

Fluorescence immunoassays using CdSe/ZnS core/shell quantum dots for the determination of progesterone in human serum

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Abstract

In this study, two heterogeneous fluorescence immunoassays using CdSe/ZnS quantum dot (QD) to label anti-progesterone antibody (P₄Ab) for the determination of progesterone (P₄) were performed in the wells of a 96-well microtiter plate. First, P₄Ab was conjugated to hydrophilic CdSe/ZnS QDs via ethyl-3-(dimethylaminopropyl) carbodiimide(EDC)-N-hydroxysuccinimide) chemistry(NHS) (QDs-P₄Ab conjugates). The QDs-P₄Ab conjugate was employed as a second antibody in a sandwich assay, where the P₄Ab was immobilized onto the 3-aminopropyltrimethoxysilane (APTMS) sol-gel membrane of the wells of a 96-well microtiter plate, and P₄ was bound between the immobilized P₄Ab and the QDs-P₄Ab conjugate. In this assay, the fluorescence intensity of the QDs increased with increasing P₄ concentrations. This assay had a detection limit of 553.9 pg/ml and a sensitivity of 18,251.96 pg/ml with a linear range of 2,184.6 – 117,082 pg/ml. In the direct binding assay, P₄ was directly bound to the QDs-P₄Ab conjugates immobilized onto the APTMS sol-gel membrane of the wells of a 96-well microtiter plate. In this direct binding assay the fluorescence intensity of the QDs decreased with increasing P₄ concentrations, and this assay had a linear range of 28.95 – 26,607.7 pg/ml with a detection limit of 3.32 pg/ml and a sensitivity of 987.24 pg/ml. These fluorescence immunoassays have been successfully applied for the determination of P₄ in real human serum, and the results were well correlated with those of a certified radioimmunoassay (RIA) method.

Keywords: Fluorescence immunoassay, sol-gel, 96-well microtiter plate, CdSe/ZnS quantum dots (QDs), progesterone

1. Introduction

Immunoassay is perhaps the methodology most frequently used to measure biological compounds in translational and clinical research [1]. The methods and categories of immunoassays have been reviewed by scientists such as Ashihara et al. [1], D.S Hage [2], and I.A. Darwish [3]. In these methodologies, a number of new techniques have been developed to quantify biological substrates that differ from the primary detection method by having high sensitivity and expanding the detection limit. One of these is fluorescence immunoassays which utilize fluorescent compounds as immuno-labels [4-6].

Human serum measurement methods, such as the direct detection and monitoring of individual labeled antibodies and their reactions with antigens, are complicated by the fact that, in addition to organic ions and hydrophilic organic substances, about 100 different proteins are present in human serum. As a result, human serum samples emit strongly when they are irradiated in blue or green regions of the spectrum [6]. This prevents the detection of single chromophores, such as fluorescein or classic rhodamine dye, in this environment. Therefore, in the analysis of bio-samples, the use of fluorescent materials with emission wavelengths out of this spectral range is preferred. The use of fluorescent materials in immunoassays creates the binding of fluorescence-labeled antibodies or antigens, and in cooperation with fluorescence spectroscopy, enables the determination of analyte concentrations in the picomole range [7].

Among many fluorescent materials, semiconductor quantum dots (QDs) belonging to groups II-VI have been studied as emerging fluorescent materials for several decades. They are attractive in many areas because of their high fluorescence quantum yield, broad excitation wavelength, long-term photostability and emission wavelength that can be controlled by the particle sizes [8]. In biomedicine research, QDs have been successfully used for cellular labeling and DNA-based assays [9]. QDs have been conjugated with biomolecules such as antibodies, nucleic acids, and peptides for use in bioanalysis and diagnostics [10]. The possibility of using QDs in immunoassays is demonstrated through their ability to function as resonant energy-donors [11,12]. The most promising applications of QDs in immunology are in optical barcodes and multiple immune responses [13,14]. However, no successful experiments based on the parallel signal integration of different colored QDs have been reported for multi-analyte immunoreactions, despite the fact that QD-based fluoroimmunoassays show high potential in the areas of bio-analysis. The limitations of QD-based fluoroimmunoassays result from the fact that most available QDs to date still suffer from several kinds of instability, such as aggregation that leads to loss of colloidal stability in bio-labelling procedures.

Progesterone (P₄) is a steroid hormone that plays an important role in maintaining pregnancy. It helps prepare the woman's body for conception and pregnancy and it regulates the monthly menstrual cycle. The most common method for P₄ determination is chromatography because of its high accuracy, reproducibility, selectivity, and sensitivity [15]. However, this is a labor-intensive method that requires expensive instruments. Immunological methods such as radioimmunoassay (RIA), enzyme linked immunoassay (ELISA) and chemiluminescence immunoassay (CLIA) are also employed for steroid detection since they are specific, easy to use, and cost-effective [16]. However, these methods have certain drawbacks such as susceptibility to interference, short lifetime of labels, less sensitivity at low concentrations of progesterone, etc.

The integration of some nanoparticles such as gold nanoparticles, graphene, and QDs with immunological methods has allowed the progesterone assay technique to achieve high specificity, rapidity, and convenience [13]. In one study, the concentrations of progesterone in

bovine serum samples were determined based on the direct attachment of P₄Ab on a modified gold disk electrode coated with gold nanoparticles [17]. P₄-coating antigen was immobilized on a glassy carbon electrode coated with thionine-graphene oxide composites in the development of a homogeneous immunoassay [18]. In the development of a homogeneous QD-based fluorescence immunoassay, QD-antigen conjugates can be employed with an unlabeled anti-progesterone antibody. Progesterone has been detected in milk by using a competitive immunoassay using QDs as fluorescent labels [19]. However, the use of QD-conjugated antigens for competition with free antigens means that the routine determination of P₄ concentrations can become expensive.

From this point of view, CdSe/ZnS core/shell QDs have been employed as the label of anti-progesterone antibody (P₄Ab) in immunoassays for P₄ detection. Hydrophilic CdSe/ZnS QDs were conjugated with the anti-progesterone antibody (P₄Ab) via the ethyl-3-(dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) coupling method. The QDs-conjugated P₄Ab (QDs-P₄Ab conjugate) was used to develop two heterogeneous fluorescence immunoassays for the determination of progesterone in human serum. One heterogeneous assay was based on the direct binding of the sample (P₄) to the QDs-P₄Ab conjugate immobilized onto the 3-aminopropyltrimethoxysilane (APTMS) sol-gel membrane of the wells in a 96-well microtiter plate, while in our previous paper the QDs-P₄Ab conjugate was immobilized onto the GA (a mixture of GPTMS (3-glycidoxypropyltrimethoxysilane) and APTMS) sol-gel membrane of the wells [20]. In the sandwich assay, the P₄Ab was immobilized onto the APTMS sol-gel membrane of the wells and the QDs-P₄Ab conjugates were employed as a secondary antibody after the progesterone was added. In this study these two heterogeneous immunoassays using the QDs-P₄Ab conjugates have been systematically characterized and applied for the determination of progesterone in human serum, and they have been compared with the commercially available progesterone assay method of RIA.

