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ORIGINAL RESEARCH

Development of a Sensitive Quantum Dot-Linked Immunoassay for the Multiplex Detection of Biochemical Markers in a Microvolumeric Format

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Purpose: For the diagnosis of various diseases, simultaneous sensitive detection of multiple biomarkers using low sample volumes is needed. The purpose of the present research was to develop sensitive multiplex detection model of QD-based ELISA (QLISA), through the spectroscopic QD-analyte complex measurements in microvolume liquid droplets on a glass microslide.

Methods: QLISA was used for the detection of cartilage oligomeric matrix protein (COMP) and human growth hormone (hGH) as model analytes. The QLISA detection method included the formation of complexes consisting of analyte antigens, biotinylated antibodies and streptavidin-coated QDs. A specific immune-complex disassembling solution was used to dissociate analyte-antibody complexes from the bottom of the 96-well plate. After dissociation, the samples were diluted with PBS, and 2 µL transferred to a reusable glass slide for fluorescence (FL) scan.

Results: The alkaline immune-complex disassembling solution that most efficiently amplified QDs FL within a prolonged 17 h time was selected. Comparison of median fluorescence intensity (MFI) of 50 nM COMP, 25 nM COMP, and 5 nM COMP detection using QD655 with the dilution of the detached samples with PBS and without dilution resulted in significant MFI differences in all cases. The FL signal readouts from QD655 in the microvolume format were from 10 to 40 times stronger than those measured directly from a 96-well plate QLISAs. In duplex analysis, two analytes COMP and hGH were measured using different QD605 and QD525 in the same well. In the respectful 96-well plate QLISA format, two different analyte concentrations can be hardly distinguishable, but the transfer to micro-volumetric detection on the glass slide highly increased the signal strength according to green and red FL intensity of QDs.

Conclusion: Our method significantly enhances detection sensitivity, as compared to measured in parallel QLISAs in a 96 well plate format, enables multiplexing and may prove very valuable for samples of limited volumes.

Keywords: quantum dots, biomarker, microvolume, FL spectroscopy, immunoassay, QLISA

Introduction

The development of biomarker immunoassays is an emerging area of research in fields of various multifactorial diseases. However, biomarker-based disease characterization is still far from a unified conceptualization. Innovation in nanotechnologies and bioconjugation techniques have enabled the application of a large diversity of nanomaterials to enhance the sensitivity of advanced immunoassays.[1–3] Semiconductor nanocrystals, also known as quantum dots (QDs), are widely used in biological research as fluorescence (FL) imaging tools.[4–7] QDs show several significant advantages over most organic fluorophore dyes.

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First of all, by changing QDs core size, QDs with the same composition, but different FL wavelengths could be synthesized. Secondly, QDs have wide excitation bands and could be easily excited with different wavelengths, while organic dyes usually have quite specific and narrow excitation bands. Furthermore, QDs FL bands are narrowed compared to most organic dye fluorescence bands, which is more suitable for multiplexing applications, when several different FL wavelength signals have to be separated. QDs typically exhibit a large Stokes shift, allowing efficient separation of the excitation and emission light. In multiplexed biomarker detection assays, this feature lowers background noise and raises the signal-to-noise ratio.

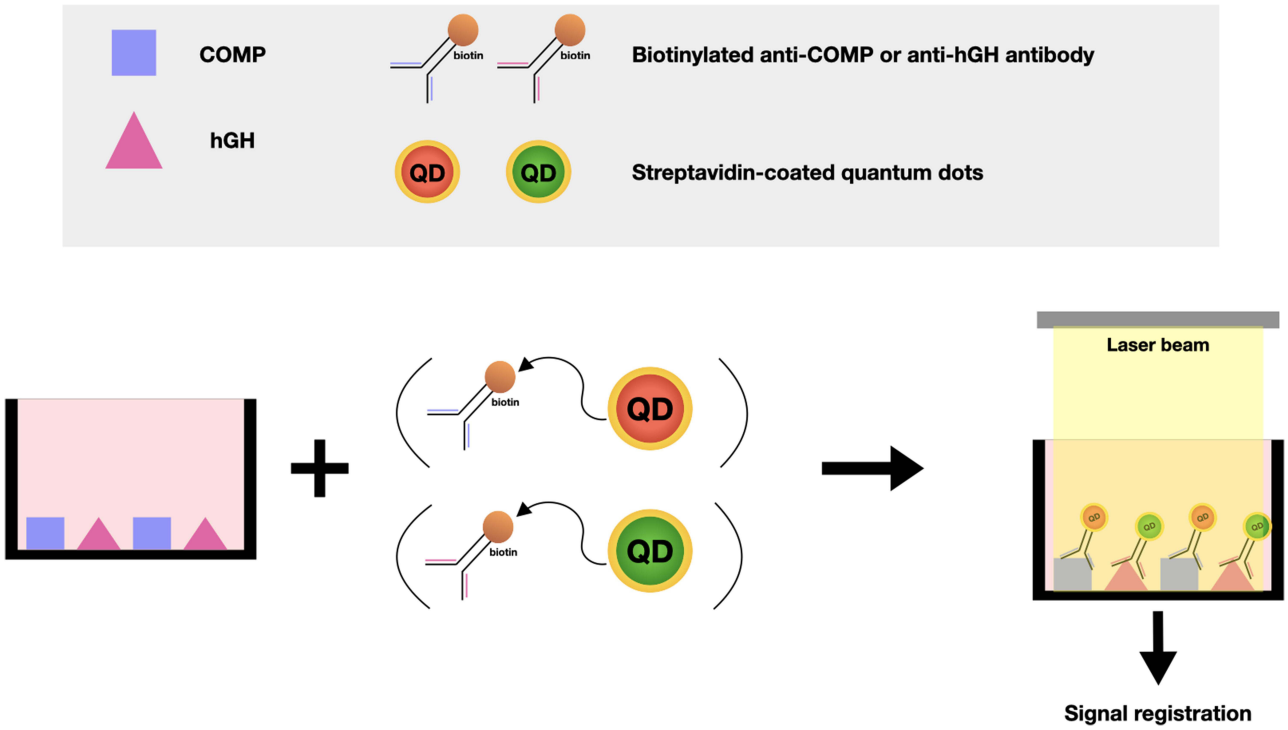
QDs have a high FL quantum yield, which allows the acquisition of high signal intensities from relatively low concentrations.[8] Moreover, QDs show far greater photostability than that of organic dyes at similar spectral regions.[9] The combination of these FL characteristics makes QDs a very powerful tool for FL multiplexing, single-molecule tracking, fluorescence resonance energy transfer, and high-throughput screening. As the surface of QDs is easily modifiable, different antibodies and other biomolecules, such as peptides, proteins, oligonucleotides can be simply attached to them. As for multiplexed immunoassays, QDs are covalently linked to streptavidin (avidin), and this allows the conjugation between streptavidin (avidin) and biotin to occur through a strong non-covalent interaction.[10]

