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A new one-step antigen heterologous homogeneous fluorescence immunoassay for progesterone detection in serum

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ABSTRACT

A new homogeneous immunoassay for the detection of progesterone was developed to measure its concentration in human serum. We utilized the weak cross-reactivity of a monoclonal anti-progesterone antibody to an analog molecule (in this case β-estradiol) to create a mixture, in which the fluorescence-labeled antibody (AbF) and quencher-labeled BSA-estradiol (eBSAq) were at optimized equilibrium. At this stage, most antibodies were bound to eBSAq and the fluorescence of AbF was quenched. After adding samples containing free progesterone to the system, these would replace the eBSAq at the antigen-binding site. The fluorescence would be released. In contrast to conventional competitive immunoassays, the fluorescence signal increases with increasing progesterone concentration, greatly simplifying detection and calibration. The performance of the assay was very simple; there was only one mixing step; and other hormones like testosterone, estradiol or dehydroepiandrosterone (DHEA) do not interfere the assay. A wide linear range from 0.1 μg/L to 100 μg/L was achieved in buffer, with a LOD of 0.1 μg/L. In human serum the LOD was 5 μg/L, and the linear range was 5–500 μg/L. For this assay it is important to find the right combination of antibody and cross-reactive antigen. If such a combination could be defined, it is conceivable to apply this assay to a wide range of analytes.

1. Introduction

Due to their simple performance homogeneous immunoassays could also be declared as “mix and measure” technique [1]. Unlike in heterogeneous immunoassays, there is no need to immobilize any reagents on a surface and there are no washing-steps. Therefore, homogeneous assays only have a few steps, in ideal case, only one step needs to be done [2,3], so work and time could be saved. Because of their simple performance homogeneous immunoassays could be easily automated and this makes homogeneous assays appropriate for high throughput applications [4,5].

The essential part of designing a homogeneous immunoassay is to find a way to observe the antibody–antigen binding in solution. Various mechanisms triggered by antigen–antibody binding can be utilized for homogeneous immunoassays [1]. A well-known method is using enzyme-reactions [6–8]. In this case an enzyme is conjugated with small analyte-molecules (haptens). When the antibodies react with the enzyme-conjugates, the enzyme reactivity would be suppressed. If a sample with free analyte is added to the system, this free analyte would react with the antibodies leading to a release of the enzyme-conjugates from the antibodies and a reconstitution of the enzyme reactivity. Another widely used method is gold-nanoparticle aggregation [9,10]. In this case large molecules like proteins could be detected. The antibodies are linked with gold-nanoparticles and will be mixed with samples. If the gold-nanoparticle-antibody conjugates react with the proteins in the sample, aggregation occurs. This aggregation induces a color-change in the solution.

Fluorescence labeling is also used for homogeneous immunoassays [11–16]. For example, some fluorophore binding antibodies are able to change the fluorescence of the fluorophores [11,15–17]. Using this character a system could be created to react with various analytes. One example was an assay containing a fluorophore–analyte conjugate and two antibodies, one binding the analyte and the other binding and quenching the fluorophore. Only one of the antibodies could bind the conjugate at the same time for sterical reasons. Addition of the sample containing the analyte was changing the binding equilibrium of the antibodies. Therefore, the fluorescence signal decreases proportionally to the

Abbreviations: LOD, level of detection; FRET, Förster resonance energy transfer; PBS, phosphate buffered saline; BHQ, black hole quencher; BSA, bovine serum albumin; NHS, *N*-hydroxysuccinimide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide

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analyte concentration [11]. Gold nanoparticles or gold surfaces can also be used as quenchers for FRET-based or fluorescence quenching assays [29,30].

In our earlier work we found out that in a conjugate of fluorescein isothiocyanate (FITC) and tetrahydrocannabinol (THC) the fluorescence of fluorescein is suppressed [12]. After binding to anti-THC-antibodies, the fluorescence could be released. Using this character we designed a new homogeneous immunoassay for THC in saliva samples. In another work of our group we described a FRET-based homogeneous immunoassay [13]. This assay could also detect THC successfully in saliva samples. In this assay we mixed a fluorescence-labeled anti-THC-antibody with saliva samples, then a THC-BSA-quencher conjugate would be added to the mixture. If there was no THC in the sample, the antibody would react with the conjugate and the fluorescence would be quenched. Otherwise the fluorescence would remain unchanged. However, this assay still needed two mixing-steps: first a preincubation of the antibody with the THC-quencher-conjugate, then the sample (containing free THC) would be added into the system to compete with the THC-quencher-conjugate. But the THC-antibody binding was so stable, that a replacement did not occur.