2. Materials and Methods

2.1 Materials and reagents

3-Aminopropyltrimethoxysilane (APTMS), cadmium acetate, 4-dimethylamino pyridine (DMAP), ethyl-3-(dimethylaminopropyl) carbodiimide (EDC), 1-hexadecylamine, hexamethyldisilathiane ((TMS)₂S), mercaptopropionic acid (MPA), N-hydroxysuccinimide(NHS), trioctylphosphine oxide (TOPO), trioctylphosphine (TOP), selenium, zinc acetate, estrogen, progesterone (P₄), mouse anti-progesterone monoclonal antibody (P₄Ab), and testosterone were purchased from Sigma–Aldrich Chemical Co. (Seoul, Korea). Chloroform, *N,N*-dimethylformamide (DMF), and methanol were obtained from Honeywell Burdick & Jackson (Morristown, NJ, USA). All other chemicals, such as sodium chloride, sodium phosphate, etc., were of analytical grade and used without further purification. Real samples of human sera were obtained from the CNU (Chonnam National University) Hormone Center (Gwangju, Korea) and used to determine the concentrations of progesterone using typical RIA and QD-based fluorescence immunoassays developed herein.

2.2 Synthesis of hydrophilic CdSe/ZnS QDs

The synthesis of the CdSe/ZnS QDs was based on previously described theories with slight modification. CdSe nanoparticles were synthesized using the modified methods of Qu and Peng [21], while CdSe/ZnS QDs were synthesized following the method of Gaunt et al. [22]. For the hydrophilic surfactant coated CdSe/ZnS QDs, MPA was capped onto CdSe/ZnS QDs by following the protocols described in the literature with minimal modification [23-24]. The absorption or emission spectra of CdSe and CdSe/ZnS QDs were respectively determined by UV-Vis spectroscopy (Multiskan Spectrum, Thermo electron Co., Finland) and a fluorescence spectrophotometer (Model: F-4500, Hitachi Co., Japan).

2.3 Preparation and purification of the QDs-P₄Ab conjugates

First, 200 μ l solution of the MPA-capped QDs, 20 μ l of EDC (30 mg/ml), and 20 μ l of NHS (32 mg/ml) were mixed with a fixed amount of P₄Ab in 10 mM phosphate buffer solution (PBS) in a glass tube. The coupling reaction was performed in the glass tube at 25 °C for 1 hr with gentle agitation. The QDs-P₄Ab conjugates were confirmed by gel electrophoresis (2% agarose). A voltage of 100 V was applied along the gel for 30 min, and the gels were then photographed under UV-light. In this study, a fixed amount of CdSe/ZnS QDs (3.2 mg in 1 ml of 10 mM PBS) was conjugated to different concentrations (0, 62.5, 125, 250, 500, and 1000 ng/ml) of P₄Ab. In addition, a fixed amount of P₄Ab (0.25 mg in 1 ml of 10 mM PBS) was conjugated to different amounts (0, 0.4, 0.8, 1.6, 2.4, 3.2 mg/ml) of QDs.

The QDs-P₄Ab conjugates were separated from unconjugated QDs or P₄Ab with a Fast Protein Liquid Chromatography (FPLC) system (ÄKTA purifier, Amersham Pharmacia Biotech Co., USA) having a Sephadex G-100 column (30 cm long and 0.9 cm diameter). The column was first pre-equilibrated with PBS (0.05 M, pH 7.4) at 0.3 ml/min. The sample, which had a total volume of 500 μ l was then loaded onto the column. A 280 nm UV-light source was used to check the flow from the column, and the eluted solution was collected for further analysis. The fluorescence spectra of the purified QDs-P₄Ab conjugates were also determined using a fluorescence spectrophotometer (Model: F-4500, Hitachi Co., Japan).

2.4 Preparation of the APTMS sol-gel membrane and immobilization of the P₄Ab or QDs-P₄Ab conjugates

The APTMS sol-gel membrane in the well of a 96-well microtiter plate was prepared in the following manners [25]: 312.5 μ l of APTMS was mixed in 687.5 μ l of 99% ethanol

solvent and polymerized by adding 40 μl of 35% hydrochloric acid. The APTMS sol-gel matrix was kept at room temperature for at least 3 hours under a shaking condition (180 rpm) prior to use. Then, 5 μl of the APTMS sol-gel solution was spread on the bottom of one well in a 96-well microtiter plate and dried at room temperature for 30 min.

To immobilize the P₄Ab (or QDs-P₄Ab conjugates) onto a well in a 96-well microtiter plate, 5 μL of the APTMS sol-gel solution was first spread onto the bottom of a well and dried at room temperature for 30 min. Next, 10 μl of glutaraldehyde solution (1 wt%) and 100 μl of the P₄Ab (or QDs-P₄Ab conjugates) in 10 mM PBS (pH 7.4) were added to the well and incubated at 4 °C for 30 hours. After incubation, the well was washed with 10 mM PBS (pH 7.4) three times. Then, 100 μl of bovine serum albumin (BSA) solution (5 mg/ml) was added to the wells to block the unoccupied sites on the well surface, incubated for 3 hours, and washed with 10 mM PBS (pH 7.4) three times. The surface morphology of the APTMS sol-gel membrane containing the QDs-P₄Ab conjugates was identified by an atomic force microscopy (AFM, Multimode IV, Veeco Instruments Inc., USA) and a scanning electron microscope (SEM, S-4700, Hitachi, Japan).

2.5 QD-based fluorescence immunoassays

2.5.1 Sandwich assay

In the sandwich assay, 100 μl of progesterone (P₄) dissolved in 10 mM PBS (pH 7.4) was added to a well that was immobilized with P₄Ab (12.5 ng/100 μl /well) and incubated at 4 °C for 24 hours. After washing the well with 10 mM PBS (pH 7.4) at least three times, 100 μl of the P₄Ab-QDs conjugates was added to the well and incubated for 24 hours. The fluorescence intensity of the well was measured with the microtiter plate reader (Sapphire², TECAN Co., Austria) at excitation/emission wavelength of 475 nm and 590 nm, respectively, with various concentrations of P₄ solution (0-20,000 pg/ml), after washing the wells at least three times. The measurements of P₄ were performed in triplicate. To dissociate the binding among P₄, the immobilized P₄Ab, and the P₄Ab-QDs conjugates, 0.1 M glycine•HCl at pH 2.5-3.0 was employed as an elution buffer.

The effects on the assay performance of different amounts of the P₄Ab immobilized onto a well surface via the APTMS sol-gel membrane were investigated with 6.25 ng and 12.5 ng in 100 μl per well in a 96-well microtiter plate.