QD-based ELISA (QLISA) suitability for biomarker detection has been shown in different fields. This technology has been used for the detection of the cluster of differentiation 20 (CD20) antigen, which is a cell surface marker overexpressed by B-lymphocyte cancer cells[11] and detection of the multifunctional cytokine interleukin 6 (IL-6).[12] Amine-functionalized QDs were conjugated with partially reduced antibodies via cross-linker to form bioconjugates used for detection. Results demonstrated that using functionalized QDs and reading the FL of the QDs from the bottom of the well, the low limit of IL-6 detection would be approximately 50 pg/mL, which is undetectable using a standard ELISA method.[12]

Different signal resolution amplification methods have been developed and investigated. For sensitive detection of QDs in liquid format, the microfluidic technology is under development.[13] To the best of our knowledge, microvolumetric analysis using QDs has not been performed previously using microslide format, which highly facilitates detection, as several samples can be run simultaneously in triplicates, whereas previous studies used small volumes for analyte detection in separate cuvettes.[14,15] It is known that in QLISA experiments, QDs themselves amplify the detectable signal due to their physical properties. However, different signal resolution amplification methods are investigated. Additional conjugation between QDs and other nanoparticles (eg, gold, silver, etc) were reported to increase the FL signal of QDs.[16,17] In all these cases, the analytes of interest were detected using a spectrophotometer in cuvettes or read from the bottom of the well.[12,18]

QDs-based microarrays were five times more sensitive than the Alexa-based microarray and seven times more sensitive than that of the ELISA for ApoE detection in Alzheimer's disease.[19] We have recently published data on ultra-sensitive surface plasmon resonance (SPR) based immunosensor for the quantitative determination of human cartilage oligomeric matrix protein biomarker (COMP) demonstrating that binding of high-mass streptavidin-coated QDs via biotin–streptavidin interaction to the surface of the immunosensor resulted in a drastic increase in the sensitivity.[20] Additional conjugation of other nanoparticles (eg gold, silver, etc.) to QDs may further enhance their FL signal.[16,17] However, the described SPR method using QDs was developed for single analyte detection, the SPR technology itself remains complicated and requires multiple resources, including equipment-specific knowledge and manipulation skills.[20,21] Whereas, for disease biomarker detection, a multiplex analyte measurement using low sample volumes as well as simple, user friendly and economically relevant method, holding scaling-up potential, is needed. Therefore, in the present study, we were seeking to address those challenges through optimization of spectroscopical analyte detection in QLISA format.

The QLISA detection method comprises capture antibodies attached to the bottom of the plate, followed by affinity interaction with analytes, and then detection antibodies conjugated with QDs, analyzed by the direct registration of their FL signal from the bottom of the plate (Scheme 1). This QLISA detection method faces the problem that immune complexes with QDs stick to the bottom of the plate emit non-specific signals and the sensitivity is questionable due to the plastic plate FL. In order to avoid these technological obstacles, our goal was to search for QD detection possibilities to measure these conjugates in a liquid format and compare the sensitivity of spectroscopical FL detection. For assay optimization, hGH and COMP were used as model biomarker analytes to develop a microvolumetric analysis method suitable for future application for the detection of various disease-specific biomarker duplex or multiplex combinations.



Scheme 1 Scheme of the classical QLISA detection method. COMP (cartilage oligomeric matrix protein) and hGH (human growth hormone) proteins are attached to the bottom of the plate, incubated with biotinylated detection antibodies, and then conjugated with streptavidin-coated QDs, followed by the direct registration of fluorescence signal from the bottom of the plate.

The purpose of the present research was to develop a sensitive multiplex detection model of QD-based ELISA, through the spectroscopic of QD-analyte complex measurements in microvolume liquid droplets on a glass microslide.

Material and Methods

Selection of an Immune Complex-Disassembling Solution

The selection of the immune complex-disassembling solution was performed using an SPR analyzer Autolab Esprit (Metrohm Autolab BV, Utrecht, The Netherlands) equipped with a continuously mixed, small sample volume (20–150 µL) and double channel cuvette. Prior to the experiment, the gold surface of the SPR sensor disc (SD AU, XanTec bioanalytics GmbH, Münster, Germany) was cleaned, modified with a self-assembled monolayer of 11-mercaptoundecanoic acid (Sigma-Aldrich, Steinheim, Germany) and capture antibodies against COMP (monoclonal mouse IgG1 clone 16F12, BioVendor, Brno, Czech Republic; Cat. No. RD182080100F1-01) were immobilized. All these procedures were performed according to the protocol described by Kausaite-Minkstimiene et al.[20] A 277.8 nM antibody solution in sodium acetate buffer, pH 4.5, was used for immobilization. During the registration of real-time sensograms of the interaction between immobilized capture antibodies and COMP (Biovendor; Cat. No. RD172080100) the surface of the antibody-modified SPR sensor disc surface was first exposed to 10 mM PBS solution, pH 7.4, for 200 s. Then, a solution of 19.99 nM COMP in PBS (Carl Roth GmbH+Co.KG (Karlsruhe, Germany)) was injected into the first channel (measurement channel) of the SPR cuvette, and pure PBS was injected into the second channel (reference channel). Interaction between COMP capture antibodies and COMP was carried out for 600 s, followed by 100 s dissociation in PBS. The formed antibody-COMP complex was disassembled by exposing the SPR sensor disc surface to an immune complex-disassembling solution (10 mM NaOH (Thermofisher Scientific; Cat. No. 11358504) and 0.5% SDS (Sigma-Aldrich; Cat. No. 436143–100G), pH 12.0; 25 mM NaOH and 0.5% SDS, pH 12.38; 10 mM glycine (AppliChem; Cat. No. A1067)/HCl (Merck; Cat. No. 1003171000) (pH 2.0) or 10 mM glycine/HCl, pH 1.0) for 300 s. The baseline was then restored by exposing the surface of the SPR sensor disc to PBS. Sensograms of the difference between the measurement and reference channels were used to evaluate the degree of immune complex disassembly. The efficiency of immune complex

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disassembly was estimated by dividing the SPR signal recorded before the interaction of immobilized capture antibodies with COMP by the SPR signal recorded after reconstitution of the baseline in PBS and multiplying the resulting value by 100%.

Spectroscopic Analysis

QDs FL spectra were measured using the FLS920 spectrophotometer (Edinburgh Instruments, UK). For all measurements disposable cuvettes with a 1 cm optical path were used (Fisher Brand). In order to investigate how different solutions affect QDs, FL spectra of 10 nM QD565 (Thermofisher Scientific; Cat. No. Q10131MP) in different solutions (distilled H_2O, PBS (Sigma-Aldrich, Steinheim, Germany)), borate-buffered saline (Thermofisher Scientific; Cat. No. Q20001MP) and an immune-complex disassembling solution composed of 50 mM NaOH and 0.5% SDS were evaluated. All samples were excited with 405 nm, and the excitation and emission slits were fixed to 5 nm. Fluorescence intensity of 10 nM QD565 in different solutions (10 mM NaOH+SDS; 25 mM NaOH+SDS; PBS) over time was measured in a 96-well plate using a spectrophotometer SpectraMax i3 (Molecular Devices, San Jose, CA, USA). The measurements were carried out after 5 min, 30 min, 1 h and 17 h. Solutions at the same concentrations but without QDs were used as controls.