In this work, we modified the homogeneous immunoassay mentioned above to make it suitable to estimate progesterone-concentrations. By using a new fluorophore–quencher-combination and new quencher-conjugates we could develop a novel one-step homogeneous immunoassay (Fig. 1). The anti-progesterone antibody we used for this assay had minimal cross-reactivity with other steroid hormones because these hormones have similar structures. This cross-reactivity is actually inevitable. For the new immunoassay we used a β-estradiol-BSA-quencher conjugate (eBSAq) to quench the labeled anti-progesterone-antibody (AbF). First we prepared a mixture of AbF and eBSAq. If eBSAq was in excess, at the dynamic equilibrium stage most AbF in the mixture was bound in the AbF–eBSAq-complex, where the fluorescence of AbF was quenched. Then progesterone samples were added to the system. Because the AbF–eBSAq binding was reversible and the progesterone-AbF binding was far stronger, free progesterone could replace eBSAq from the complex. The fluorescence would then be released. In our earlier work we used the Dyomics dyes DY481 and DYQ661 as fluorophore–quencher-pair [13]. Other common fluorophore–quencher pairs include Cy5/Cy5.5, Cy5/BHQ2, Cy3/BHQ2 or Texas Red/BHQ1 [27,28]. For the assay described here we chose the novel red fluorescence dye S2314 (Fig. 3) to label the antibody and the Dyomics dye DY800 to make conjugate eBSAq. The fluorophore S2314 emitted at 664 nm when coupled on antibodies, which is a wavelength region with less background signals than around 510 nm or 580 nm. The fluorescence was much stronger than DY481. S2314 is also available for commercial applications. The fluorophore DY800 had weak fluorescence and is therefore an appropriate quencher. After the AbF–eBSAq-formation the fluorescence could be quenched up to 70%. This is much better than the old fluorophore–quenching-pair (about 40%) (Fig. 2).

2. Experimental section

2.1. Instruments and reagents

The fluorescence measurement was performed with a LS-55 spectrofluorimeter (PerkinElmer, Coventry, UK). The extinction measurement was performed with NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, USA). The NHS ester of the fluorescence dye S2314 was purchased from FEW Chemicals GmbH (Wolfen, Germany). The quencher DY800-NHS was purchased from Dyomics GmbH (Jena, Germany). Bovine serum albumin was purchased from Applichem GmbH (Darmstadt, Germany). β-Estradiol-6-one 6-(*O*-carboxymethyloxime), 4-Pregnene-3,20-dione (progesterone), *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma-Aldrich Co. (Taufkirchen, Germany). Bronopol was purchased from Molekula Ltd. (Shaftesbury, UK). Human male serum was purchased from Lonza (Cologne, Germany).

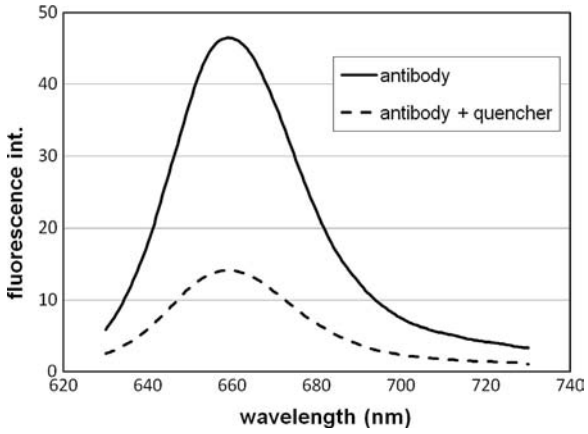

Fig. 2. Fluorescence spectrum of AbF before and after adding eBSAq. After binding of the dye labeled antibody to the quencher labeled antigen complex, the fluorescence intensity is reduced significantly.

