Quantum Dot Self-Assembly for Protein Detection with Sub-Picomolar Sensitivity

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Received December 31, 2007

A novel approach to sensitive and rapid antigen detection is described. In the presence of a specific antigen, quantum dot—antibody conjugates rapidly self-assemble into agglomerates that are typically more than 1 order of magnitude larger than their individual components. The size distribution of the agglomerated colloids depends on, among other things, the relative concentration of quantum dot conjugates and antigen molecules. Quantum dot agglomerates mediated by antigen recognition were characterized by measuring their light scattering and fluorescence characteristics in an unmodified flow cytometer. Protein antigens angiopoietin-2 and mouse IgG were detected to sub-picomolar concentrations using this method. This simple technique enables the potential simultaneous detection of multiple antigenic biomarkers directly from physiological media and could be used for early detection and frequent screening of cancers and other diseases.

Introduction

The growing proteomic understanding of disease processes can be a powerful tool for diagnosing, prognosing, and monitoring cancers and other medical conditions. An assay that detects multiple specific molecules from the complex mixture present in serum, and is rapid, sensitive, and simple to administer, would be ideal for such an application. At present, this is a practical, clinical challenge. Antibody-based recognition remains one of the most promising strategies for such applications, but conventional approaches such as parallelized enzyme linked immunosorbent assay (ELISA), gel electrophoresis, and protein microarrays are suboptimal for point-of-care molecular profiling on the basis of cost, complexity, and speed.

Detection of pathogens by molecular recognition-based self-assembly of microspheres was proposed as far back as 1956 in the form of the semiquantitative latex agglutination test,1 which is still used for detecting bacteria.2–4 Nanoparticle-based agglutination tests utilizing surface plasma resonance (SPR) shift have been recently demonstrated for the detection of DNA fragments5 and proteins.6 Biomolecules have also been detected using fluorescent semiconductor nanoparticles (quantum dots) by either spatial7 or temporal8 coincidence of multicolored quantum dot—antibody conjugates. Flow cytometric microsphere-based immunoassays (FMBA) has been proposed for the simultaneous detection of multiple proteins.9,10 FMBA assays are essentially antigen capture sandwich immunoassays on microspheres, and they use microsphere size and/or fluorescence intensity as discriminating characteristics for multiplexing. Compared to these techniques, antigen mediated quantum dot agglomeration combined with flow-based detection on a microfluidic device has the potential to offer better sensitivity, ease of use, speed, and cost of testing.

We demonstrate here this novel method for sensitive, simple, and rapid fluid phase detection of proteins based on simultaneous measurement of multiple properties of the colloidal mixture. Quantum dots (QDs) are conjugated with polyclonal antibodies (Ab) using a streptavidin—biotin interaction.11–13 In the presence of the appropriate antigen molecules, these QD—Ab conjugates rapidly self-assemble into colloidal structures with sizes that are 1–2 orders of magnitude larger than the constituents (Figure 1). The size, structure, and fluorescence characteristics of these self-assembled structures are a function of the relative concentrations of the QD—Ab conjugates and the antigen molecules, among other factors. These attributes of the colloidal structures can be characterized by several techniques, including flow cytometry, dynamic light scattering, and electrical sensing zone or Coulter counter method. Flow cytometry is a powerful technique routinely used in biomedical laboratories for rapidly assessing multiple characteristics of a large population of cells and other microparticulates suspended in a hydrodynamically focused fluid stream.14 The use of flow cytometers is common in clinical medicine, and the protein detection strategy described here could be adopted into clinical practice relatively easily. The basic principle of flow cytometric analysis can also be implemented on a microfluidic chip,15,16 implying the ability to create a compact point-of-care diagnostic or field deployable analyte detection
individual events and detailed multiparametric mapping of the whole population. The detection of extremely rare events is thus possible, enabling detection of molecular analytes with high sensitivity and resolution. Furthermore, since flow cytometry is capable of detecting multiple fluorophores, simultaneous QD-based detection of multiple analytes could be achieved using different QD populations targeted for detection of different antigens.