QD–Streptavidin Conjugate Size and Conjugation Evaluation

A hydrodynamic diameter of QD605 (ThermoFisher Scientific; Cat. No. Q10101MP) was measured using the dynamic light scattering technique. QD-streptavidin conjugates were measured using particle size and zeta potential analyzer Zeta Plus PALS (Brookhaven Inc., USA). It was the size of a large macromolecule or protein (~15–20 nm) (Supplementary Figure 1). Other QDs size vary slightly within a few nanometers. The conjugation efficacy was also confirmed with SPR experiments (Supplementary Figure 3).

Preparation of Biotinylated Antibodies

COMP (Biovendor; Clone: 17C10; Cat. No. RD182080100C1-01) antibodies were biotinylated with sulfosuccinimidyl biotin (sulfo-NHS-biotin). Antibodies were mixed with sulfo-NHS-biotin at a molar ratio of 1:50 in 10 mM PBS pH 7.4, and incubated for 30 min. Dialysis using a Slide-A-Lyzer™ MINI Dialysis Device (20 K MWCO, Thermo Fisher Scientific, Pittsburgh, Pennsylvania, USA) was used to remove free sulfo-NHS-biotin molecules. The volume ratio of sample to PBS (pH 7.4) was maintained at least at 1:1000. Dialysis was continued overnight at 4 °C. The solution of biotinylated antibodies was stored at 4 °C. Human growth hormone antibodies (biot-anti-hGH) (R&D systems; Cat. No. BAF1067) were purchased already biotinylated.

Quantum Dot-Linked Immunosorbent Assay

Instead of horseradish peroxidase (HRP), conventionally used in ELISA, QDs were used as fluorescent signal carriers and immune reaction indicators for the analyte detection and quantification. COMP or hGH (R&D systems; Cat. No. 1067-GH-025/CF) at different concentrations were applied in duplicates to the bottom of the well (50 µL/well) of 96-well plates and incubated at 4 °C overnight. Each well was washed 3 times with PBS solution (250 µL/well) and blocked with 4% bovine serum albumin (BSA) (in PBS, 100 µL/well) for 2 h at room temperature (RT). After 3 washes with PBS (250 µL/well), biotinylated detection antibodies (anti-COMP 17C10) (Biovendor; Cat. No. RD182080100C1-01) or anti-hGH (R&D systems; Cat. No. BAF1067) were conjugated with streptavidin-coated QDs (ThermoFisher Scientific) in "low protein binding" tubes (Eppendorf AG) for 1 h separately and then the formed QD-Ab complex solution was added to the wells (50 µL/well) for 1 h in RT. After 3 washes with PBS (250 µL/well), 80 µL/well of PBS was added for FL intensity measurement from the bottom of the plate. Measurement parameters: excitation – 400 nm; emission – depending on the QDs wavelength; spacing – 0.40; density – 9 (resulting in a total of 69 scans/well). FL intensity was measured using the spectrophotometer SpectraMax i3. All streptavidin-coated QDs, QD625 (Cat. No. A10196), QD655 (Cat. No. Q10123MP), QD525 (Cat. No. Q10141MP), QD605 (Cat. No. Q10101MP), were used for QLISA experiments and were purchased from Thermofisher Scientific.

Immune-Complex Disassembling and Microvolumeric Fluorescence Quantification

PBS (80 µL/well) from the 96-well plate was discarded after FL measurements and an immune-complex disassembling solution (50 mM NaOH and 0.5% SDS, 12 µL/well) was added for 5 min at RT to dissociate QD-Ab complexes from the

bottom of the well. An equal volume (12 µL/well) of PBS was added to each well, followed by thorough mixing with a pipette. A 2 µL of the mixture (in triplicates) was further used for FL intensity measurement on a reusable glass microslide of the spectrophotometer, 8 testing samples in triplicates/per glass microslide, see (Scheme 2).

Multiplex Analyte Detection in a Microvolume Format

Our next step was to demonstrate the possibility of simultaneous detection of several analytes in the same sample using microvolumeric QLISA technology. For that, the plate was coated with 50 nM, 25 nM and 5 nM of both COMP and hGH proteins. QD525-streptavidin conjugates were incubated (Thermofisher Scientific; Cat. No. Q10141MP) with biotin-anti-hGH antibodies, QD625-streptavidin (Thermofisher Scientific; Cat. No. A10196) with biotin-anti-COMP antibodies for an hour. After 1 h, the solutions were mixed and added at a volume of 50 µL to each well. Singleplex detection of each analyte was performed in parallel for comparison of reference FL intensity. Following the QD fluorescence intensity measurements, the QD-Ab were dissociated from the bottom of the well and transferred for microvolumetric measurement on a reusable glass microslide of the spectrophotometer, as described above.

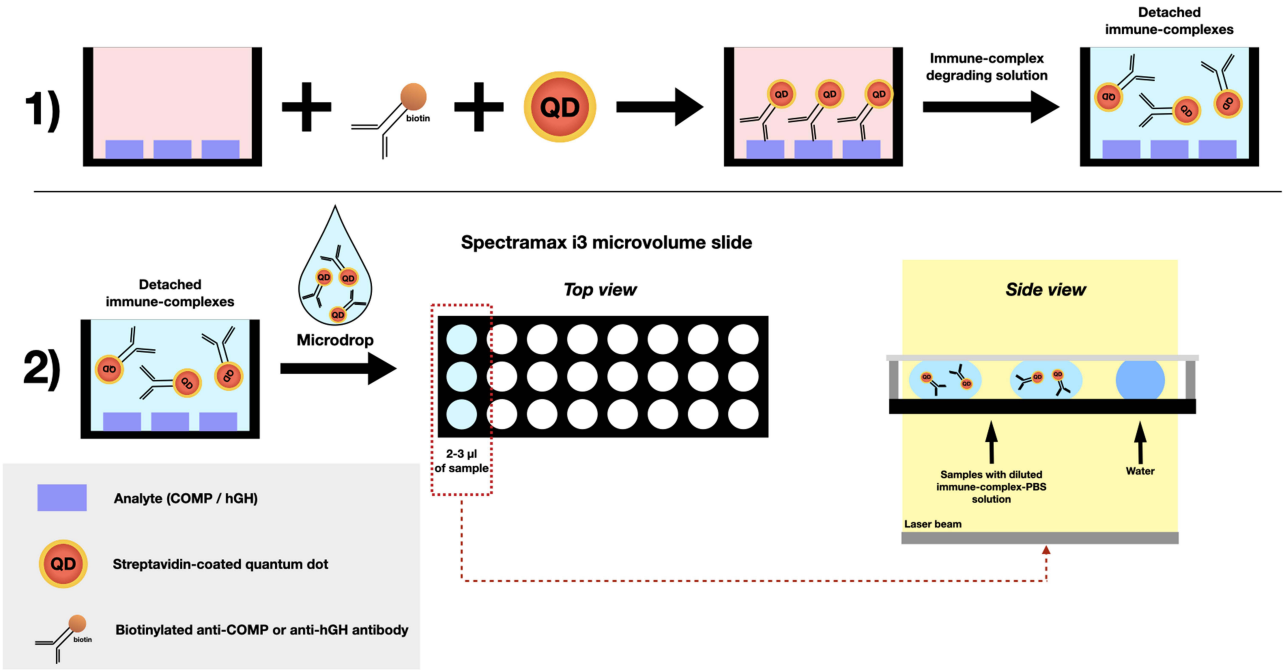
Statistical Analysis

All experiments were analyzed using GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). Statistical differences were determined using two-way ANOVA tests and as per the requirement of the analysis $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$. *P*-value of less than 0.05 was considered statistically significant.