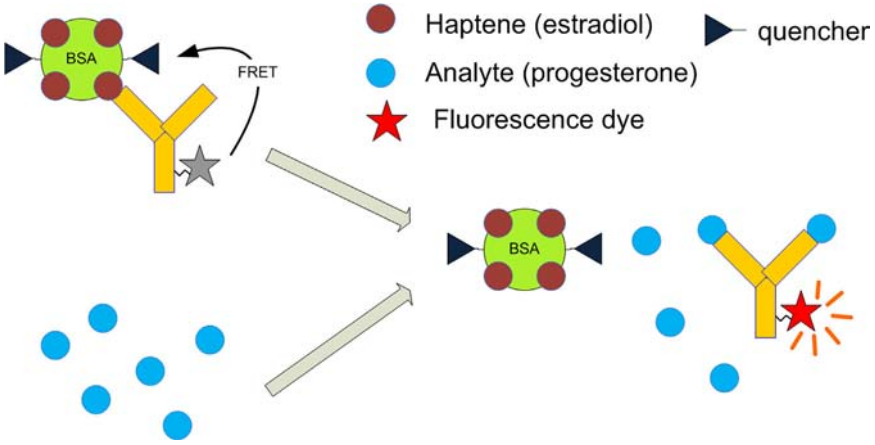

Fig. 1. Schematic representation of the new homogeneous immunoassay: The equilibrium of the complex of dye-labeled antibody and quencher labeled antigen-BSA conjugate is disturbed by free antigen from the sample. Then molecules of the dye containing antigen–antibody complex are destroyed and the fluorescence of the dye is released according to the concentration of free analyte.

2.2. Generation of anti-progesterone antibodies

Monoclonal anti-progesterone antibodies were raised using hybridoma technique [18]. For this purpose Balb/c mice were immunized three times with conjugates of bovine serum albumin and progesterone (4-Pregnen-3, 20-dione 3-*O*-carboxymethyloxime: BSA; Steraloids, London, UK). Immunization started with 100 μg conjugate using Freund's complete adjuvant. Booster immunizations were carried out six and eight weeks after the first immunization using 50 μg conjugate without adjuvant. Four days after the final booster immunization electrofusion of spleen cells with myeloma cells (P3 × 63Ag8.653, ATCC CRL-1580) in the presence of polyethylene glycol 8000 was performed as described [19]. Selected hybrids were cultivated in RPMI 1640 medium (containing 10% fetal calf serum, 2 mM glutamine and 50 mM β-mercaptoethanol) and subcloned by limiting dilution on mouse peritoneal feeder cells. Culture supernatants of clones and subclones were tested in an enzyme immunoassay (ELISA) for antigen binding. The class and subclass of monoclonal antibodies were determined as described [19]. Purification of antibody from culture supernatant was performed by protein A affinity chromatography [20].

2.3. Preparation of eBSAq

Carboxyl-β-estradiol was dissolved in DMF to create a 10 g/L solution. EDC was dissolved in Ethanol to create a 20 g/L solution. 20 mg of NHS was then dissolved in 1 ml of EDC-solution to create EDC-NHS mix-solution (20 g/L for both). For the activation of carboxyl-β-estradiol, 338 μL of EDC-NHS-solution and 112.5 μL of carboxyl-β-estradiol solution were mixed together. After incubation for 15 min at room-temperature, BSA (20 g/L in 0.1 M bicarbonate buffer, pH 8.5) was added to the mixture. After 24 h the mix-solution was dialyzed against 5% ethanol in water for 4 h. Dialysis was repeated three times for 2 h against water.

In the next step the quencher DY800 was coupled to estradiol-BSA. The method followed the specifications provided by Dyomics with a few modifications. Briefly, 10 μl bicarbonate buffer (1 M, pH 8) was added to prepared estradiol-BSA solution (the volume and concentration of estradiol-BSA solution could vary from 10 to 50 μL, in order to achieve various coupling-ratios). DY800 was diluted in DMSO (dry) to create 5 g/L solution, which then (volume could vary depending on the amount of estradiol-BSA used) was added to the estradiol-BSA solution. The mixture was incubated overnight. At the end the eBSAq was extracted with pro-spin CS-800 protein-purification columns (emp Biotech, Berlin, Germany). The Dye/BSA ratio could be calculated with the form provided by Dyomics:

$$\frac{\text{dye}}{\text{protein}} = \frac{A_{800} \times \varepsilon_P}{\varepsilon_F (A_{280} - A_{800} \times k)}$$

with A: absorption, ε_P: extinction coefficient of BSA-estradiol at 280 nm, ε_F: extinction coefficient of fluorescence-dye at 800 nm, k: correction factor (from manufacturer). The values of absorption were measured with NanoDrop Spectrophotometer.

