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An alternative to Western blot analysis using RNA aptamer-functionalized quantum dots

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ABSTRACT

To make full use both of optical properties of quantum dots (QDs) and of specific interactions between aptamers and their ligands of interest, we employed QD-conjugated RNA aptamer interactions with histidine tag. QDs offer revolutionary fluorescence performance due to their long-term photostability, brilliant colors, fixability, and narrow, symmetrical emission spectra, and aptamers are known to specifically bind to their target molecules, including metal ions, small molecules, and macromolecules. In this study, we have synthesized RNA aptamer-functionalized QDs, and demonstrated their application to specific protein detection, as an alternative to the conventional Western blot analysis. We observed that our RNA aptamer-functionalized QD system dramatically reduced the time and effort required for conventional Western blot analysis, whereas the selectivity was comparable to that of the conventionally available anti-histidine tag antibody and the sensitivity was comparable to that of the Coomassie blue staining method. In principle, owing to the remarkable optical properties of QDs and a wide versatility of aptamers for selection, our system can harness the high brightness, stability and reusability to quantitatively detect aptamer-recognizable proteins. Furthermore, multiplex detection for several proteins on a single blot can be achieved by our new method, which thus may be able to facilitate and simplify the routinely used protein detection procedure, and make a variety of proteomics analysis possible.

While recent advances in bioanalytical sciences and bioengineering have led to the development of DNA chips,[1] miniaturized biosensors,[2] and microfluidic devices such as microelectromechanical systems or MEMS,[3] there are several conventional but indispensable methods that are still routinely used in biological research. Among them, Western blot analysis, also known as protein immunoblot, is an analytical technique used to detect and quantify specific proteins in a given sample of tissue homogenate or extract.[4] The most common and established procedures for Western blots involve SDS–PAGE to separate the proteins according to their size, a transfer to a membrane for immobilization and the use of two antibodies for their detection, which is the reason there is an initial requirement for the generation of a specific antibody that can recognize and specifically bind to the protein of interest. However, the production of a specific antibody is time-consuming and not a foolproof procedure. And the procedures usually use radioactively labeled secondary antibodies for sensitivity purposes.[5] Nonradioactive systems, such as luminescent systems that reach similar performance without the disadvantages of working with radioactivity, make use of an enzymatic reaction that amplifies the signal through a turnover of detectable substrates at the site of recognition,[5] but this strategy poses a significant caveat for quantitative purposes, since it involves formation of a ternary complex at the recognized site and an amplification step through an enzymatic reaction. Both processes contribute to a potential variance of the signals created by a specific amount of sample. Additionally, the linearity of the signal over an applicable range of sample amount is compromised especially by the amplification reaction.[6]

To avoid these potential problems, several recombinant protein tagging methods have been developed, enabling the detection of the protein of interest with a commercially available anti-tag-specific antibody. The most widely used tagging strategy is the histidine tag (His-tag), consisting of six consecutive His residues, which was initially developed for protein purification.[7] Recent advances in His-tag detection technology led to the generation of nickel–nitrilotriacetate (Ni–NTA) conjugates with either alkaline phosphatase or horseradish peroxidase that can selectively detect the His-tagged protein of interest without the need for a protein-specific antibody (e.g., anti-His antibody selector kit, Qiagen). Importantly, this method does not require secondary antibody treatment. However, although Ni–NTA conjugates reduce the time and effort involved, they still require a final enzymatic reaction for signal generation, which prompted us to employ quantum dots (QDs) as a direct signal probe.

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QDs are semiconducting nanoparticles, which can be prepared with interesting optical properties.[8] Of the several available types of QDs, CdSe/ZnS QDs have recently emerged as a useful and alternative material for biomolecular labeling due to their unique optical advantages, including photostability, narrow emission bands, broad excitation spectra, and accessibility to versatile functionalization that allows site-specific targeting.[8] These fluorescent properties of QDs are one of the key advantages for their use in Western blot applications over currently used detection technology,[9] since QDs absorb light over a broad spectral range and fluoresce at wavelengths determined by their physical sizes, producing precise and narrow spectral emissions, and QDs are extremely efficient at absorbing light and converting it to a highly stable fluorescent emission, making them up to approximately 50 times brighter than conventional organic fluorophores.[8] This high level of brightness and excellent photostability bring the added value of high sensitivity and ruggedness. Moreover, multiple functionality can be introduced to exploit the binding cooperativity.[10] Recently, antibody-conjugated QDs have been successfully used in Western blots to detect the tracer proteins.[11]