Results

Immune-Complex Disassembling Solution Selection

The results of SPR studies showed that the efficiency of immune-complex degradation using a solution consisting of 10 mM NaOH and 0.5% SDS was about 98.97%, which was the highest, as compared to the degradation efficiency of the other buffers tested, see Table 1. Basic solutions are also more compatible with QDs as they increase fluorescence



Scheme 2 Singleplex detection of an analyte in microvolume format. Formation of COMP (cartilage oligomeric matrix protein), or hGH (human growth hormone) complexes with biotin-labeled anti-COMP or anti-hGH antibodies, respectively, and streptavidin-coated QDs of different spectra. Immune-complex disassembling solution dissociates the complexes through an analyte-biotinylated antibody connection. After dissociation, microdrops are transferred to microvolume reusable glass slide-based spectrophotometric scan.

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Table 1 Immune-Complex Disassembling Solution Efficiency

Immune-Complex Disassembling Solution	Degradation Efficiency, %
10 mM glycine/ HCl, pH 2.0	78.41
10 mM glycine/ HCl, pH 1.0	85.72
10 mM NaOH and 0.5% SDS, pH 12.0	98.97
25 mM NaOH and 0.5% SDS, pH 12.38	98.29

Note: Surface plasmon resonance analysis conditions: 277.78 nM of 16F12; 19.99 nM of COMP; duration of degradation – 300s.

intensity as compared to acidic solutions. Therefore, further experiments were performed using a NaOH-based immune-complex disassembling solution.

Fluorescence Intensity Comparison

FL intensity comparison of 10 nM QD565 showed that immune-complex disassembling solution composed of 50 mM NaOH and 0.5% SDS amplifies QDs FL, as compared to other commonly used solutions (distilled H_2O, PBS, borate-buffered saline) (Figure 1). The FL intensities of QD565 in 50 mM NaOH and 0.5% SDS, diluted or not with PBS, and borate buffer (MFIs of $1.96x10^5$ and $1.15x10^6$, respectively) are stronger than of the same concentration of QD565 in PBS or distilled water (MFIs of $1.12x10^6$ and $1.15x10^6$, respectively) (see Figure 1, insert). However, the borate buffer is not suitable because it foams up easily, and does not form a required drop shape reaching microslide. The signal of 10 nM QD565 in 10 mM NaOH and 0.5% SDS is slightly reduced by the dilution of the detached samples with PBS 1:1 v/v when measured on a microslide, whereas in this case the drop shape was appropriate and reached the cover glass.

FL of QD565 (10 nM) in both 10 mM and 25 mM NaOH + 0.5% SDS buffers is stronger than in PBS and more stable over time. Within a prolonged 17 h time, the QD565 nm FL signal remains sufficiently strong in these two buffers (Figure 2).

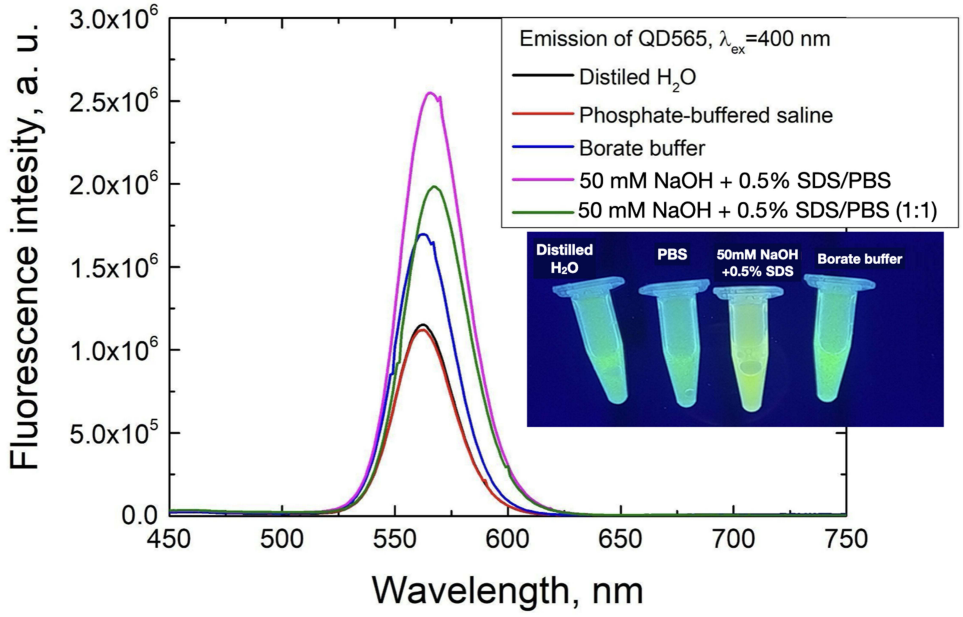

Figure 1 Comparison of 10 nM QD565 fluorescence spectra in different solutions.