The self-assembled quantum dot structures generated in this study were detected based on their fluorescence intensity. The forward light scatter, side light scatter, and fluorescence emission at multiple wavelengths were recorded for each particle detected. The fraction of all events above a size threshold corresponding to 0.5 µm latex size standard spheres was proportional to the antigen concentration. One antigen used in these experiments was human angiopoietin-2 (ang-2), a 66 kDa protein involved in neo-angiogenesis.\textsuperscript{17,18} Due to the importance of neo-angiogenesis in the proliferation of various cancers, ang-2 is a protein of interest as a cancer marker and therapy target\textsuperscript{19,20} and has also been shown to correlate with the invasiveness and growth of various cancers.\textsuperscript{17,21–23} Detection of mouse IgG (mus) is also demonstrated using the same method.

The antigens in this study were detected to sub-picomolar concentration, comparable to the detection of the same molecules by conventional techniques such as ELISA or Western blot. Thus, the agglomeration-based detection strategy is already comparable to conventional immunorecognition techniques in terms of sensitivity. The flow-based detection approach also enables characterization of multiple properties of individual self-assembled structures, thus providing a tool for investigating self-assembly in a novel and powerful manner, which may lead to insights into this important nanoscale phenomenon.

**Experimental Section**

Streptavidin-coated quantum dots with 705 nm (#Q10161MP), 585 nm (#Q10111MP), and 525 nm (#Q10141MP) emission wavelengths were purchased from Invitrogen (Carlsbad, CA) and used as received for flow cytometry, bulk agglomeration fluorescence, and dynamic light scattering experiments, respectively. Biotin conjugated anti-angiopoietin-2 polyclonal antibody (AA2) (#BFA623) and recombinant human angiopoietin-2 (ang-2) (#623-AN-025) were purchased from R&amp;D Systems (Minneapolis, MN) and reconstituted in Tris-buffered saline (TBS) containing 0.1% bovine serum albumin (BSA). Mouse IgG (mus) (#23873), human IgG (hum) (#23872), and rabbit IgG (rab) (#23874) were purchased from Polysciences, Inc. (Warrington, PA) and reconstituted in 1x phosphate-buffered saline (PBS). All reconstituted samples were aliquoted and stored at −20 °C. The aliquots were thawed and diluted to appropriate concentration using PBS with 0.1% BSA (PBS-BSA) immediately prior to use. Biotin conjugated anti-mouse IgG (GaM) (#553999) was purchased from BD Biosciences (San Jose, CA) and stored at 4 °C. All other chemicals used were ACS reagent grade. Borate buffer (10 mM) was used for Zetasizer and bulk fluorescence measurement experiments. Buffers were prepared in deionized water and filtered through a 0.2 µm filter prior to use.

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Flow cytometric measurements were carried out on a Beckton Dickinson (BD) FACSCalibur instrument. BD FACSaria and BD LSR II flow cytometers were also used for optimizing detection parameters. Bulk fluorescence was measured in a BioTek (Winooski, VT) Synergy HT multidetection microplate reader. Dynamic light scatter (DLS) measurements were carried out on a Malvern (Malvern, U.K.) Zetasizer Nano ZS instrument. Fluorescence measurements were carried out in a Nanopod Technologies (Wilmington, DE) ND-3300 fluorospectrometer.

Quantum Dot–Antibody Conjugation. The quantum dot–streptavidin conjugates (QD) and biotinylated anti-angiopoietin-2 polyclonal antibody (AA2) or biotinylated goat-anti-mouse polyclonal antibody (GaM) were mixed in PBS-BSA at a QD/antibody molar ratio of 1:3 and 1 nM QD concentration. The conjugation was monitored by particle size estimation in the reaction mixture by DLS. The conjugate was diluted to appropriate concentrations and used immediately after synthesis.

Antigen Induced Self-Assembly. The QD–antibody (QD–Ab) conjugate suspension and the antigen or control solution at the appropriate dilutions and volumes were added to PBS-BSA for a total volume of 1 mL. BSA, similar to ang-2 in terms of molecular weight, also acted as a negative control for ang-2. Rab and hum were used as negative controls for mus. The reaction mixtures were incubated at room temperature for 60 min and then analyzed by flow cytometry. The baseline event distribution of QD–Ab dispersed in PBS-BSA was also analyzed.