The binding between P₄ and the QDs-P₄Ab conjugates was studied at different incubation times and with various P₄ concentrations. First, the sample (P₄) was incubated at 4 °C for 12 hours to bind to the immobilized P₄Ab and the wells were washed with 10 mM PBS (pH 7.4). Then, the QDs-P₄Ab conjugates were introduced into the wells and incubated for 3, 6, and 24 hours.

The interference of some components normally existing in real blood samples such as ascorbic acid, Na⁺, Ca⁺⁺, K⁺, and different hormones (estrogen, testosterone) on the samples was examined in the P₄ concentration range from 0 to 5,000 pg/ml. Ascorbic acid (0.1, 0.5, and 1.0 mM) and some ions (K⁺, Na⁺, and Ca²⁺ in the concentrations of 50 mM, 100 mM, and 150 mM, respectively) in a sample were also investigated.

Three elution buffers were studied to separate the bonds among P₄ and immobilized P₄Ab and P₄Ab-QDs conjugates. Specifically, 0.1 M glycine•HCl (pH 2.5-3.0) and 0.2 M NaOH (pH 13.1) were prepared and used as acidic and alkaline elution buffers, respectively. 50 mM Tris-HCl (pH 7.0) containing 2% sodium dodecyl sulfate (SDS) and 50 mM dithiothreitol (DTT) was also made and used for the dissociation. Each elution buffer was incubated in a well for either 5 or 10 minutes.

In this sandwich immunoassay a significant difference in the fluorescence intensities was

also found in the P₄ concentration range of 50 to 10,000 pg/ml. The fluorescence intensity for each sample was measured after an incubation time of 3 hours.

2.5.2 Direct binding assay

In this assay, 100 µl of progesterone (P₄) dissolved in 10 mM PBS (pH 7.4) was added to each well immobilized with the QDs-P₄Ab conjugates (e.g. conjugation of 0.32 mg CdSe/ZnS QDs added to 500 ng/ml P₄Ab). After incubating the samples (P₄) for 24 hours, the wells were washed with 10 mM PBS (pH 7.4) at least three times. The 96-well microtiter plate was then inserted into the measurement chamber of the microplate reader (Sapphire², TECAN Co., Austria), and the fluorescence intensity of each well was measured at excitation/emission wavelengths of 475 nm and 590 nm, respectively, in the P₄ concentration range from 0 to 20,000 pg/ml. The measurement of P₄ was performed in triplicate. The dissociation of the binding between P₄ and the QDs-P₄Ab conjugates was carried out with 0.1 M glycine•HCl at pH 2.5-3.0 as an elution buffer.

The long-term stability of the immobilized QDs-P₄Ab conjugates was also evaluated using a well immobilized with 100 µl of the QDs-P₄Ab conjugates. After incubating the samples (P₄) at different P₄ concentrations for 24 hours and washing each well with 10 mM PBS (pH 7.4) several times, the fluorescence intensity of the well was measured every week. When the microplate was not being used, it was kept in a refrigerator.

Optimum amounts of the QDs-P₄Ab conjugates immobilized onto the wells in a 96-well microtiter plate were also investigated with 50 µl, 100 µl, and 150 µl of the QDs-P₄Ab conjugates. After immobilizing 50 µl, 100 µl, and 150 µl of the QDs-P₄Ab conjugates in wells, samples (P₄) at different concentrations were introduced into the wells and incubated for 3 hours at room temperature. The fluorescence intensity of the wells was measured after washing each well with 10 mM PBS (pH 7.4) several times.

The binding between P₄ and the QDs-P₄Ab conjugates was also studied at different incubation times (3, 6, and 24 hours) and with various P₄ concentrations.

The interference of some components such as ascorbic acid (0.1, 0.5, 1.0 mM), Na⁺ (50, 100, 150 mM), estrogen, and testosterone (500 and 5000 pg/ml) on the binding between P₄ and the immobilized P₄Ab was investigated in the P₄ concentration range from 0 to 5,000 pg/ml. The effects of pH on the progesterone measurement were also investigated.

The same three elution buffers as those used in the sandwich assay (0.1 M glycine•HCl (pH 2.5-3.0), 50 mM Tris-HCl (pH 7) containing 2% SDS and 50 mM DTT, and 0.2 M NaOH (pH 13.1)) were prepared and studied to dissociate the binding among the sample (P₄) and the QDs-P₄Ab conjugates. The elution buffer was also incubated in a well for either 5 or 10 minutes.

To remove some unbound components from a sample, such as other proteins, several washing buffers were prepared with the addition of surfactants such as Tween X20 (0.05 v/v%) and X100 (0.05 v/v% and 0.1 v/v%) to 10 mM PBS.

In this direct binding assay, a significant difference in the fluorescence intensities was found in the entire range of progesterone concentrations (50 - 10,000 pg/ml). To ensure that the affinity reaction was completely finished, the fluorescence intensity for each sample was measured after an incubation time of 3 hours.

2.6 Radioimmunoassay (RIA)

Radioimmunoassay (RIA) for progesterone was performed using Progesterone ¹²⁵I RIA Kit (ICN, Biochemical Inc., Diagnostic Division, Costa Mesa, CA, USA) according to the manufacturer's protocol with minor modifications [26]. Radioactivity was determined using a gamma counter (Packard, Cobra II 5005, Meriden, CT, USA). All samples were run in triplicate and two sets of progesterone standards were included in each assay. The concentrations of

progesterone were calculated with smart RIA (Packard, Meriden, CT, USA). The progesterone antiserum was developed in a rabbit using testosterone-17- β -hemisuccinate: BSA as an immunogene [27]. The lower limit of assay sensitivity for progesterone was 5 pg/ml.

2.7 Data analysis

Each set of data was obtained from at least three independent measurements and presented as mean \pm standard deviation (SD). The difference in the sensitivity between the different assays or in the fluorescence intensity in the presence of different ions (K^+ , Na^+) was evaluated by one-way analysis of variance (ANOVA). The differences observed between samples were considered to be significant at p -values (probability) less than 0.05. The statistical data tests were conducting using the software InStat (vers.3.01, GraphPad Software Inc., San Diego, CA, USA). The normalized fluorescence intensity (Norm. Fl. intensity) represents the ratio of the fluorescence intensity enhancement, which is calculated by dividing the difference between the fluorescence intensity (FI) at a given progesterone concentration and the fluorescence intensity (FI₀) at a concentration of 0.0 pg/ml by the fluorescence intensity (FI₀) at a progesterone concentration of 0.0 pg/ml, i.e. $(FI - FI_0)/FI_0$.

