Sensitive and multiplexed detection of proteomic antigens via quantum dot aggregation

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Abstract

A rapid, single-step, solution-phase method for quantifying multiple proteomic biomarkers is described. Nanoscale quantum dot–antibody conjugates self-assemble into microscale aggregates in the presence of a specific antigen through antibody-antigen molecular recognition. These aggregates are easily discriminated from the individual components by flow cytometry. Quantum dot (QD) aggregates can be quantified and correlated to the antigen concentration. Two QD populations with distinct emission spectra are used for detecting two proteomics antigens in a single reaction volume. Multiplexed detection of vascular endothelial growth factor A and angiopoietin-2 is demonstrated at the physiologically relevant, picomolar concentration range. Nonmultiplexed detection of the antigens is also demonstrated, with a femtomolar sensitivity limit. This technique may be optimized for low-cost early detection and frequent screening of cancers and other diseases as well as detection of the biological response to therapy.

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Research in functional genomics and proteomics is leading to the discovery and clinical validation of a large number of molecular biomarkers. These markers are thought to be associated with specific diseases, variants, and disease progression states. Biomarker-based understanding of many pathologies is poised to markedly improve patient outcomes for many major diseases including cancers, Alzheimer’s disease, vascular pathologies, and others. It is expected that techniques based on molecular biomarkers will be used for screening, early diagnosis, frequent monitoring, and personalized therapy management of diseases in the coming decades.

Conventional protein detection technologies that are currently used in research settings are not optimal for frequent diagnostic applications. These methods, such as mass spectrometry, enzyme-linked immunosorbent assays (ELISAs), two-dimensional polyacrylamide gel electrophoresis, and others, are used for identifying and validating proteomic biomarkers but fall short on one or more of the important parameters of speed, sensitivity, multiplexing capability, or production of quantitative data. Perhaps most important from the point of view of clinical utility, ease of implementation and cost are not favorable for these techniques because of the requirement for highly trained personnel and difficulties in automation.

A variety of nanoscale and microscale technologies have been proposed and demonstrated over the last few years for efficient and cost-effective detection of molecular biomarkers from biological samples. These techniques take advantage of a variety of nanostructures including microcantilevers\textsuperscript{1}; metallic, semiconductor, or polymer nanoparticles\textsuperscript{2}; carbon nanotubes,\textsuperscript{3} and more.\textsuperscript{4}

In a recent publication we have demonstrated a sensitive, rapid, single-step, solution-phase antigen detection technique based on nanoparticle self-assembly and flow cytometry.\textsuperscript{5} Quantum dots (QDs) were conjugated with polyclonal antibodies (Abs) using a streptavidin-biotin interaction.\textsuperscript{6} In the presence of the appropriate antigen molecules (mouse IgG and angiopoietin-2) these QD-Ab conjugates rapidly self-assembled into colloidal structures with sizes one to two orders of magnitude larger than the
constituents (QD-Ab conjugates and proteomic antigens). The proportion of these self-assembled structures to the total events counted by flow cytometry was shown to be a function of the relative concentrations of the QD-Ab conjugates and the antigen molecules, among other factors. The quantification of these microscale aggregates was easily achieved on a conventional flow cytometer. We also proposed that because of the multiparametric characterization capabilities of flow cytometers, it would be possible to detect multiple antigens from a single sample using QDs with distinct emissions spectra.

In the present article we extend our previous work to demonstrate multiplexed detection of two proteomic antigens. Angiopoietin-2 (Ang2) and vascular endothelial growth factor A (VEGF) were detected simultaneously in the 1- to 100-pM concentration range using two QD-Ab conjugates with distinct emissions spectra. The detection sensitivity limit for nonmultiplexed detection is also improved by an order of magnitude as compared with the previous work, to 50 fM. The detection of two distinct aggregate populations was achieved by optimizing flow-cytometric data acquisition parameters to minimize the baseline noise that occurs because of nonspecific antigen-Ab reactions as well as uncertainties in measuring QD aggregate fluorescence with the flow cytometer. It is expected that multiplexed antigen detection sensitivity can be improved to approach the individual antigen detection sensitivity by improving sample preparation and modifying data acquisition parameters.