In addition to QDs, we made use of RNA-based aptamers in the present study, instead of Ni–NTA (anti-His antibody selector kit, Qiagen) or antibodies[11] for specific His-tagged detection. Aptamers are known to be a special class of nucleic acids that can specifically bind, with high affinity, to a target molecule.[12] Emerging as alternatives to antibodies, a wide range of aptamers have been found to bind specifically to proteins.[12] Thus, they have been used in many bioanalytical applications, such as for specific detection of proteins,[12,13] metal ions,[14] and small molecules,[15] and for target-specific delivery.[16] The most popular techniques for signal production caused by aptamer-biomolecule interactions use electrochemical- and optical-detection platforms. These usually have sensitivities that allow detection of micromolar to nanomolar concentrations. In the previous studies, DNA-based aptamer-functionalized gold nanoparticles and QDs have been used for the detection of thrombin and adenosine.[12,13,15] Aptamer-functionalized nanoparticles were synthesized by direct chemisorptions of thiolated oligonucleotides, by biotin-streptavidin interaction, or by simple adsorption.[12–14]

Among the wide variety of potential biofunctional groups, we used the highly selective interaction of an RNA aptamer complex with oligohistidine (K_d ~3.78 pM).[17] The affinity between RNA aptamer and oligohistidine has been known to be comparable or superior to that of protein-antibody (K_d ~10^{-5}–10^{-12} M).[18] Therefore, the use of such an interaction could provide a simple, selective and sensitive method that would overcome the limitation of conventional antibody-based protein labeling and also could be applied across a wide range of applications. And since both RNA aptamer and QDs have many benefits separately, it was expected that by combining aptamers and QDs, a simple, time-saving, and sensitive methodological technique for Western blot analysis to detect His-tagged proteins would be developed. Due to the extensive use of His-tagged proteins in biological research, a simple Western blot system would be a valuable alternative for the detection of recombinant proteins.

To accomplish this task, we first generated RNA aptamer conjugated QDs. Amino-modified QDs were conjugated to thiol-containing RNA aptamers using sulfo-SMCC cross-linker (Fig. 1) according to the literature.[19] In brief, QDs, Qdot® 655 ITK™ amino (PEG) quantum dots (Invitrogen, CA) were resuspended in 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.2. Cross-linker (100-fold excess) was added to QDs and allowed to react for 1 h. Samples were filtered on a NAP-5 gravity column (to remove excess cross-linker) into similar buffer supplemented with 10 mM EDTA. 5′-Thiol-containing RNA aptamer[19] was added to filtered QDs and allowed to react overnight at 4 °C. Using three Amicon filters, product was filtered twice with Dulbecco's phosphate-buffered saline (PBS), twice with a high salt buffer (1.0 M sodium chloride, 100 mM sodium citrate, pH 7.2), and twice again with PBS. High salt washes were required to remove electrostatically bound RNA, which was not removed with PBS washes alone. The prepared QDs were stable and maintained strong fluorescence in depc-treated deionized water.

To check the specificity and sensitivity of QDs against His-tagged proteins, we used partially purified His-tagged thioredoxin in fusion with the recombinant human Fas-associated factor 1 (FAF1) UBX domain (residue 571–650),[20] which is known to bind to the N domain of p97/VCP, a multifunctional AAA^+-family ATPase that is involved in a variety of cellular processes, such as nuclear envelope reconstruction, the cell cycle, post-mitotic Golgi reassembly, suppression of apoptosis.[21] The His-tagged protein sample was overproduced and partially purified through the same experimental procedures as described in the previous study.[20]

Total partially purified protein concentration was measured using Bradford solution, and then the indicated amounts of sam-