3. Results and discussion

3.1 Properties of the QDs-P₄Ab conjugates

The luminescence and monodispersity of the QDs-P₄Ab conjugates are essential for a QD-based fluorescence immunoassay to achieve high sensitivity and performance. In Fig. 1(a), gel images of the QDs-P₄Ab conjugates are shown clearly under UV light. The fluorescence emission intensity of the QDs-P₄Ab conjugates changed with different amounts of the anti-progesterone antibody (P₄Ab) and QDs with fixed amounts of QDs (3.2 mg/ml) or P₄Ab (250 ng/ml). In the gel image, the QDs-P₄Ab conjugates moved slower than QDs alone because of the decrease in negative charge on the surface of QDs after conjugation with P₄Ab. An adequate combination of the QDs and P₄Ab for conjugation could be selected from the gel image with a high emission intensity at the band site, as shown in Fig. 1(a), where few QDs and P₄Ab have been washed without conjugation. In this work, a combination of 1.6 mg/ml QDs and 250 ng/ml P₄Ab could be used for the optimal conjugation of the QDs to P₄Ab.

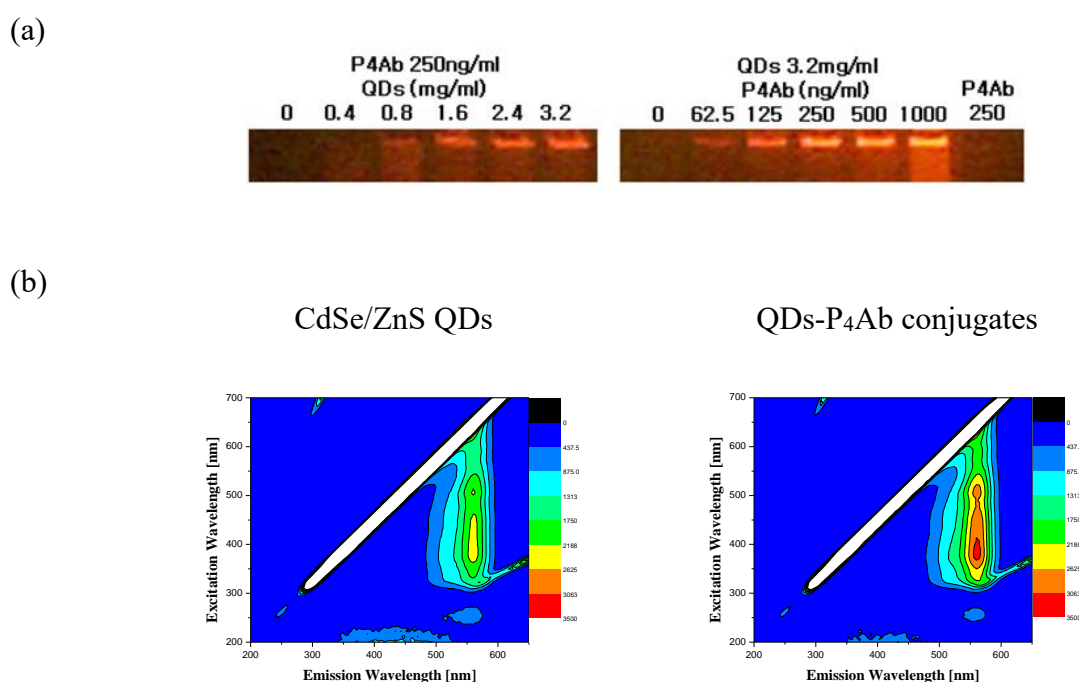


Fig. 1. (a) Photo images of gel electrophoresis (2% agarose) for the conjugation of QDs to P₄Ab. left) QDs-P₄Ab conjugates with different amounts of CdSe/ZnS QDs (QDs = 0, 0.4, 0.8, 1.6, 2.4, and 3.2 mg/ml) with a fixed amount of P₄Ab (250 ng/ml). right) QDs-P₄Ab conjugates with different concentrations of P₄Ab (P₄Ab = 0, 62.5, 125, 250, 500 and 1000 ng/ml, right most only P₄Ab 250 ng/ml) with a fixed amount of QDs (3.2 mg/ml); (b) 2D fluorescence spectra of CdSe/ZnS QDs and the purified QDs-P₄Ab conjugates measured with a fluorescence spectrophotometer (Model: F-4500, Hitachi Co.)

After conjugation of the QDs to P₄Ab via EDC/NHS coupling chemistry, the QDs-P₄Ab conjugates were separated and purified from a number of components such as EDC, NHS, unconjugated QDs, and P₄Ab by using ultrafilter-centrifugation with YM-100 and a fast protein liquid chromatography (FPLC) system equipped with a Sephadex G-100 gel column. Then, the QDs-P₄Ab conjugates were concentrated with a freeze dryer and used either for immobilization onto a well in a direct binding assay or for affinity binding with the sample as a second antibody in a sandwich assay.

As shown in Fig. 1(b), there were no differences in the fluorescence spectra between the CdSe/ZnS QDs and the purified QDs-P₄Ab conjugates after conjugation. The excitation wavelength is in the range of 300-530 nm and the emission band edge is 590 nm.

3.2 Surface morphology of the APTMS sol-gel membranes

The surface morphologies of the APTMS sol-gel membranes in the absence and presence of the QDs-P₄Ab conjugates were characterized by AFM and SEM, respectively, and they are shown in the images in Fig. 2. In the absence of QDs-P₄Ab conjugates, the mean height (Ra, 0.333 nm) and the mean roughness (Rq, 0.404 nm) indicated that the APTMS sol-gel membrane had a flattened and smooth surface (Fig. 2(a)). The APTMS sol-gel membrane in the presence of QDs-P₄Ab conjugates had a larger mean height (Ra, 1.410 nm) and a larger mean roughness (Rq, 1.701 nm) than the sol-gel membrane without the QDs-P₄Ab conjugates (Fig. 2(b)). SEM images at different scales were also taken to show the surface morphology of the APTMS sol-gel membrane in the presence of the QDs-P₄Ab conjugates (Fig. 2(c) & 2(d)).

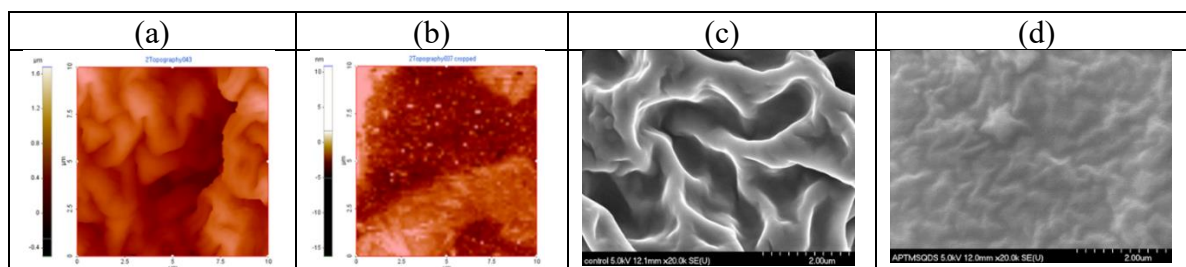


Fig. 2. AFM images of the APTMS sol-gel membrane in the absence (a) and presence (b) of the QDs-P₄Ab conjugates, as well as SEM images of the APTMS sol-gel membrane in the presence of the QDs-P₄Ab conjugates with different scale bars: (c) 2.0 μm and (d) 0.2 μm.