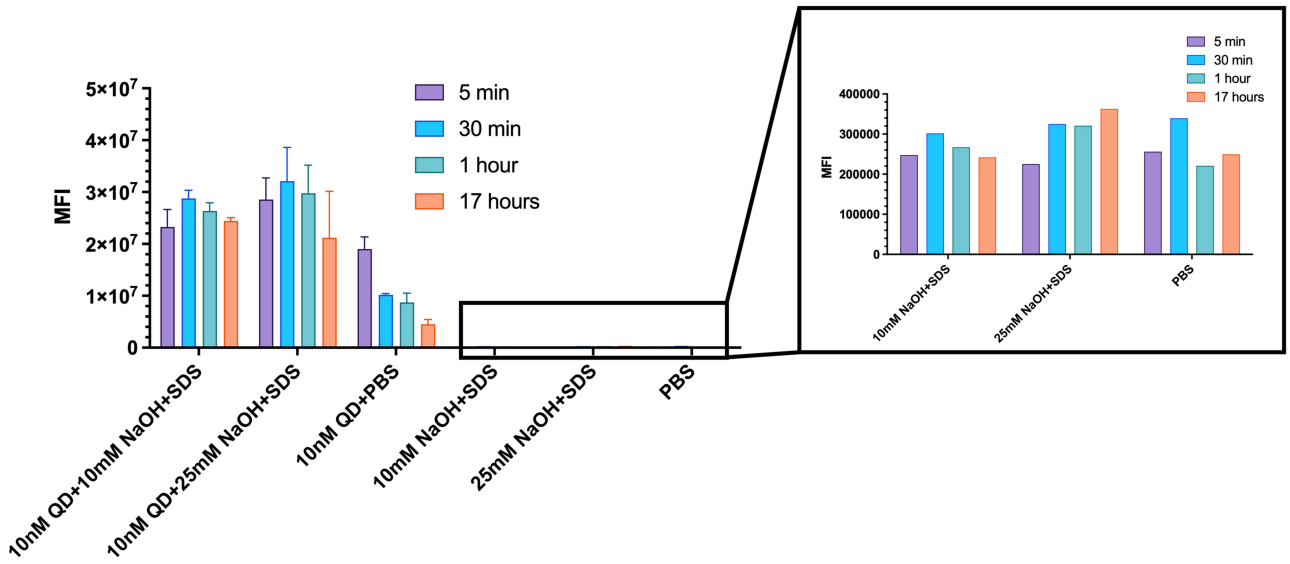

Figure 2 Choice of immune-complex disassembling solution. Fluorescence of QD565 (10 nM) in NaOH+SDS buffers and PBS was measured with a spectrophotometer at different time points in a 96-well plate. 10 mM and 25 mM NaOH and 0.5% SDS (immune-complex disassembling solutions) and PBS without the addition of QDs were measured as control groups respectively. Error bars represent the standard deviation of the average value (N=2).

Sample Dilution and Optimization of Analyte Detection Using a Glass Microslide

Even though FL spectrometry has shown that FL intensity is higher in non-diluted solution before transfer to microvolumes, the dilution with PBS is a necessary step to avoid foaming and to ensure the formation of round-shaped microdrop (Scheme 2 and Figure 3). MFI of 50 nM COMP detection using QD655 with and without dilution resulted in MFI of 764824 and 560299, respectively (1.36-fold increase in diluted samples) (***P ≤ 0.001). A 25 nM COMP detection MFI difference with diluted vs non-diluted was 403247 and 189127, respectively (2.13-fold increase in diluted samples) (***P ≤ 0.001). A 5 nM COMP detection MFI difference was 32031 with diluted and 18217.5 with non-diluted (2.5-fold increase in diluted samples). Increased FL intensity in samples diluted in PBS 1:1 is related to the ability of the solution to form a round-shaped drop reaching a glass microslide (Figure 3).

Evaluation of QLISA in a Standard 96-Well Plate and Microvolume Formats

Two different analytes – COMP and hGH were used to compare the detection methods. 17C10 anti-COMP biotinylated antibody (biotin-anti-COMP) and anti-hGH biotinylated antibody (biotin-anti-hGH) and streptavidin-coated QD655 were

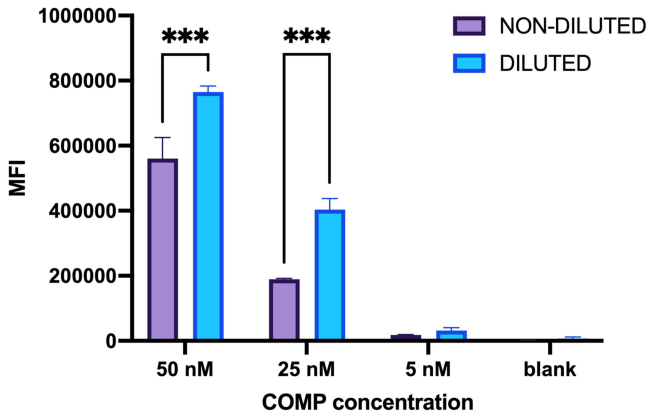

Figure 3 Optimization of COMP analyte detection on the microslide. Non-diluted and diluted 1:1 (v/v) with PBS 10 mM NaOH and 0.5% SDS buffer on a glass microslide. At concentrations 50 nM, 25 nM, and 5 nM COMP was analyzed using 17C10 anti-COMP-biotinylated Ab and streptavidin-coated QD655. MFI – median fluorescence intensity; Blank – sample without COMP protein coating, followed by all of the other protocol steps. *P*-values indicate statistical significance (***$P \leq 0.001$). Error bars represent the standard deviation of the average value (N=3).

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used. The efficacy of immune-complex disassembling solutions was tested and the maintenance of FL intensity of QDs in different solutions analyzed using a spectrophotometer.

The FL signal of QDs for COMP detection at the highest concentration (50 nM) was around 144723 MFI and the lowest (3.125 nM) was 8427 MFI, indicating the direct correlation between analyte concentration and FL signal intensity in a 96-well plate format (Figure 4).

The signal of the highest COMP concentration (50 nM) was detected 5.5 times lower in 96-well format plate as compared to the microvolume format in a glass microslide after detachment from the bottom (144723 MFI vs.796305 MFI, respectively) (****P ≤ 0.0001) (Figure 4). This data demonstrates that QDs transfer to the supernatant and further to microvolumes on microslide increases detection sensitivity, as well as decreases the background signal (blank). The difference between the lowest COMP concentration tested and the control blank is about 1.3-fold in the 96-well plate format, while in the microslide format, this difference increases up to 14.4-fold, imposing the possibility of detection of even lower concentrations of the analyte.

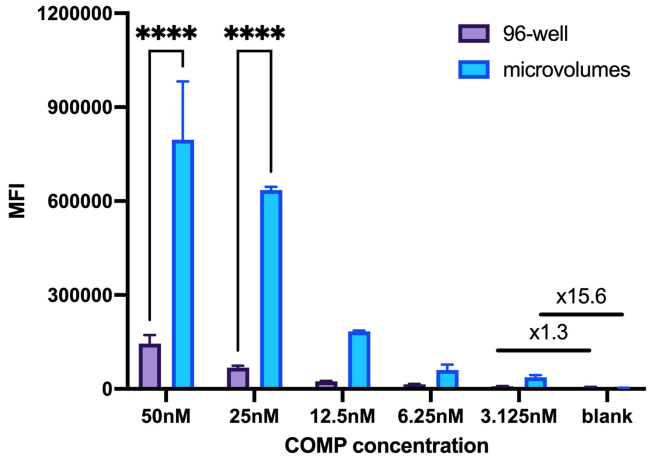


Figure 4 Different COMP concentration (50 nM to 3.125 nM) detection in 96-well plate vs microvolumetric format. 17C10 anti-COMP-biotinylated Ab and streptavidin-coated QD655 were used. Purple bars indicate the signal detected from the bottom of the plate, excitation 400 nm, emission 655 nm, 80 µL volume. Blue bars indicate the signal detected on a microvolume glass slide (2 µL/sample), excitation 400 nm, emission 655 nm. Blank – every protocol step followed, excluding coating the bottom of the well with COMP (non-specific signal). *P*-values indicate statistical significance (****$P < 0.0001$). Error bars represent the standard deviation of the average value (96-well plate N=2; microvolumes N=3).