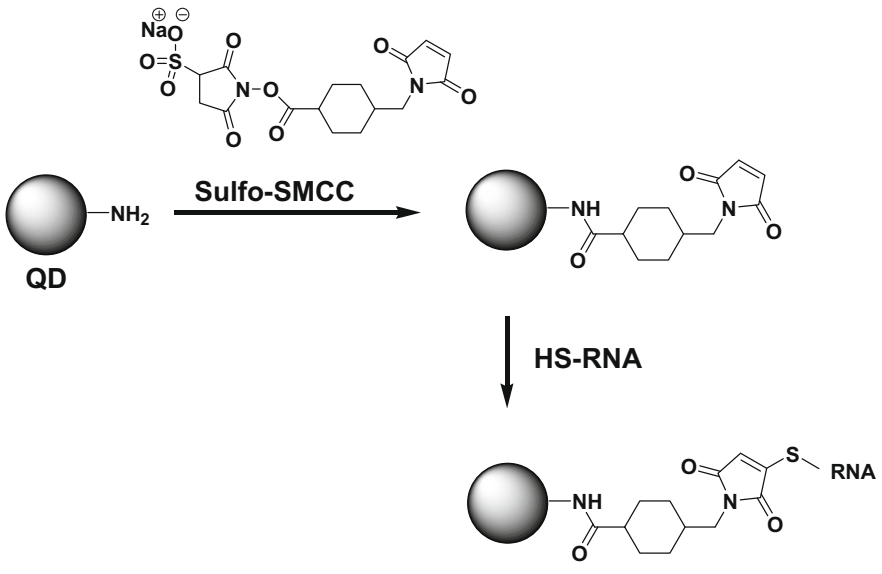

Figure 1. Scheme of RNA aptamer conjugation on quantum dots (QDs).

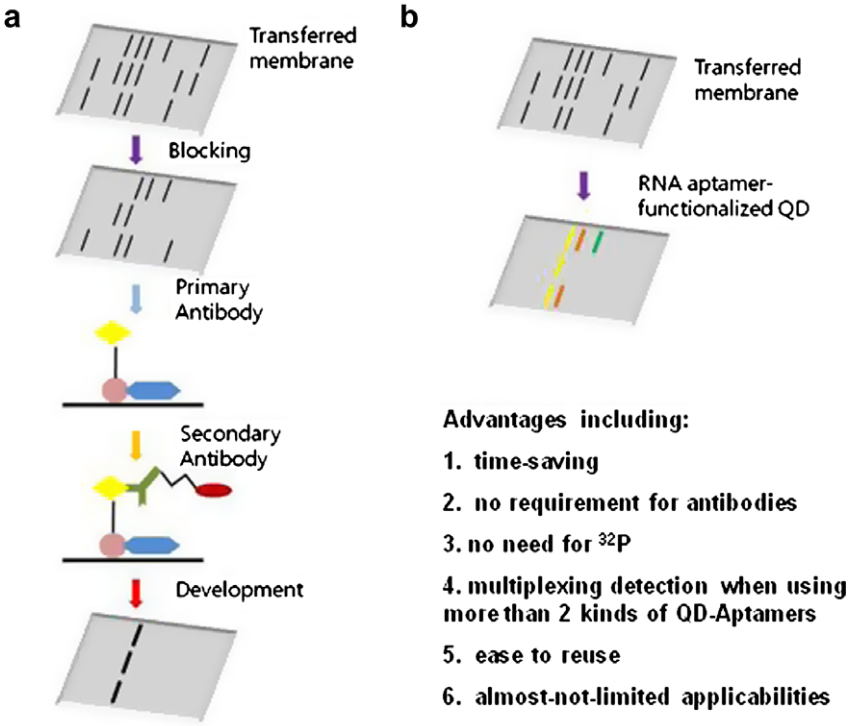

Figure 2. Procedure comparison of our method (right) to the conventional Western blot analysis (left). Possible advantages of our method over the conventional Western blots are listed.

ples were loaded onto a 15% SDS–PAGE gel (see Fig. 2 for procedure comparison of our QD-based Western blotting system to the conventional Western blot analysis). The proteins and molecular weight markers (Fermentas, Ontario) were separated according to size, followed by transfer to a Protran nitrocellulose membrane (Whatman, NJ), using a Mini Trans-Blot Cell electroblotter (Bio-Rad, CA). Subsequently, the membrane was incubated in Ponceau S solution for 5 min at room temperature. The membrane was rinsed three times with 0.1% phosphate-buffered saline Tween-20 (PBST) and incubated with RNA aptamer conjugated QDs containing 0.1% PBST for 2 h at room temperature. After incubation, the membrane was rinsed three times with depc-treated deionized water. Images were captured using a BIO-RAD ChemiDoc XRS system with UV transillumination mode.