3.3 Development of QDs-based fluorescence immunoassays

Two heterogeneous immunoassays have been developed by immobilizing either the anti-progesterone antibody (P₄Ab) or the QD-P₄Ab conjugates onto a well surface of a 96-well microtiter plate via the APTMS sol-gel membrane. These two assays have been characterized for the determination of progesterone in human serum, and their results are compared with those of a commercially available progesterone assay method, RIA.

3.3.1 Sandwich immunoassay

The amount of P₄Ab immobilized on the well via the APTMS sol-gel membrane affects the performance and sensitivity of a sandwich assay. Therefore, different amounts of P₄Ab were immobilized on the well surface, and their effects on progesterone measurements are shown in Fig. 3. The normalized fluorescence intensity with progesterone concentrations was higher at 12.5 ng P₄Ab/100 μl/well than 6.25 ng P₄Ab/100 μl/well. Therefore, in this sandwich immunoassay, the concentration of 12.5 ng P₄Ab/well led to immobilization on the well surface through the APTMS sol-gel membrane.

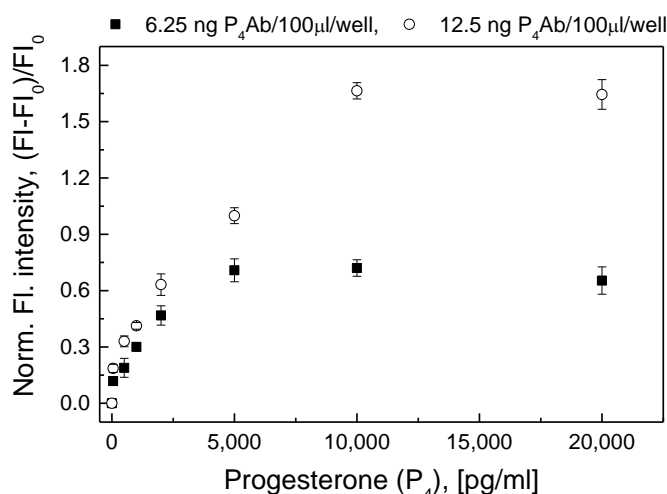


Fig. 3. Change in the normalized fluorescence intensity of the wells with various progesterone concentrations at different amounts of P₄Ab immobilized per well. Data are represented as mean±SD (n=3).

The performance of a sandwich immunoassay can be affected by the binding capability between the immobilized P₄Ab and P₄ as well as that between P₄ and the QD-P₄Ab conjugates. The binding capability is related to the incubation time, buffer pH value, amounts of immobilized P₄Ab, etc. The effects of the binding between P₄ and the QDs-P₄Ab conjugates on the assay performance were investigated with different incubation times (3, 6, and 24 hours) at various P₄ concentrations. Fig. 4 shows the normalized fluorescence intensity with different progesterone concentrations and at different incubation times. The incubation time between P₄ and the QDs-P₄Ab conjugates did not significantly affect the change in the normalized fluorescence intensity, i.e. the assay performance.

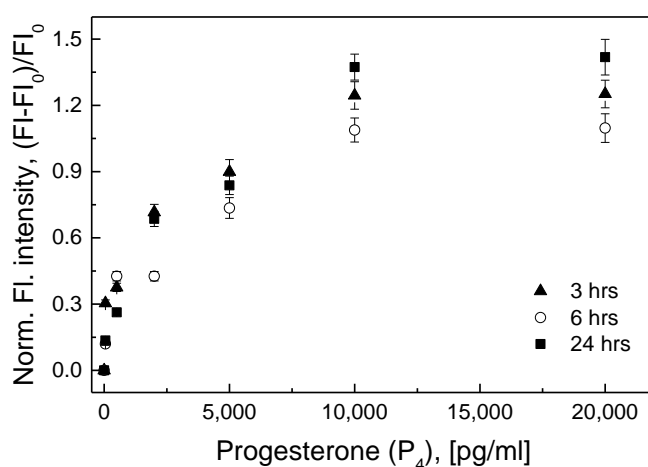


Fig. 4. Change in the normalized fluorescence intensity of the wells with various progesterone concentrations at different incubation times between P₄ and the QDs-P₄Ab conjugates. Data

are represented as mean \pm SD (n=3).

Samples containing ascorbic acid (up to 1.0 mM), Na⁺ ion (to 150 mM) or estrogen and testosterone were introduced into a well immobilized with P₄Ab, then the QDs-P₄Ab conjugates were added to the well. After incubating and washing the well with 10 mM PBS (pH 7.4), the normalized fluorescence intensity decreased for samples containing 0.1 mM ascorbic acid. This indicates that the presence of ascorbic acid in the samples interfered with the binding between P₄ and P₄Ab in the sandwich immunoassay. However, in the samples with other components, the normalized fluorescence intensity was not significantly changed (less than 10%) (data not shown).

The dissociation of the binding among the samples (P₄), the immobilized P₄Ab, and the QDs-P₄Ab conjugates in the sandwich assay were investigated using three elution buffers, as in the direct binding assay. The changes in the normalized fluorescence intensity with different progesterone concentrations are shown in Fig. 5 with three elution buffers and elution times. When no elution buffer was used, the normalized fluorescence intensity increased with increasing progesterone concentration. The normalized fluorescence intensity stayed almost constant at 0.12, with 0.1 M glycine•HCl at an elution time of 5 or 10 minutes. The results show that 0.2 M NaOH did not dissociate the binding between P₄ and the QDs-P₄Ab conjugates at an elution time of 5 minutes or more. The 50 mM Tris-HCl buffer dissociated the binding very effectively at an elution time of 10 minutes, but at an elution time of 5 minutes, the normalized fluorescence intensity with 5,000 pg/ml of progesterone (P₄) remained at about 0.23. This indicates that 0.1 M glycine•HCl can be used to dissociate the binding between P₄ and the immobilized P₄Ab in the sandwich immunoassay.