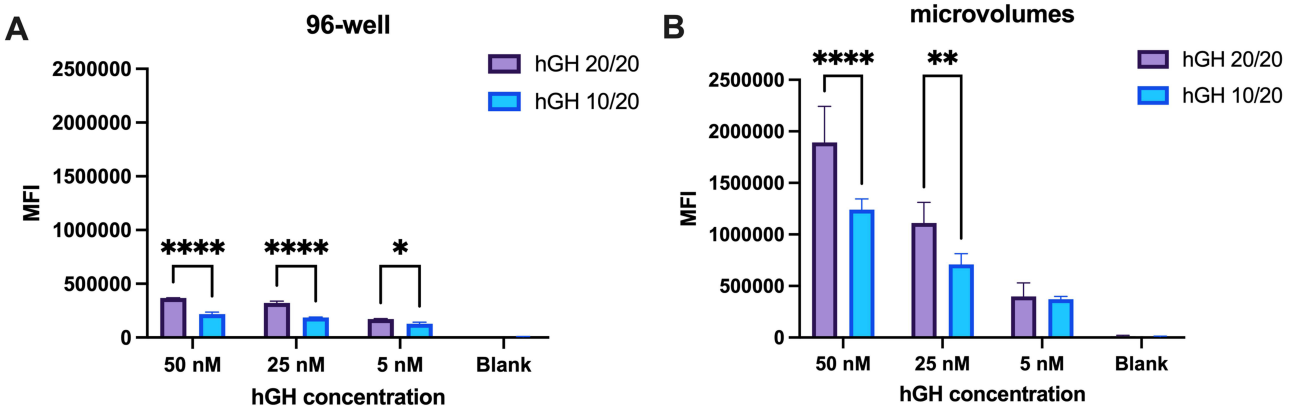


Figure 5 96-well (**A**) and microvolumetric (**B**) detection of different hGH concentrations (50 nM to 5 nM) with anti-hGH-biotinylated Ab (20 nM or 10 nM concentration) and streptavidin-coated QD625 (20 nM concentration). A - The signal was detected from the bottom of the 96-well plate, hGH 10/20 or 20/20 represent nM concentrations of Ab/QD. B - The signal was detected on a microvolume glass slide (2 µL sample size, immune complexes degraded with 50 mM NaOH and 0.5% SDS and diluted with PBS, 1:1 v/v). hGH 10/20 or 20/20 represent nM concentrations of Ab/QD. Horizontal lines represent the difference between the lowest hGH concentration and blank. Blank – every protocol step followed excluding coating of the bottom of the well with hGH (non-specific signal). *P*-values indicate statistical significance (*$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$). Error bars represent the standard deviation of the average value (96-well plate N=2; microvolumes N=3).

The results also show increase of QDs FL intensity at a higher concentration of biotin-anti-hGH (20 nM), as compared to 10 nM (Figure 5A). 50 nM, 25 nM and 5 nM hGH detection signal was significantly higher with the use of 20 nM anti-hGH/20 nM QD625 (****P ≤ 0.0001, ****P ≤ 0.0001, *P ≤ 0.05, respectively). However, the use of both, 20 nM anti-hGH/20 nM QD625 vs 10 nM anti-hGH-/20 nM QD625 concentrations results in relatively strong and distinguishable signals between the different concentrations of analyte (Figure 5A).

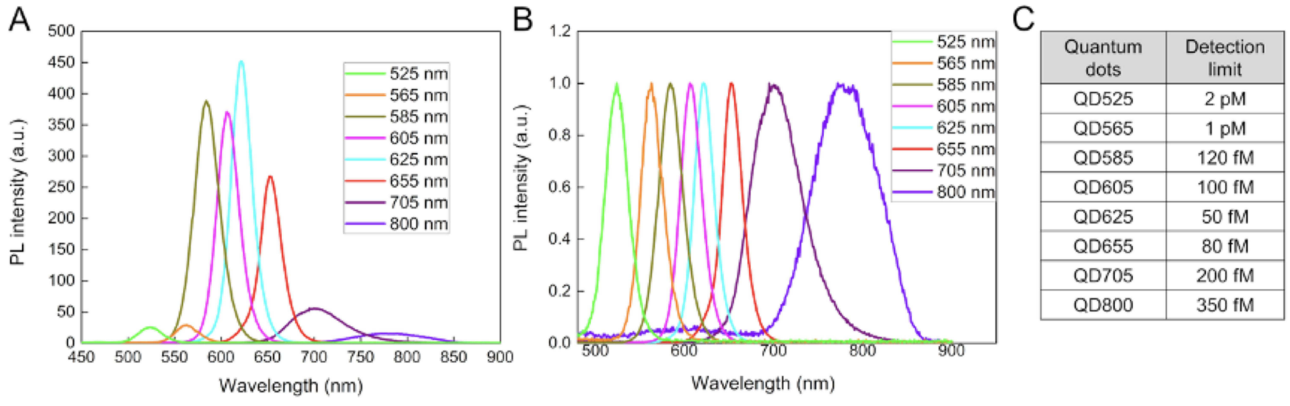
Transfer to microvolumes amplified MFI as compared to 96-well format plate, and the use of a higher concentration of thebiotin-anti-hGH also increased the detection signal at all concentrations. The MFI of the lowest hHG concentration analyzed (5 nM) was relatively high when either 20 nM and 10 nM concentrations of anti-hGH were used (30- and 40-fold higher MFI compared to blanks, respectively), which was 1.6 and 2.4-fold higher as compared to the corresponding differences in the 96-well format plate (Figure 5B).

Multiplex Analyte Detection in a Microvolume Format

To choose the best combinations of streptavidin-coated QDs for biomarker multiplex analysis, their optical properties were investigated and detection limits were determined. Fluorescent in the UV-NIR spectra CdSe/ZnS QDs of different sizes were used in the study (Figure 6A and B).

The investigation of streptavidin-coated QDs optical properties revealed that CdSe/ZnS QDs of different sizes exhibit different FL quantum yields, and the antigen detection limit will depend on the excitation wavelength and the sensitivity of the FL detector in the specific spectral range. QDs detection limits were evaluated and determined: QD525 – 2 pM; QD565 – 1 pM; QD585 – 120 fM; QD605 – 100 fM; QD625 – 50 fM; QD655 – 80 fM; QD705 – 200 fM; QD800 – 350 fM (Figure 6C). The QD-streptavidin conjugation was verified for size distribution with dynamic light scattering (example in Supplementary Figure 1). In this research, a successful interaction of streptavidin-coated QD625 with the biotinylated antibodies and conjugates formation was verified by ultra-sensitive surface plasmon resonance (SPR) analyzer (data shown in the Supplementary Figure 3).