Detection of pathogens by molecular recognition-based self-assembly was proposed as far back as 1956 in the form of the latex agglutination test,7 which is still used for detecting bacteria,8-10 and for determining blood types, but is unsuitable for sensitive detection of low-concentration small molecules. Nanoparticle-based variants of the agglutination test have been demonstrated for small molecules such as DNA fragments11 and proteins.12 In both these demonstrations the change in plasmon resonance due to self-assembly of the metal nanostructures was spectroscopically monitored. Small molecules have also been detected using QDs using Ab-mediated coincidence of multiple QDs with different emission wavelengths.13,14 Flow-cytometric microsphere-based immunoassay has been proposed for the simultaneous detection of multiple proteins.15,16 Flow-cytometric microsphere-based immunoassays are essentially antigen capture sandwich immunoassays on microspheres, and use microsphere size and/or fluorescence intensity as discriminating characteristics for multiplexing. Compared with these techniques, antigen-mediated QD agglomeration demonstrated here, combined with flow-based detection on a microfluidic device, has the potential to offer better sensitivity, ease of use, speed, and cost of testing.

The multiplexed detection of two candidate cancer biomarkers involved in angiogenesis is demonstrated in this article. It has been demonstrated that the use of biomarker panels—multiple biomarkers evaluated concurrently—results in a significantly improved diagnostic and prognostic performance as compared with the use of a single proteomic biomarker single discriminating protein.17-19 One of the biomarkers evaluated in this article is VEGF, a 21-kDa protein. VEGF is a strong angiogenetic factor, and elevated serum levels have been detected in melanoma, pituitary, colorectal, breast, and prostate cancers.20 In the control population, mean plasma VEGF concentration quoted in the literature is 1–9 pM, and in the presence of cancer the mean value increases to 15–24 pM, based on the type and stage of the cancer studied.20,21 The other biomarker evaluated in this article, Ang2, a 66-kDa protein, is involved in neo-angiogenesis.22,23 Because of the importance of neo-angiogenesis in the proliferation of various cancers, Ang2 is a protein of interest as cancer marker and therapy target.24,25 and has also been shown to correlate with the invasiveness and growth of various cancers.22,26 The normal and elevated concentrations in the plasma for Ang2 are also in the low picomolar range, comparable to those for VEGF.27

The molecular recognition-mediated self-assembly used in this technique is not directly affected by the choice of nanoparticle in the nanoparticle-antibody conjugate probe used. However, QDs present an ideal combination of properties that make them an excellent choice for this application. The unique fluorescence properties of QDs28-31 facilitate detection of aggregates as well as discrimination from other microparticulates that may be present in solution. Surface modification for biological applications, including conjugation with proteins and Abs,6,32,33 and multiplexed biological applications,34-36 have been well explored. Hence, QDs are widely recognized to be suitable for ex vivo diagnostic technology.7

Methods

Materials

Streptavidin-coated QDs with 705 nm (QD705, no. Q10161MP) and 585 nm (QD585, no. Q10111MP) fluorescence emissions were purchased from Invitrogen (Carlsbad, California) and used as received. Biotin-conjugated anti-Ang2 polyclonal antibody (aA2, no. BAF623), biotin-conjugated anti-VEGF polyclonal antibody (aVEGF, no. BAF293), recombinant human Ang2 (Ang2, no. 623-AN), and recombinant human VEGF (no. 293-VE) were purchased from R&D Systems (Minneapolis, Minnesota), reconstituted in Tris-buffered saline containing 0.1% bovine serum albumin (BSA), and stored at −20°C. Appropriate dilutions of all Abs and antigens were prepared in phosphate-buffered saline (PBS) with 0.1% BSA immediately before use. All buffers were filtered through 0.2-μm filters. All other reagents were ACS reagent grade. Deionized water with 18 MΩ resistance was used for preparing buffers. Flow-cytometric measurements were carried out on a Beckton Dickinson (BD Biosciences, San Jose, California) FACSCalibur.
Quantum dot–antibody conjugation