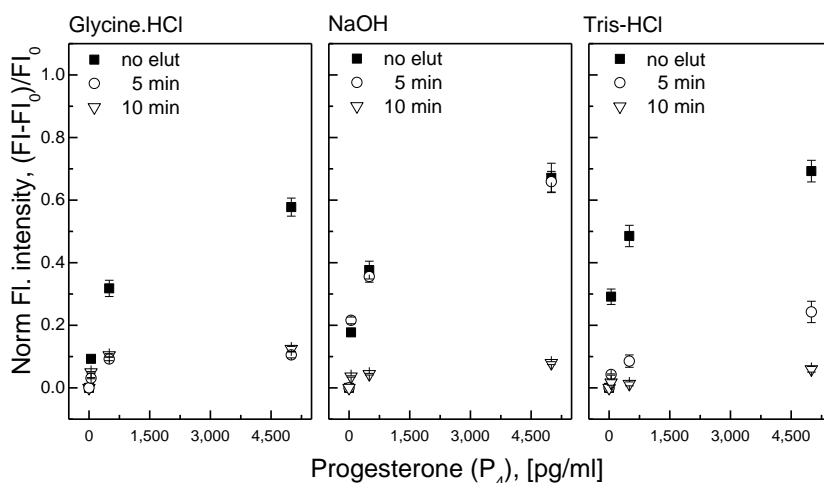


Fig. 5. Effects of three elution buffers on the dissociation of the binding among P₄, the immobilized P₄Ab, and the QDs-P₄Ab conjugates in the sandwich assay. Data are represented as mean \pm SD (n=3).

A number of other experimental conditions, including immobilization time, were also investigated and optimized. Table 1 summarizes the final experimental conditions that were

ultimately used in the sandwich immunoassay.

Table 1. Final experimental conditions of the sandwich immunoassay

Step		Volume/well	Procedure
1	Pretreatment	5 μ l	Addition of APTMS sol-gel solution and incubation for 5 min
2	Immobilization	100 μ l	P4Ab at 4 °C for 24 hours
3	Washing	200 μ l	PBS three times
4	Blocking	100 μ l	BSA
5	Washing	200 μ l	PBS three times
6	Immunoassay	100 μ l	P ₄ incubated at 4 °C for 24 hours
	Washing	200 μ l	PBS three times
	Incubation	100 μ l	QDs-P ₄ Ab conjugates at 4 °C for 3 hours
7	Washing	200 μ l	PBS three times
8	Measurement	---	Fluorescence intensity at 475 nm/590 nm
9	Elution	200 μ l	Glycine•HCl for 5 min
10	Washing	200 μ l	PBS three times

Under the final experimental conditions of the sandwich assay, the performance of the assay was assessed with a series of standard solutions with different progesterone concentrations. The calibration curve is shown in Fig. 6. The regression equation and parameters were obtained from a 4-parameter-logistic curve by using OriginPro Software (OriginLab Co., USA).

The plot indicates that linear concentration range of P₄ was from 2184.6 to 117,082 pg/ml. The detection limit, which was calculated from 10 % of the maximum normalized fluorescence intensity (i.e., 3.28578), was is 553.9 pg/ml. The sensitivity of the assay was 18,251.96 pg/ml. The precision of the assay was estimated from the relative standard deviation (RSD) of the signals obtained for the analysis of a triplicate of samples containing 2,000 pg/ml, and it was ultimately found to be 3.7 %.

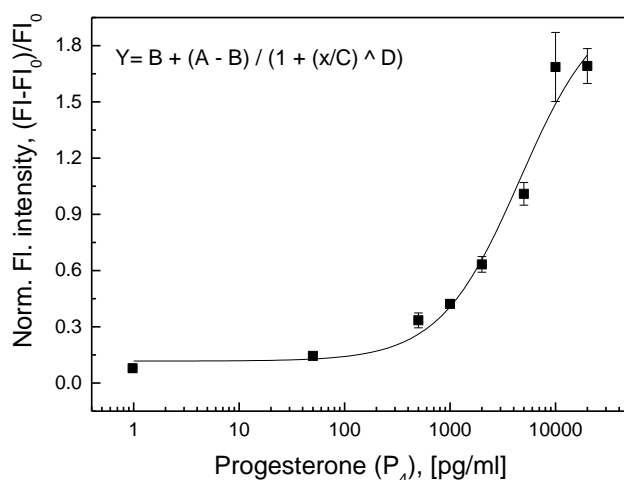


Fig. 6. Calibration curve for P₄ concentrations in the sandwich immunoassay. Each point on the curve is the average of three measurements. The error bars correspond to ± 3 SD. The RSD in all points is <5%. Parameter A is 0.09517, which is the background of the normalized fluorescence intensity; parameter B is 3.28578, which is the maximum normalized fluorescence intensity obtained; parameter C is 18,251.96 pg/ml, which is the sensitivity of the assay; and parameter D is 0.72629, which is the slope of the curve. The regression coefficient (R^2) of the curve is 0.99235. Data are represented as mean \pm SD (n=3).

3.3.2 Direct binding immunoassay

The immobilization capacity of the QDs-P₄AB conjugates onto the well surface affects the performance and sensitivity of the immunoassay. The immobilization of the QDs-P₄AB conjugates can be accomplished in several ways: a) encapsulation of the QDs-P₄Ab conjugates into the APTMS sol-gel membrane on the well surface, b) peptide binding between the carboxyl group of the water-soluble QDs in the QDs-P₄Ab conjugates and the amine group of the APTMS in the APTMS sol-gel membrane etc.

The encapsulation of the QDs-P₄Ab conjugates into the APTMS sol-gel membrane can be influenced by the gellation time of the APTMS sol-gel solution introduced into the well of a 96-well microtiter plate. The effects of the gellation time on the immobilization capacity of the QDs-P₄Ab conjugates were investigated by spreading the sol-gel solution into wells and incubating the samples for 5, 10, 20, and 30 minutes at room temperature. The measurement results of the fluorescence intensity at various P₄ concentrations showed that the intensity decreased as the gellation time of the sol-gel solution increased. That is, due to their soft texture, the QDs-P₄Ab conjugates were more successfully encapsulated into the APTMS sol-gel membrane at a short gellation time (e.g. 5 min) than at a long gellation time (e.g., 30 min) (data not shown).

In this assay, the direct binding of P₄ to the immobilized QDs-P₄Ab conjugates decreased the fluorescence emission intensity of the QDs, because it changed the negative charge distribution around the QDs [28].

The long-term stability of the QDs-P₄AB conjugates immobilized in a well was investigated at different P₄ concentrations. After 7 weeks the fluorescence intensity of the well was mostly maintained, i.e., over 80% of its initial value (data not shown). This showed that the immobilization of the QDs-P₄Ab conjugates into the APTMS sol-gel network created a special

strong texture.

Different amounts of the QDs-P₄Ab conjugates were immobilized onto a well surface and their effects on progesterone are shown in Fig. 7. There was no significant difference in the normalized fluorescence intensity in the wells with 50, 100, and 150 μ l of the QDs-P₄Ab conjugates. Therefore, in this direct binding assay, 100 μ l of the QDs-P₄Ab conjugates have been immobilized into the APTMS sol-gel membrane on a well surface.