We were further choosing the best potential combinations of QDs to avoid FL spectral overlapping in the 525–705 nm range. Different QDs have different quantum yields and detection limits and thus also have to be carefully chosen when combining them for multiplexed detection of the analytes, especially of different abundance. QDs with high quantum yield and lowest detection limit have to be used for the detection of the analyte of low abundance and vice versa 3. In the multiplexing experiments using three QDs in a spectral range 525–705 nm, five different combinations (QD525/QD605/QD795; QD565/QD625/QD705; QD525/QD625/QD705; QD525/QD585/QD705; QD525/QD585/QD655) with the same 405 nm excitation wavelength were identified (Figure 6; Supplementary Figure 2). For further analysis, a combination of streptavidin-coated QD525 and QD605 was chosen (Supplementary Figure 2). The bottom of a 96-well plate was coated with two analytes (COMP and hGH), which were analyzed using two corresponding QD-detection antibody conjugates.



Quantum dots	Detection limit
QD525	2 pM
QD565	1 pM
QD585	120 fM
QD605	100 fM
QD625	50 fM
QD655	80 fM
QD705	200 fM
QD800	350 fM

Figure 6 CdSe/ZnS QDs fluorescence in the VIS-NIR region. (**A**) – different size CdSe/ZnS QDs FL spectra; (**B**) – normalized FL spectra with excitation using 400 nm wavelength. (**C**) – QDs FL detection limits.
Abbreviation: FL- fluorescence.

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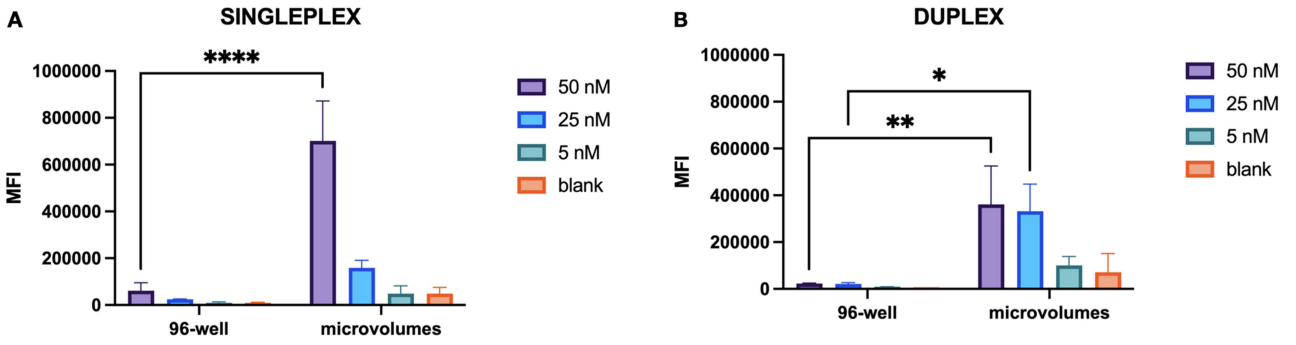

Figure 7 Singleplex (COMP coating) (**A**) and duplex (both COMP and hGH coating) (**B**) detection of different COMP concentrations (50 nM, 25 nM, 5 nM) in 96-well plate vs microvolumetric format. In 96-well format, signal was detected from the bottom of the plate (80 µL volume) and in microvolumes (signal detected on a microvolume glass slide (2 µL/sample). Blank – every protocol step followed, excluding coating of the bottom of the well with analytes (non-specific signal). *P*-values indicate statistical significance (**P* ≤ 0.05, ***P* ≤ 0.01, *****P* ≤ 0.0001). Error bars represent the standard deviation of the average value (96-well plate N=2; microvolumes N=3).

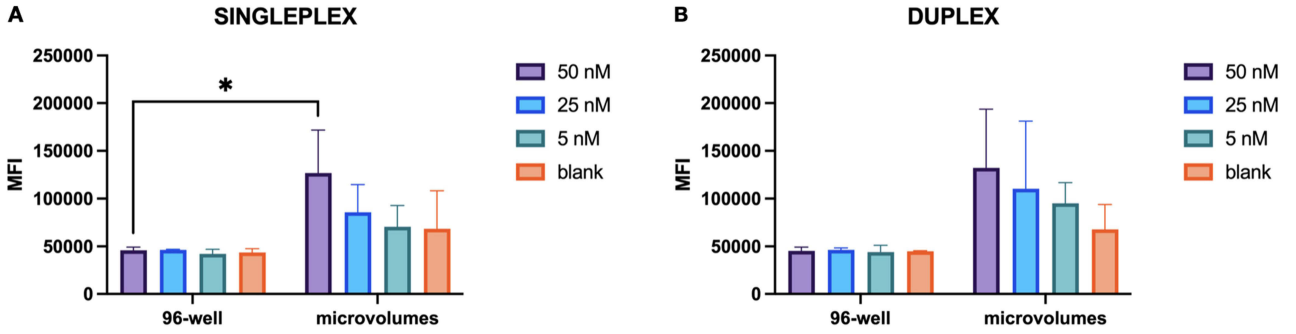

Figure 8 Singleplex (hGH coating) (**A**) and duplex (both hGH and COMP coating) (**B**) detection of different hGH concentrations (50 nM, 25 nM, 5 nM) in 96 well plate vs microvolumetric format. In 96-well format, signal was detected from the bottom of the plate (80 µL volume) and in microvolumes (signal detected on a microvolume glass slide (2 µL/sample). Blank – every protocol step followed, excluding coating of the bottom of the well with analytes (non-specific signal). *P*-values indicate statistical significance (**P* ≤ 0.05). Error bars represent the standard deviation of the average value (96-well plate N=2; microvolumes N=3).

In singleplex detection, MFI difference of the highest COMP concentration (50 nM) from blank (every protocol step followed without initial coating the bottom of the well with COMP (non-specific signal)) in 96-well and microvolumeric detection was 6.3 and 14.5-fold, respectively. For singleplex hGH detection, in 96-well format plate different concentrations were not distinguishable from blank signal, but after transfer to microvolumes, the difference between 50 nM and blank increased up to 1.86-fold. In duplex detection (two analytes detected with different QDs in the same well) 50 nM of COMP to blank ratio in 96-well measurement was 3.1-fold and 5.6-fold, respectively, for hGH – as in singleplex, was not distinguishable from blank, but increased to 2-fold when transferred to microvolumes (Figure 7). For hGH, in 96-well format plate neither in singleplex nor duplex analysis different concentrations (detected using QD525) were not distinguishable from blank potentially due to lower FL quantum efficiency and autofluorescence of the plastic 96-well plate (Figure 8).

The results indicate that our proposed method can be used for two analyte detections in the same sample using different FL wavelength QDs. In 96-well format plate, different analyte concentrations were hardly distinguishable, but the use of the immune-complex disassembling solution and transfer to microvolumeric detection increases the signal strength and can detect different analyte concentrations. Thus, a low concentration of the analyte, undetectable in a 96-well plate, could be detected after transferring the assay into microvolumeric format on a glass slide.

Discussion

Previously published data on QLISA application for single or multiplex biomarker analysis detect analytes of interest in standard 96-well plates or cuvettes at 50–100 µL sample volumes.[12,22–24] Our study aimed to optimize the sensitivity of QLISA and to reduce sample volume through the application of the newly developed microvolumeric (2 µL) analysis format.