Results and Discussion

The candidate cancer biomarker protein, ang-2, was detected by flow cytometry to 0.5 pM concentration. Event classification between aggregates and individual particles was based on the observed bimodal population distribution. The forward scatter histogram (Figure 3b) suggests that a forward light scatter intensity of 10 au is a suitable threshold value for this demarcation. The optimal side scatter threshold value of 10 au was similarly identified. The fraction of events classified as aggregates was 10.1 ± 2.2%, compared to the background control aggregate formation of 1.2 ± 0.2%. The relationship between aggregate formation and concentration of ang-2 ([ang-2]) followed a log-linear correlation over the range of antigen concentrations from 0.5 to 100 pM when detected with 10 pM QD-AA2 and from 500 to 50 000 pM when detected with 100 pM QD-AA2 (Figure 2). The slopes of these relationship enabled resolution of [ang-2] between 0.5 and 50 000 pM.

The maximum detected [ang-2] was 50 000 pM, limited primarily by the concentration of ang-2 stock available. Literature reports of [ang-2] detected in serum range from 23 to 44 pM, which is within the high-resolution range of measurement with this QD agglomeration technique. Similarly, serum concentrations of other biomarkers are in the multi-picomolar range. Hence, exceeding the sensitivity of ELISA significantly is not an objective of the work presented, but it is being pursued separately.

Mus, which was used as a model protein in the initial experiments to optimize the instrument detection parameters and experimental conditions, was also detected by flow cytometry to 0.5 pM concentration. The fraction of events classified as aggregates was 1.0 ± 0.3%, compared to the negative control aggregate formation of 0.7 ± 0.1%. Two different log-linear regimes were observed for aggregate formation, in a manner similar to that documented for ang-2. A concentration of QD-GaM was used to detect mus from 0.5 to 500 pM. A concentration of 100 pM QD-GaM was used to detect mus from 500 to 500 000 pM. The slope of these relationships effectively enabled resolution of [mus] between 0.5 and 500 000 pM.

The use of multiple reactions to detect a large range of analyte concentrations is comparable to other antibody-based techniques, where separate reactions with optimized protocols and stoichiometry are often required to detect a low concentration and high concentration of the same analyte. The incubation time for these experiments was optimized in preliminary studies. Initial experiments were carried out to characterize the kinetics of agglomeration by quantifying the fraction of all events indicating large agglomerates at several time points up to 4 h. A 1 h incubation period was determined to be optimal based on rapid initial agglomeration leading to suitably high detection sensitivity.

Figure 2. Ang-2 was detected down to 0.5 pM using the QD agglomeration technique. The percent of total events detected that were categorized as agglomerates (Y axis) is a log-linear function of the antigen concentration (X axis). Since the number of agglomerates in the two component reaction is limited by the availability of either or both of the components, the function is linear over a limited range. Hence, the agglomeration behavior of the lower concentration range of Ang-2 (0.5–100 pM) was linear when detected with 10 pM QD–AA2, while the higher concentration range of ang-2 (500–50 000 pM) exhibited a log-linear agglomeration behavior with 100 pM QD–AA2. Data points are mean ± standard deviation, n = 3.

in this time period. Since a differential signal is obtained with identical reaction conditions for all samples, this detection protocol is suitable for quantitative detection of antigens.