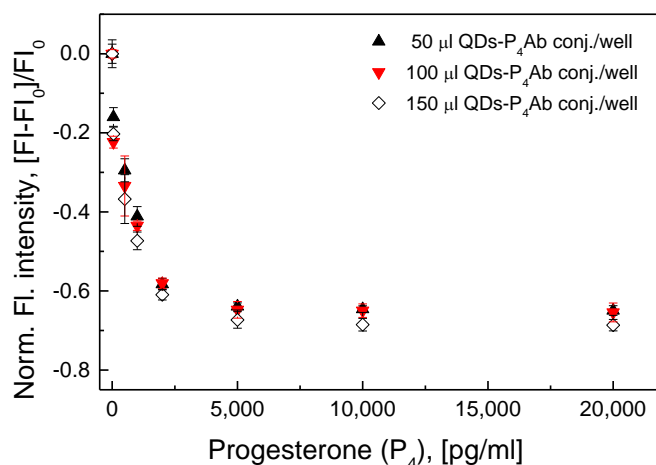


Fig. 7. Change in the normalized fluorescence intensity of the wells immobilized with 50, 100, and 150 μ l of the QDs-P₄Ab conjugates with respect to progesterone (P₄) in a direct binding assay. Data are represented as mean \pm SD (n=3).

In an immunoassay, Biomolecular samples should be analyzed as quickly as possible. This is because the analysis time of a sample is affected by the binding between the antigen and antibody, and the binding of P₄ to the immobilized QDs-P₄Ab conjugates depends on the incubation time of P₄. The change in the normalized fluorescence intensity with different incubation times and at various P₄ concentrations is shown in Fig. 8. The normalized fluorescence intensity decreased in the concentration range from 0 to 5,000 pg/ml, regardless of the P₄ incubation time.

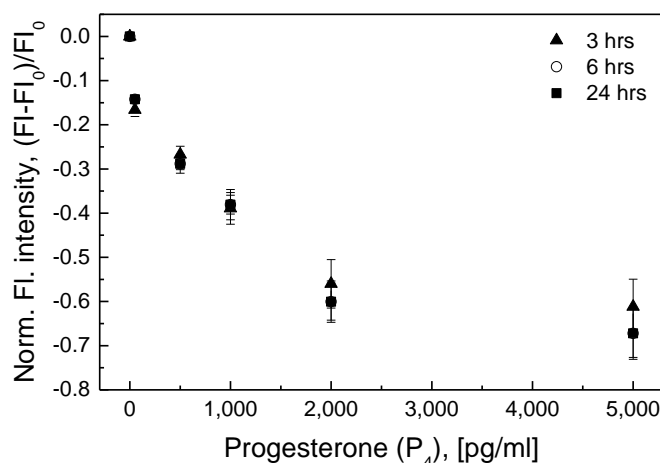


Fig. 8. Change in the normalized fluorescence intensity with various progesterone concentrations at different incubation times. Data are represented as mean \pm SD (n=3).

Next, the interfering effects of ascorbic acid (up to 1.0 mM) as well as Ca⁺⁺, K⁺, and Na⁺ ions (to 150 mM) on the binding between P₄ and the QDs-P₄Ab conjugates were investigated. The fluorescence intensity of the samples with additives did not change significantly compared to the intensity of the sample without additives. The addition of estrogen and testosterone to the sample did not interfere with the binding of P₄ to the QDs-P₄Ab conjugates. (data not shown). However, there may be some amino acids and proteins in samples which would interfere with the binding of the samples (P₄) to the QDs-P₄Ab conjugates.

The dissociation of the binding between sample (P₄) and the QDs-P₄Ab conjugates plays an important role in the repeated use of a well immobilized with the QDs-P₄Ab conjugates. The effects of three elution buffers on the dissociation are shown in Fig. 9 at a few concentrations of progesterone. When no elution buffer was used, the normalized fluorescence intensity decreased with increasing concentrations of progesterone.

It was also decreased with 0.1 M glycine•HCl for an elution time of 5 or 10 minutes, where the binding between P₄ and the QDs-P₄Ab conjugates was not dissociated completely. However, the binding between P₄ and the QDs-P₄Ab conjugates was not eluted with 0.2 M NaOH. From these results, we used 0.1 M glycine•HCl to effectively dissociate the binding between P₄ and the QDs-P₄Ab conjugates immobilized in a well.

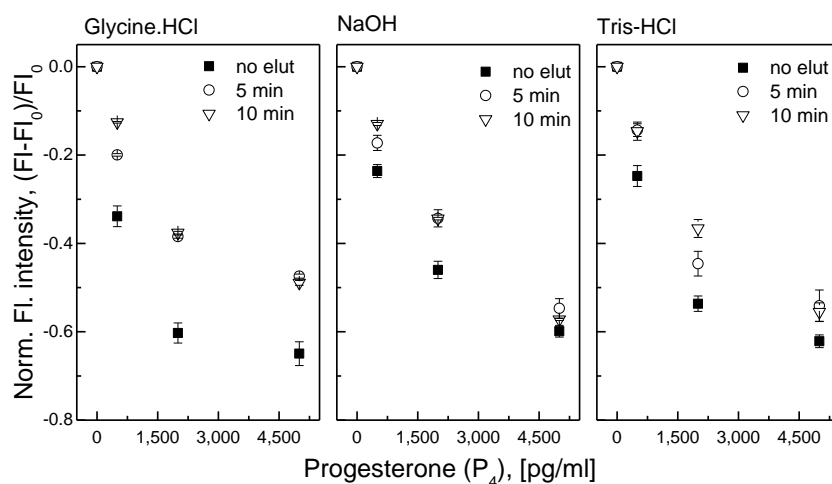


Fig. 9. Effects of three elution buffers on the dissociation of the binding between P₄ and the QDs-P₄Ab conjugates in the progesterone concentration range of 0 - 5,000 pg/ml. Data are represented as mean \pm SD (n=3).

To wash away the proteins in the samples and in some of the unbound samples, 10 mM PBS with surfactants (Tween X20 and X100) was employed after incubating the sample in a well immobilized with the QDs-P₄Ab conjugates. The fluorescence intensity did not change significantly (less than 10%) with PBS containing surfactants between different progesterone concentrations.

Some other experimental conditions, such as sample volume, were investigated, and the final experimental conditions ultimately used for the assay are summarized in Table 2.

Table 2. Final experimental conditions for the direct binding assay

Step		Volume/well	Procedure
1	Pretreatment	5 μ l	Addition of APTMS sol-gel solution and incubation for 5 min
2	Immobilization	100 μ l	QDs-P ₄ Ab conjugates at 4 $^{\circ}$ C for 24 hours
3	Washing	200 μ l	PBS three times
4	Blocking	100 μ l	BSA
5	Washing	200 μ l	PBS three times
6	Immunoassay	100 μ l	P ₄ incubated at 4 $^{\circ}$ C for 24 hours
7	Washing	200 μ l	PBS three times
8	Measurement	---	Fluorescence intensity at 475 nm/590 nm
9	Elution	200 μ l	Glycine•HCl for 5 min
10	Washing	200 μ l	PBS three times

Under the final experimental conditions, the performance of the direct binding assay was assessed as a series of standard solutions with different progesterone concentrations. The calibration curve was adjusted to an exponential decay curve [29], as shown in Fig. 10.