The transfer of the 96-well bottom-linked immune complexes to a liquid phase was performed using an immune-complex disassembling alkaline solution. After 17 h of incubation, the fluorescence of the QD565 in the disassembling buffer remained relatively strong compared to other buffers used. The proposed alkaline solution for disassembling immune complexes might be also applied in the future for detection of QDs conjugated to analytes on other surfaces and/or detection systems, for instance, glass, nanoparticle or magnetic bead (MB) – bound immune complexes. For instance, the application of 8 different separation buffers (pH ranging from 7 to 11) for dissociation of QD-antibody complexes from MB has been previously reported, suggesting that the alkaline detachment buffers are the most suitable for QDs quantification in MB-based QLISA, as the lower pH acidic solutions cause QDs dissolution.[25] Although the mechanism is not completely clear, one of the explanations could be the higher stability of QDs in the basic pH. Basic solutions are used for other SPR experiments to detach antibody complexes from sensors.[20] The next step for this new method is the transfer of solid-phase immune complexes to a liquid-phase and microvolume format. Dilution of the detached immune complex with a buffer or diluent appears to be a very important step for round-shaped low viscosity drop formation on a glass microslide for further QDs spectroscopy measurements. Stronger QD FL signal was determined in borate saline buffer as compared to PBS, see Figure 1. Although the QDs signal is slightly reduced by the dilution with PBS, this step is necessary to avoid foaming and to ensure the formation of a liquid interface between two glass surfaces.

Low sample and assay reagent volume are also big advantages of this QLISA format. Cuvette or 96-well plate-based measurement formats require a higher sample volume, at least 50–100 µL/well. For the method proposed hereby, the sample and the whole assay volume can be as low as sufficient to cover the bottom of a well in a 96-well plate, ie, 12 µL. Furthermore, FL measurement on a reusable glass slide requires 2 µL of the sample, allowing the application of the 2 µL samples in triplicates which further ensures better accuracy of the measured data.

Originally, such spectrophotometric measurement on a reusable glass slide was used for quantitative DNA, RNA or protein detection, but the application of the same methodology to detect QDs FL increases the sensitivity and reduces nonspecific FL, which is a major drawback of plastic ware. Additionally, the same glass slide can be used numerous times, as the sample droplets are wiped out from the glass surface after the MFI measurement.

The potential of QDs has been investigated for multiplexed biological imaging[4] and toxin analysis in a single well of a microtiter plate.[26] Due to wide excitation spectra, it is possible to simultaneously excite QDs of various sizes at a single wavelength and determine emission at a variety of wavelengths. This capability significantly enhances the efficiency and throughput of QD application for multiplexed biomarker analysis. QDs FL spectral analysis revealed that the detection limits of different QDs range between 50 fM (QD625) and 2 pM (QD525). Additionally, the narrow emission spectra of individual QDs ensures very little spectral overlap. Therefore, our proposed method of analysis, comprising the use of immune complex disassembling solution and transferring samples to microvolume reading format (2 µL on glass slides) can be used not only for single analyte detection (singleplex) but also for quantification of two or more analytes (multiplex) in the same well, using different wavelength QDs.

For the potential triplexed detection in a spectral range of 525–705 nm, five different combinations were identified. For multiplexed detection of 4 analytes, to minimize overlapping of FL spectra, additional QD with an emission peak at around 800 nm should be used. For demonstration of the multiplexing strategy different QDs coupled with antibodies against COMP and hGH served as duplex detection probes separate analytes. In the 96-well plate, different analyte concentrations can be hardly distinguishable, but the transfer to micro-volumetric detection highly increased the signal strength and sensitivity, revealing the differences in analyte concentrations. Thus, the simultaneous sensitive detection of COMP and hGH antigens according to green and red FL intensity of QDs in the same sample was possible only in microslide format. These data demonstrate that our proposed analytical method of QLISA enables not only single analyte detection (singleplex) but also the quantification of two or more analytes (multiplex) within the same well.

Study Limitations

We have shown the microvolumetric QLISA's effectiveness with COMP and hGH, this optimized protocol is adaptable for detecting various other analytes and their combinations. However, in the present study, we have used recombinant proteins, instead of patient body fluid samples, where precise detection may be further complicated due to the presence of

high levels of proteins and higher risk of non-specific signal. Wider analysis using lower doses to identify the minimal detection limits should also be performed in the future.

Conclusion

The sensitivity of QLISA can be amplified by transferring the well bottom-linked immune complexes to a liquid phase, using an immune-complex disassembling solution, followed by dilution with PBS and analysis in a microvolume format. Although demonstration of our technology primarily focused on COMP and hGH, this in vitro QDs microvolume detection protocol holds promise for FL spectrophotometry registration of multiplexed biomarkers simultaneously. The FL signal readouts from QDs in the microvolume format were 10 to 40 times stronger than those from a standard QLISA in 96-well plate.

Notably, this method ensures reduction in sample size and assay reagent volume, it is suitable for FL spectrophotometry, while the microvolume format facilitates a cost-effective measurement of analytes. Taken together, our method significantly enhances detection sensitivity of QLISA, and enables multiplexing of analytes in samples of limited volumes.

Abbreviations

Ab – antibody; BSA – bovine serum albumin; CD – cluster of differentiation; COMP – cartilage oligomeric matrix protein; ELISA – enzyme-linked immunosorbent assay; FL – Fluorescence; hGH – human growth hormone; HRP – horseradish peroxidase; IL – interleukin; MB – magnetic bead; MFI – median fluorescence intensity; PBS – phosphate-buffered saline; QD – quantum dot; QLISA – Quantum dot-linked immunoassay; RT – room temperature; SDS – sodium dodecyl sulfate; SPR – surface plasmon resonance; Sulfo-NHS-Biotin – sulfosuccinimidyl biotin; UV-NIR, ultraviolet near-infrared; VIS-NIR, visible near-infrared. biot-anti-COMP, 17C10 anti-COMP biotinylated antibody; biot-anti-hGH, anti-hGH biotinylated antibody; strep-QD, streptavidin-coated quantum dots.

Data Sharing Statement

The data supporting these findings can be found at State Research Institute Centre for Innovative Medicine, Department of Regenerative Medicine, Santariskiu str. 5, Vilnius, Lithuania.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

Ms Ursule Kalvaityte, Dr Edvardas Bagdonas, Dr Gailute Kirdaite, Prof. Dr. Asta Kausaite-Minkstimiene, Prof. Dr. Almira Ramanaviciene, Dr Anton Popov, Dr Jaroslav Denkovskij and Prof. Dr. Eiva Bernotiene report a patent EP23154900.7 pending to State Research Institute Centre for Innovative Medicine. Dr Gailute Kirdaite, Prof. Dr. Asta Kausaite-Minkstimiene, Prof. Dr. Almira Ramanaviciene report grants from European Research Executive Agency, during the conduct of the study. Prof. Dr. Eiva Bernotiene reports grants from Innovative Medicine Centre, during the conduct of the study. The authors report no other conflicts of interest in this work.

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