Using the optimized protocol described earlier, QD-streptavidin conjugates (QDs) and biotinylated anti-Ang2 polyclonal antibody (aA2) or anti-VEGF polyclonal antibody (aVEGF) were mixed in PBS-BSA at a QD/Ab molar ratio of 1:3 and 1 nM QD concentration. The conjugate was used as synthesized after incubation for 30 minutes at room temperature (18-23°C).

Antigen-induced self-assembly

The QD-antibody (QD-Ab) conjugate solution and the antigen or control solution at the appropriate dilutions and volumes were added to PBS-BSA for a 500-μL final volume. BSA, similar to Ang2 in terms of molecular weight, acted as a negative control for Ang2 and VEGF. The reaction mixtures were incubated at room temperature for 60 minutes and then analyzed by flow cytometry. Baseline event distribution of QD-Ab's dispersed in PBS-BSA was also analyzed. Nonmultiplexed detection was carried out by reacting 5 fM to 500 pM VEGF with 10 pM QD705-aVEGF conjugates, and 5 fM to 500 pM Ang2 with 10 pM aA2, both in 500-μL final volumes. Ten different concentration values of each antigen were examined, and three data sets of each antigen were acquired from three different experiments. For multiplexed detection the antigen mixture was prepared in PBS-BSA by sequentially adding appropriate volumes and dilutions of the VEGF and Ang2 stock solutions. The mixture of QD705-aVEGF and QD585-aA2 were added to these antigen solutions for a final concentration of 10 pM of each of the QD-Ab populations in 500-μL final volumes. Experiments with 5 fM to 500 pM VEGF concentrations with no Ang2 or 1 pM, 10 pM, or 100 pM Ang2 were carried out. The inherent variability of the QD-Ab dilution caused a large effect on the fraction of agglomerates observed. Hence, only the data sets with base QD-Ab count within 2000 ± 200 were used for analysis.

Flow-cytometric characterization

The size, fluorescence, and number of the aggregates in the incubated samples were characterized by flow cytometry. The basic flow-cytometric protocol followed was similar to the one used in our previous publication. Briefly, signal amplification for the flow cytometer parameters forward scatter (FSC) and side scatter (SSC) detectors and fluorescence detectors with 585 ± 21 nm (FL2) and 650 nm long-pass filters (FL3) were optimized for characterizing small particles. The FSC and SSC performance was calibrated by using 0.2-, 0.5-, 1-, 2-, and 2.8-μm latex calibration particles. In Figure 1, A, the regions indicated by R1, R2, R3, R4, and R7 correspond to the 0.2-, 0.5-, 1-, 2-, and 2.8-μm latex calibration particles, respectively. Aggregates were defined as particles with FSC greater than 10 a.u. This corresponds to a nominal size greater than approximately 0.5 μm, as well as the observed differentiation of the individual QD-Ab conjugates from the large aggregates inherent in the