The regression equation and parameters were obtained from a 4-parameter-logistic curve by using OriginPro Software (OriginLab Co., USA). The plot indicates that the linear concentration range of P₄ was from 28.95 to 26,607.7 pg/ml. The detection limit, which was calculated from 10% of the minimum normalized fluorescence intensity (i.e. -0.90743), was 3.320 pg/ml. The sensitivity of the assay was 987.24 pg/ml. The precision of the assay was estimated from the RSD of the signals obtained for the analysis of a triplicate of samples containing 2,000 pg/ml, and it was ultimately found to be 4.5 %.

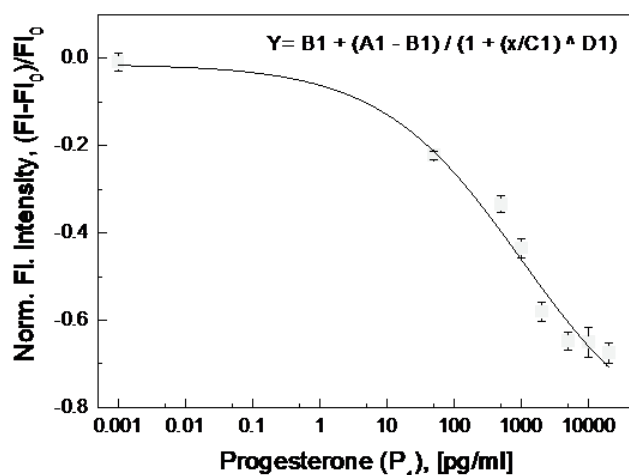


Fig. 10. Calibration curve for P₄ concentrations in the sandwich immunoassay. Each point on the curve is the average of three measurements. The error bars correspond to ± 3 SD. The RSD in all points is <5%. Parameter A1 is -0.01391, which is the background of the normalized fluorescence intensity; parameter B1 is -0.90743, which is the maximum normalized fluorescence intensity obtained; parameter C1 is 987.24 pg/ml, which is the sensitivity of the assay; and parameter D1 is 0.41518, which is the slope of the curve. The regression coefficient (R^2) of the curve is 0.99709. Data are represented as mean \pm SD (n=3).

3.4 Applications to real samples

Two types of heterogeneous immunoassay, i.e., a direct binding assay and a sandwich assay, were applied to determine the P₄ concentrations in several real samples of human serum, and these were compared with the results obtained using a commercial RIA kit. A calibration curve from a commercial RIA kit was constructed, and the expression was obtained using a least square procedure as follows:

$$\text{CPM} = 407.24 + 2561.02 * \exp(-0.015 * X)$$

where CPM is the arbitrary unit for the gamma counter and X is the P₄ concentration in the

range from 0 to 10,000 pg/ml. The regression coefficient (R^2) of the curve was 0.9916.

Fig. 11 shows the correlations of the results obtained from two heterogeneous immunoassays and the RIA method. Some samples (Nos.7-9) were spiked by adding a standard P_4 solution (5,000 pg/ml) to real samples. The mean difference in P_4 concentrations determined using the three methods was within 0.15, i.e. 15.0 %, resulting from the measurement errors and regression equations of each calibration curve. That is, the standard deviation among the three assays was not high and was within the range of acceptability, i.e. less than 20%. This clearly indicates that the fluorescence heterogeneous immunoassays developed here may be comparable and acceptable alternative tools for the determination of P_4 concentrations in clinical diagnosis.

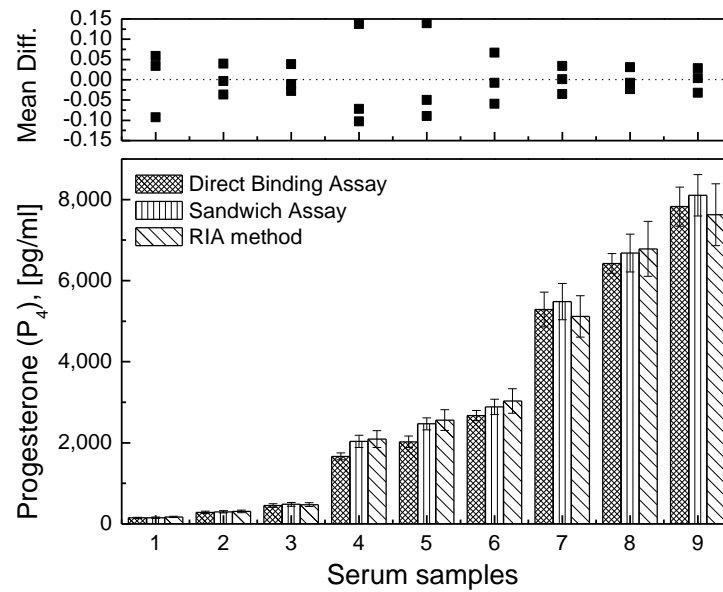


Fig. 11. Comparison of P_4 concentrations in human serum determined by using a direct binding assay, sandwich assay, and the RIA method. The mean difference represents $((P_4$ concentration measured with an assay - mean value of P_4 concentrations measured with three assays) / mean value of P_4 concentrations measured with three assays). Data are represented as mean \pm SD (n=3).

4. Conclusion

Fluorescence heterogeneous immunoassays using CdSe/ZnS core/shell QDs were newly developed in this study to determine the concentrations of progesterone in human serum. The detection scheme was based on the direct change in the fluorescence intensity of QDs on the binding of the QDs-P₄Ab conjugates to P₄. Hydrophilic CdSe/ZnS QDs were conjugated to an anti-progesterone antibody (P₄Ab) via EDC/NHS chemistry. Two types of heterogeneous assay were studied in a 96-well microtiter plate. While the direct binding assay was based on the direct binding of P₄ to the QDs-P₄Ab conjugates immobilized onto the APTMS sol-gel membrane of the wells, the sandwich assay was based on the binding of P₄ to P₄Ab immobilized onto the APTMS sol-gel membrane of the wells, and then on the binding of P₄ to the QDs-P₄Ab conjugates as a second antibody. The change in the fluorescence intensity of the QDs-P₄Ab conjugates was correlated to the P₄ concentrations, as the intensity increased with increasing P₄ concentrations in the sandwich assay, but it decreased with increasing P₄ concentrations in the direct binding assay. While the sandwich assay had a detection limit of 553.9 pg/ml and a sensitivity of 18,251.96 pg/ml with a linear range of 2184.6 – 117,082 pg/ml, the direct binding assay had a detection limit of 3.32 pg/ml and a sensitivity of 987.24 pg/ml with a linear range of 28.95 – 26,607.7 pg/ml. This indicates that the developed heterogeneous assays have been successfully applied for the determination of P₄ in real human serum. The results showed a good correlation with the accredited RIA, suggesting that the developed assays meet the demands for clinical diagnosis.

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