These results indicate that the sensitivity of the QD agglomeration technique for ang-2 and mus is comparable to the limit using ELISA and Western blot.\(^\text{(25,26)}\) Mass spectroscopy, which is currently used for recognition of protein expression patterns, detects protein from clinical samples only semiquantitatively,\(^\text{27}\) and it is hence not directly comparable with the QD agglomeration technique. Furthermore, the sensitivity for target analytes in mass spectrometry is also highly variable with respect to the molecular mass of the analyte, relative abundance compared to the overall sample, and other factors. The clinical utility of any biomarker detection method depends on both sensitivity and selectivity, especially in complex mixtures such as serum. Positive results can be obtained when data are statistically different compared to the natural population variation in biomarker concentration, or by establishing personalized baseline values for significant biomarkers. The use of multiple biomarkers correlated with a single physiological state also increases the predictive power of biomarkers significantly, compared to that of a single biomarker.\(^\text{(28-31)}\) The suboptimal performance of ELISA, Western Blot, two-dimensional gel electrophoresis, and mass spectrometry. These advantages suggest the possibility of further developing this method for sensitive, rapid, and economical point-of-care proteomic diagnostics. The possibility of harnessing other types of intermolecular recognition reactions such as DNA–DNA, ligand–receptor, and aptamer–target suggests wider utility of this method, and it is currently under investigation. Since the QD–Ab construct is modular, other types of nanoparticles may also be used in place of QDs and could enable alternate detection methods.

Polyvalent molecular recognition interactions between the reactants are required for the formation of self-assembled structures in this manner. If only monoclonal antibodies were used, the QD–Ab–antigen structures formed would be similar in size to the constituents and thus harder to detect. To achieve polyvalent interactions, we have utilized quantum dots conjugated with polyclonal antibodies. However, monoclonal antibodies are also likely to be useful if the target antigen presents multiple copies of the epitope on its surface. While quantum dots with a single emission wavelength were used in these experiments to detect a single protein, preliminary evidence suggests that multiplexed protein detection using multiple quantum dot populations is also feasible.

The studies reported here were conducted in samples of controlled and well-known composition. Biomarker detection capabilities in complex mixtures such as blood plasma are unknown. The performance of this approach for sensing biomarkers in clinical samples may be partially inferred from the control data and from the well-known characteristics of immunorecognition methods such as ELISA which has a number of commonalities with the QD agglomeration method. Additional studies are required to characterize biomarker detection in serum.
using functionalized QDs, and these are under way but are not part of this work.

The percentage of self-assembled agglomerates in a colloidal mixture can presumably be determined by flow cytometry using a variety of parametric combinations. We have utilized a combination of forward light scatter threshold and side light scatter threshold to demarcate agglomerates from smaller particles. The fraction of total events corresponding to the agglomerated subpopulation serves as a metric correlated with antigen concentration. An example of the significant difference in the approximate size distribution of QD agglomerates mediated by ang-2 antigen in comparison with the BSA control appears as panels b and e, respectively, in Figure 3. Forward light scatter intensity (FSC) is an approximate surrogate that is positively correlated with event diameter, suggesting that the addition of ang-2 mediates the formation of many aggregates significantly larger in diameter than can be triggered by the BSA control antigen. The correlation between forward light scatter and event size for this instrument is identified in panels a and d in Figure 3 as the gated regions R1, R2, R3, and R4, which correspond to latex calibration sphere diameters of 0.2, 0.5, 1.0, and 2.0 μm, respectively. Events of these sizes are significantly larger than the diameter of antibody-functionalized QDs (Figure 5, approximately 0.045 μm or 45 nm). Quadrant gating in the forward light scatter and side light scatter (SSC) space highlights events with diameters greater than approximately 0.5 μm (500 nm).