Although reagents and gel imaging system need to be further optimized in future works for higher sensitivity and lower background signal, our QD system could successfully detect only the His-tagged protein from the partially purified protein mixture as low as 375 ng with 2-h incubation time, and the obtained signals were by large linear over a range of 0.375 μg to 3.75 μg (Fig. 3). However, with the same incubation time, the blot that had been probed with the anti-histidine antibody did not generate any visible signal. Detectable bands appeared only on the blot with overnight incubation (data not shown). This clearly demonstrates that our QD-based Western blotting system exhibited an even better signal with similar linearity as Coomasie blue staining method, although more sophisticated image acquisition and analysis methods are likely to improve the results in the present study. In theory, QD-based Western blot imaging is flexible and can be done at several levels of sophistication. At a minimum, for example, a handheld blue or ultraviolet light source and a high-quality color digital camera can be used to illuminate the blot and capture images of the fluorescent protein bands. With a gel imaging or documentation workstation, detection varies with the illumination source and the installed filters on that particular model of the instrument, since most gel imagers have a source sufficient to excite QD conjugates, such as an epi-UV illuminator. In addition, we found that labeled blots could be stored in a buffer at 4 °C with minimal loss of signal for imaging at a later date (data not shown).

The minimum amount of protein that can be detected with our method is thought to be a function of aptamer affinity and the source of background fluorescence in the image, such as the nonspecific binding of the given aptamer and the auto-fluorescence of the blotting membrane, considering several protein-antibody

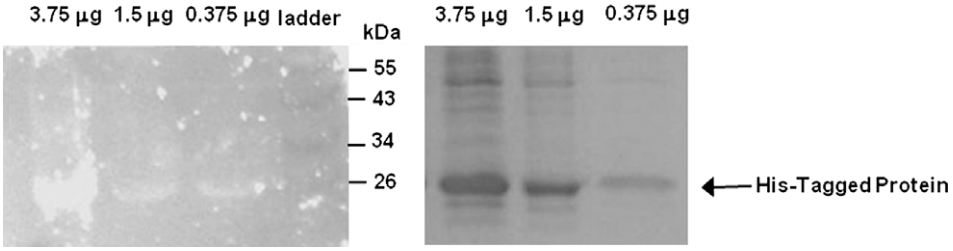

Figure 3. Results of QD-based Western blot analysis for His-tagged recombinant protein detection. At right, a result from the commercially available Coomassie blue staining (BIO-RAD, CA) is shown in comparison with our QD system.

combinations in the literature that have shown sensitivities to the low pictogram level with a 100-fold linear concentration range.

The benefits of QDs in biological applications are being appreciated more than ever and hence, scientists are introducing QDs in biological applications. However, to date there have been no reports on the direct use of RNA aptamer-conjugated QDs, by which antibody binding or enzymatic reaction steps can be excluded, in a Western blotting application. Our RNA aptamer conjugated QDs Western blotting system dramatically reduced the number of steps required, as well as the time and cost involved, compared with the traditional system. In addition, the specificity and signal strength were similar to or better than those with the traditional methods. Moreover, we obtained a linear signal over a range of 0.375 μg to 3.75 μg of His-tagged proteins making quantitative analysis of Western blots easier and more reliable. It should be also noted that in principle, owing to the optical properties of QDs and a wide versatility of aptamers for selection, our system can harness the high brightness, extreme stability and reusability to quantitatively detect aptamer-recognizable proteins, and due to the versatility of RNA aptamers, this method can be applied to any protein, nucleic acids, or even small molecules and metal ions. Furthermore, sensitive multiplex detection for several proteins on a single blot can be achieved by our new method, which thus may be able to facilitate and simplify the routinely used protein detection procedure, and make a variety of proteomics analysis possible, including analyses of differential expression and post-translational modifications such as glycosylation and phosphorylation or dephosphorylation activity.

In summary, for the first time to our best knowledge we employed QD-conjugated RNA aptamer interactions with His-tag, leading to development of an alternative to the conventional Western blots. Our present study brings the power of QD fluorescence technology to a work-horse application in proteomics. The advantages of high sensitivity, multiplex labeling, photo- and chemical stability and the availability of many compatible gel imaging platforms may accelerate information flow in proteomics.

Acknowledgments

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