300 fluorescence microscope equipped with a Chroma 61002 triple-band filter set and a CCD camera. MetaMorph software was used to acquire and process the photomicrographs.

Capture of a biotinylated protein on TentaGel beads and detection by using Streptavidin Quantum dots: Peptides immobilized on TentaGel beads (about 1 mg) were preblocked with 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS; 1 mL) for four hours at 4 °C. After excess liquid had been decanted, the beads were incubated with biotinylated protein (500 μL, 1 μM) in NaCl (1 M), BSA (1%), and Tween 20 (1%) at room temperature for 30 minutes. After washing with PBS at room temperature (five minutes, three times, 1 mL) the beads were incubated with Streptavidin-coated Qdots 605 (50 nM, 250 μL) in the blocking solution provided by the manufacturer at room temperature for 30 minutes. After excess liquid had been decanted, the beads were washed with PBS at room temperature (five minutes, three times, 1 mL). An aliquot was placed in a standard 96-well microtiter plate and analyzed under a fluorescence microscope.

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Keywords: combinatorial chemistry · protein capture · proteomics · quantum dots · on-bead assay

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