The events in the upper right quadrant are highlighted in red and are defined to be QD agglomerates in this method. This gating also corresponds to the bimodal population distribution in the aggregated sample, as seen from the FSC histogram (Figure 3b). The addition of 10 pM ang-2 resulted in an agglomerate subpopulation of 44% (Figure 3a), significantly greater than the 1.2% mediated by addition of the control BSA antigen (Figure 3d). The agglomerates identified by forward light scatter intensity are also fluorescent in the FL3 wavelength range (650 nm and longer), consistent with the fluorescence of QDs with emission maxima of 705 nm (Figure 3c). The fluorescence emission intensity of QD agglomerates is not a direct summation of the fluorescence emission intensities of each of the estimated 2500 QDs per agglomerate, suggesting that modulation of the excitation and/or fluorescence emission occurs in these agglomerates. Based on the expected structure of the agglomerates, the QDs are likely to be separated by about 20 nm. Hence, the cause of the quenching is unlikely to be based on fluorescence resonant energy transfer. However, the formation of a network of QDs may reduce the intrinsic fluorescence quantum yield of each QD through modulation of the nanoscale surface characteristics. The large size and complex structure of the agglomerates may shield many of the QDs from the excitation light source, as well as trap emitted radiation, and hence, part of the reason for the observed fluorescence quenching could be optical screening. While the exact mechanism of the fluorescence quenching is unclear, quenching upon agglomeration was confirmed separately by measuring the bulk fluorescence intensity (Figure 4) during the agglomeration reaction. The bulk fluorescence of reaction mixtures with different [mus] was observed over time in a plate reader. The fluorescence intensity of the mixture decreased with time for [mus] = 666 nM, the highest concentration of antigen used, while the reaction mixture with the negative control [hum] = 666 nM did not exhibit decreasing bulk fluorescence intensity.

The graphical representation of the flow cytometric data (Figure 3) also suggests that further improvement in the detection sensitivity may be achievable by fine-tuning the threshold used here to demarcate agglomerates from nonagglomerates. Furthermore, while measuring the fraction of particles larger than a threshold is a simple way of quantifying agglomeration, it does not take into account the full complexity of the self-assembly phenomenon. Detection sensitivity and resolution may also be improved by creating sophisticated data processing algorithms that correlate antigen concentration with other measurable parameters such as agglomerate size distribution, population distribution in the light scatter versus fluorescence intensity space, and kinetic measurement of the agglomeration. Using flow cytometry, these parameters can be easily measured for each of the particles. This may enable sophisticated and quantitative

![Figure 4](Image)

Figure 4. The suspension fluorescence intensity of 1 nM QD–GaM suspension decreases after addition of mus, consistent with aggregation-modulated fluorescence quenching observed by flow cytometry. The largest decrease is seen in the sample containing the highest concentration of mus. Flow cytometric characterization of the same sample showed a very high fraction of agglomerates. Optical screening of excitation and emission due to the large size and complex structures of the agglomerates as well as modulation of nanoscale surface properties upon agglomeration are thought to be responsible for the observed fluorescence quenching. Data from one experiment, typical of n = 3.

The FL3–FSC representation (Figure 3c and f) provides an example of how the multiparametric data obtained from the flow cytometer enable sophisticated analysis of the sample and may increase the signal-to-noise ratio and sensitivity of detection. In this instance, two different populations of particles appear in the upper-right quadrant of the FSC–SSC space (Figure 3a) but cannot be distinguished from each other. However, in the forward scatter–fluorescence space (Figure 3c), the nonspecific agglomerates can be easily separated from the antigen mediated agglomerates. Most QD–AA2–ang-2 agglomerates have high forward scatter and low fluorescence intensity (Figure 3c). While the volume of these agglomerates is about 250-fold greater than the individual QD–AA2 agglomerates, the fluorescence intensity is only 3-fold greater. A very small fraction of particles (less than 0.1%) in this agglomerated sample show high FSC as well as high FL3 intensities. These anomalous events are likely due to electronic noise as well as nonspecific agglomeration between QD–AA2 conjugates. While these two populations appear in the same region on the FSC–SSC plot (Figure 3a), they can be easily distinguished from each other in the FL3–FSC representation (Figure 3c). In samples where a higher concentration of the QD–Ab conjugate is used, the number of these anomalous events is even larger. Combined with the smaller overall fraction of the agglomerate population in these samples, the increased utility of the multiparametric characterization to increase signal-to-noise ratios and detection sensitivity is apparent.

![Graph](Image)
characterization of the agglomeration and hence sophisticated, automated, and simultaneous detection of multiple antigens.