Figure 1. The QD-Ab/antigen aggregates were characterized by flow cytometry. The panels show the raw data acquired from a multiplexed sample containing 10 pM Ang2 and 10 pM VEGF as well as the appropriate QD-Ab conjugates. (A) Optimized flow-cytometric detection parameters permit the resolution of individual QD-Ab conjugates and small aggregates (black dots, bottom left quadrant) from the micron-scale aggregates (red dots, upper right quadrant). The regions labeled R1, R2, R3, R4, and R7 indicate 0.2, 0.5, 1, 2, and 2.8 μm calibration particles, respectively. (B, C) FL versus FSC data representation of the events representing aggregates acquired with FL3 and FL2 triggers, respectively. The regions R8 and R9 demarcate areas on these charts corresponding to the aggregates (FSC > 10, as in A). The events in R8 and R9 are counted as single-antigen aggregates mediated only by VEGF and Ang2, respectively. The bottom edges of R8 and R9 are specified such that false positives are reduced but not more than 1% of the events in an equivalent nonmultiplexed experiment are counted as false negative.
agglomeration process and the measurement protocol. The data were acquired at low flow rate (12 ± 3 μL/min) for 1 minute, and either the FL2 or FL3 channels individually for nonmultiplexed detection or both sequentially for multiplexed detection were used as triggers to minimize noise from nonfluorescent microparticles in the reaction mixture. In our previous publication the fraction of aggregates was defined as the event fraction appearing in the upper right quadrant of the FSC versus SSC plot. Because the aggregates appear in this quadrant regardless of the fluorescence intensity in the FL2 and FL3 channels, detection of multiple QD populations requires an approach influenced by QD fluorescent color. To achieve multiplexed detection, the fraction of aggregates was instead characterized in the FSC versus FL3 plot in region R8 for the QD705 QD population (Figure 1, B) and FSC versus FL2 plot in region R9 for the QD585 QD population (Figure 1, C). The lower boundary of regions R8 and R9 was raised to minimize the QD585 aggregates count in the QD705 population and vice versa. The gating was adjusted to limit this loss of aggregate events to 1% of total number of events in the triggering population, as determined by characterizing nonmultiplexed samples.

Statistical analysis of the data sets was carried out using the SigmaStat 3.1 software (SigmaStat and SigmaPlot-Systat Software Inc., San Jose, California). The Student’s $t$-test was used to determine whether experimental parameters, including the concentration of the nonspecific antigen in the multiplexing mixture, signal acquisition optimization, and gating geometry, had a statistically significant effect on the relationship between antigen concentration and agglomerate percentage. When $t$ values for two data sets compared are close to zero, the difference between the two data sets and the effect of the experimental parameters is statistically insignificant.

Results

Nonmultiplexed detection of VEGF and Ang2 was achieved from 50 fM to 100 pM. Figure 2 shows the correlation between aggregates as a percentage of total number of events counted and antigen concentration. In these nonmultiplexed aggregation experiments the concentrations of either antigen from 50 fM to 100 pM produced a log-linear correlation with the fraction of events detected as aggregates. The background aggregate concentration caused by nonspecific assembly of QD-Ab conjugates in the PBS-BSA buffer has a value of 1.39% ± 0.3% for QD705-Ang2. The Student’s $t$-test comparison for the current Ang2 data with previously published data indicates no statistically significant difference. Thus, the change in gating strategy to accommodate multiplexed detection has no effect on the quantification of aggregates. Percentage aggregates formed for antigen concentrations less than 50 fM were within three standard deviations of the mean nonspecific aggregate count in the absence of the specific antigens (i.e., the background noise). Aggregate formation in the presence of 500 pM antigen was close to background level, suggesting a quenching of the agglomeration process due to the excess antigen. Data represent the mean and standard deviation of values observed from $n = 3$ experiments.
aVEGF and 1.56% ± 0.38% for QD585-aA2. A t-test comparison of the QD585-aA2/Ang2 agglomeration percentage from 500 fM to 100 pM [Ang2] in the current data and in the previously published data\(^5\) indicates no statistically significant difference based on a \(t\) value of 0.073. Over the range of antigen concentrations measured, the two lower antigen concentration values of 5 fM and 10 fM generate aggregates that are within three standard deviations of the background nonspecific aggregate count. The samples with 500 pM antigen concentration also deviate from the log-linear correlation.