Other parameters including antigen—antibody binding energies, fluid mixing energy and duration, and presence of nonspecific reactants are also important to the characteristics of the agglomerates formed. In this work, the time and relative concentration of QDs and antigen were optimized based on measured aggregative formation, but the optimization of the reaction with respect to the other parameters is ongoing. The current data represent a system in equilibrium, but the final state may not provide optimum biomarker detection sensitivity. The kinetics of QD aggregate formation are presumably influenced in a multivariate way that is currently under investigation. Temporal differences in QD aggregate formation mediated by specific and nonspecific interactions are expected based on the range of immunorecognition kinetic parameters. The translation of a traditional kinetic ELISA approach to the present system is particularly elegant, since each QD aggregate event is time-stamped during flow cytometric detection. In this way, additional discrimination between QD aggregation mediated by specific biomarker and nonspecific interactions may be achieved, improving the sensitivity and selectivity of the proposed method. Parameters such as temperature, pH, and ion concentration are also expected to have some effect on the agglomeration behavior. The characterization of the effects of these parameters is currently under way; however, the current reaction media were chosen to closely approximate the properties expected from a physiological sample such as serum.

In addition, the flow cytometric detection strategy influences the sensitivity of QD aggregate detection; optimization of triggering and amplification settings has been performed to obtain the results presented here, but additional improvements are likely with continued refinement. Preliminary investigation indicates that increased laser power, shorter excitation laser wavelengths, and better detection optics may provide significantly improved biomarker detection sensitivity. Improved understanding of QD aggregate detection by flow cytometry, a nonlinear optical process, may yield new approaches to improved biomarker detection performance through adjustment of instrument settings.

The AA2-ang-2 and GaM-mus systems were both characterized by flow cytometry as well as DLS. Particle size and aggregation data measured by both modalities correlate well within each system. The data from flow cytometric characterization of the AA2-ang-2 system and DLS characterization of the GaM-mus system are shown in the interest of brevity. Preparation of the QD-Ab conjugates was monitored using a DLS particle sizer. Successful preparation of the QD-GaM conjugates was indicated by a shift in particle diameter from 25 nm representing the unmodified QDs to 45 nm indicative of the formation of QD-GaM conjugates (Figure 5). This shift was not observed when QDs were treated with the same volume of TBS-BSA. A similar change in particle size distribution was observed upon conjugation of AA2 and QDs. DLS measurements were also used to verify agglomeration in the presence of the antigen. The addition of mus to the QD-GaM suspension resulted in the formation of a new peak at 2000 nm, indicating the formation of large agglomerates. The agglomerates are up to 2 orders of magnitude larger than the individual constituents, as verified also by other methods including flow cytometry and the electrical sensing zone method (data not shown). When the negative control antigen (hum) was added, this large peak was not observed in the DLS data, verifying the hypothesis that agglomeration occurs primarily by specific antibody—antigen recognition rather than by other nonspecific interactions.

In conclusion, we have demonstrated a novel antigen detection technique based on fundamental nanoscale phenomena. This technique has several advantages over conventional antigen detection strategies due to the use of solution-phase biofunctionalized quantum dots and a microfluidics-based detection strategy. Sensitive detection was carried out completely in the fluid phase, in a single step, and with minimal incubation. In this work, we have separately detected two different proteins with high sensitivity and specificity. Simultaneous detection of multiple proteins from a complex mixture such as a serum is currently being investigated. The cross reactivity of antibodies as well as nonspecific reactions both lead to agglomeration and may limit sensitivity in these scenarios. These limits may be overcome through the use of sample processing techniques from conventional immunorecognition methods as well as sophisticated methods for acquisition and processing of the data, including kinetic measurements, which is not possible with other detection methods. Our technique requires minimal sample volume, is amenable to multiplexing, automation, and implementation in a microfluidic chip, and is completely modular, making it an ideal candidate as a platform technology for frequent and low cost proteomic testing and monitoring of cancers, as well as an advanced point-of-care diagnostic based on a diverse array of intermolecular recognition-based biomarker sensing.

Acknowledgment. We acknowledge assistance with flow cytometric detection optimization by J. Higginbotham. Figure 1 was prepared by D. Dorset.

Supporting Information Available: Animation of QD agglomeration (avi format). This material is available free of charge via the Internet at http://pubs.acs.org.

LA704078U