Quantitative multiplexed detection of both antigens in the physiologically relevant concentration range was also achieved. Multiplexed detection of the two antigens was demonstrated over the 1- to 100-pM concentration range. The relationship between the VEGF concentration and percentage aggregates (R8\%) is log-linear even in the presence of Ang2 (Figure 3). The slope ± standard error values of the correlation for the multiplexed detection of VEGF at 0, 1, 10, and 100 pM Ang2 are 3.9 ± 0.33, 4.0 ± 0.28, 4.1 ± 0.33, and 4.3 ± 0.35, respectively. The slope for nonmultiplexed detection of VEGF is 4.6 ± 0.31. The t-test comparisons of the QD705-aVEGF/VEGF aggregate percentages from 1 pM to 100 pM [VEGF] in all experiments have a range of \(t\) values from -0.039 to 0.077, indicating no statistically significant effect of Ang2 concentration on VEGF detection (Table 1). The VEGF-negative control experiments with Ang2 added at the appropriate concentration to the multiplexed QD-Ab mixture determined the limit of VEGF detection. The highest percentage of nonspecific aggregates + three standard deviations (\(P = 99.7\%\)) formed in the negative control experiment with 100 pM Ang2 resulted in a sensitivity limit of 1 pM for VEGF. The background aggregate percentage increased with increasing Ang2 concentration. At 100 pM Ang2 background nonspecific aggregate percentage was 6.4 ± 0.87%, compared with the background aggregate percentage in the nonmultiplexed experiments (1.4 ± 0.1%).
Quantitative multiplexed detection of Ang2 was also achieved from the same set of samples, as measured by R9% (Figure 4). The change in VEGF concentration in the samples does not have a statistically significant effect on the detection of Ang2 in the range of concentrations examined. The \( t \) values for these data range from -0.046 to 0.043. The \( t \) tests for the multiplexed sample data sets were carried out for R9% values of the four concentrations of Ang2 (0, 1, 10, and 100 pM), for each concentration of VEGF, and for the nonmultiplexed Ang2 detection data set.

Discussion

Multiplexed detection of proteomic antigens with molecular recognition-mediated nanoparticle self-assembly is feasible in the physiologically relevant concentration range. Because this technique is based on antibody-antigen interaction, it is expected that detection sensitivity similar to that demonstrated here would be achievable for a variety of proteomic biomarkers subject to the dissociation constant \( (k_d) \) ranges for the specific polyclonal antibody-biomarker
pairs. The reproducibility of the equilibrium aggregate percentage suggests the feasibility of self-assembly–based quantification of antigens, even though individual QD-Ab/Ag interactions are presumably random. The fundamental feasibility of QD self-assembly for multiplexed protein quantification has been demonstrated here as a proof of concept. Significant improvements in the materials and methods, including adoption of microfluidic-based instrumentation, are appropriate to translate this technique into a routine clinical, personal, or research proteomic profiling tool. The results also establish that the adjustments made to the data acquisition process and gating strategies to permit multiplexed detection do not have a statistically significant effect on the quantification of aggregates.

As expected from the mechanism of antibody-antigen interaction, the presence of a large excess of antigen in the solution rapidly associates with antibody binding sites and inhibits the QD aggregation process. This is manifested in the low aggregate count for both antigens in the nonmultiplexed experiments at the highest concentration (500 pM, Figure 2). This antigen concentration corresponds to a 50:1 antigen/QD-Ab ratio. At the 3× antibody/QD molar ratio used to prepare the QD-Ab conjugates, we can calculate that the ratio of antigens to antibody binding sites is approximately 8:1. This is comparable to the 10-fold molar excess of antigens commonly used for antibody blocking in conventional antibody-antigen based reactions. As a result of this effect, for clinical implementation, optimized QD-Ab concentrations may be required for each target antigen based on the expected concentration range. We have earlier demonstrated that it is possible to extend the dynamic range of this technique by using multiple QD-Ab concentrations. We anticipate that such multiple antigen:QD-Ab stoichiometries can be easily implemented at low cost on second-generation microfluidic-based instrumentation.

Another interesting feature observed from the nonmultiplexed data is that the lower limit of detection is far lower than what would be expected based on simple stoichiometric considerations. Using the 10-pM QD-Ab concentration, it is possible to detect 50 fM antigen in the nonmultiplexed reaction, an antigen/QD-Ab ratio of 1:200, or antigen/Ab binding site ratio of 1:600. Although such ratios are not uncommon in conventional Ab-antigen based reactions, stoichiometric considerations suggest that the large excess of antibody binding sites would limit the formation of large aggregates. However, the presence of a statistically significant number of aggregates at this antigen concentration indicates that the aggregate formation is strongly favored. This may result from the lower free energy of the aggregates compared with the individual components. Theory-based calculations and simulations will be performed to test this hypothesis but are not a part of this experimental study.

The difference in the fraction of agglomerates observed for the two antigens at identical concentration, and consequently, the different slopes for the two detection curves, indicate that the number of aggregates formed is affected not only by QD-Ab/antigen ratio but also by other characteristics, potentially including Ab-antigen affinity and the size of the antigen. We have also reported this effect previously; the slope for the detection curves of Ang2 and mouse IgG is different. Similar effects are also observed in other antibody-based protein detection methods. For example, different characteristic detection curves are obtained in commercial ELISA methods for different antigen-antibody pairs.

We have also confirmed that multiplexed detection of both of the antigens tested is feasible in the physiologically relevant concentration range. The aggregate fraction is not significantly affected by the presence of the multiple antigens in the reaction mixture. t tests show that the effect of multiplexing on the detection curve within the 1- to 100-pM concentration range is statistically insignificant for both the antigens. The effect of multiple antigens in the reaction mixture on the agglomeration of a QD-Ab/antigen pair is of critical significance for a multiplexed antigen profiling technique. Because there are numerous antigens present in a complex biological sample, measurement robustness against these nonspecific reactions is an important factor in determining the suitability of a technique for application as a multiplexed diagnostic. The antigen sensitivity limit is reduced in a multiplexed sample relative to the sensitivity in nonmultiplexed equivalent due to increased background noise. The adverse characteristic may be through antibody-antigen cross-reactivity and/or nonspecific nanoparticle interactions. In the technique demonstrated here, the characterization of the aggregates was carried out with flow cytometry, and the aggregates were counted in the FL versus FSC space. The aggregates with high intensity in one fluorescence channel appear as low-fluorescence aggregates in the detection channel. The gating strategy permits the exclusion of a large number of these false-positive detections. Once the gating strategy is optimized, it need not be adjusted for the set of antigens.

In summary, we have established that the QD agglomeration-based protein detection technique is capable of multiplexed quantification of proteomic antigens. We have demonstrated that two antigens can be detected simultaneously at physiologically relevant concentrations in a simple physiological buffer. The present work discusses one approach to develop the methods and materials for multiplexed biomarker detection, a fundamental aspect of future molecular diagnostics. Although quantitative detection sensitivity in complex physiological samples is currently not suitable for direct clinical application, the principles of biomolecular recognition used in this approach are comparable to those used in ELISA and other clinically applicable molecular diagnostic methods. Hence, the optimizations developed over the past few decades for ELISA and recent advances in other molecular diagnostic methods are informing the efforts to improve the clinical utility of the new technique described here. Optimization of the reagents and biological samples, as well as of the incubation and data acquisition protocols, may provide the ability to quantify
proteins from physiological samples. These optimizations may include improvements to the surface chemistry and nonspecific interaction properties of the reagents, amended incubation and characterization protocols (including the characterization of nanoparticle agglomeration kinetics\cite{39}), sample preparation for removing the high-concentration carrier proteins and antibodies and customized instrumentation optimized for small particle detection. With such improvements, this simple, quantitative, and low-cost method may be suitable for clinical application.

References