To achieve various coupling-ratios, 6 syntheses was performed. Dye-NHS/estradiol-BSA ratios during the coupling and Dye-BSA ratios reached after the coupling are shown in Table 1.

Table 1
Various dye-BSA ratios achieved by using various dye-NHS/estradiol-BSA ratios in synthesis.

Ref. No.	1	2	3	4	5	6
Dye-NHS/BSA used	0.00	2.07	4.79	9.57	19.14	31.90
Dye-BSA reached	0.03	0.77	1.85	3.74	7.00	13.22

2.4. Labeling of antibodies

The method was analog to the BSA-quencher conjugation described above. 1.1 mg of each antibody (protein A purified monoclonals EC9, CE7 and BF12) in 80 μL PBS-buffer was prepared. 10 μL bicarbonate buffer (1 M, pH 8) was added. 5 mg of the lyophilized S2314-NHS were diluted in 1 mL DMSO, from this solution 13 μL were added to the antibody solution, respectively. The reaction took 2 h. Then the labeled antibodies (AbF) were purified with CS-800 protein-purification columns (emp Biotech, Berlin, Germany).

2.5. Fluorescence measurement

To find out the optimal eBSAq/AbF ratio for the assay a titration was performed: 1 mL of AbF (1 mg/L) was prepared in a cuvette and the fluorescence was measured. Then stepwise 5 μL of eBSAq (500 mg/L) was added into the AbF-solution and the fluorescence-quenching was measured. After 15 μg of eBSAq was added into the AbF solution, the fluorescence did not reduce any more. In the assay the AbF concentration was 0.4 mg/L and the eBSAq concentration was 9 mg/L.

For the assay a reagent-mixture was prepared: The three labeled antibodies (EC9, CE7 and BF12) were diluted to 40 mg/L in PBS-buffer (with 0.05% Bronopol as preservative), respectively. 220 μL of each diluted antibody solution was then mixed with 22 mL PBS-buffer and 33 μL eBSAq solution. The mixtures must stay for at least 30 min before the first use. This mixture could be stored in the refrigerator for one week.

The control module "time driver" of LS55-control software was chosen for the fluorescence intensity measurement. The settings of the equipment were the following: wavelength Ex.: 644 nm; Slit Ex.: 10 nm; Wavelength E_m.: 664 nm; Slit E_m.: 16 nm; Integration time 5 s.

Progesterone was dissolved and diluted in a mixture of DMSO and ethanol (1:1 v/v) to create standard solutions with concentrations ranging from 1 μg/L to 1 g/L.

For the progesterone assay 990 μL of the eBSAq and AbF mixture was transferred into a quartz cuvette. The background fluorescence was measured for 3 min (4 data points). Then the progesterone sample (10 μL of progesterone standard solution) was added into the cuvette and the solution was well mixed using a spatula. The fluorescence was measured for another 10 min. For the calibration with progesterone standards the average of the last three data points before adding the sample applied as original fluorescence signal (S_0). The average of the first three data points after adding the sample applied as measuring signal (S_m). The raising of fluorescence due to progesterone was calculated as percentage ($S_m/S_0 \times 100\%$).

2.6. Progesterone test in serum samples

Progesterone standard solutions were added into male serum-samples to create concentrations from 1 μg/L to 625 μg/L (with dilution factor 5). The measurement procedure was similar to the calibration measurement. The differences were: only 950 μL of the eBSAq and AbF mixture was prepared in the cuvette and 50 μL of serum sample was added. For the calculation the last three data points of the 10-min-measurement was used for S_m.

3. Results and discussion

Several hybridoma cells producing monoclonal antibodies against progesterone were generated. Three monoclonal antibodies, namely EC9, CE7 and BF12, all of them were of IgG1 isotype, were evaluated to build up the homogenous immunoassay.

For an immunoassay, the test sensitivity depends on affinity and concentration of the antibody. If the affinity is high enough – for example, when the antigen–antibody-complex was formed, and dissociation does not occur – then the test sensitivity depends on the antibody-concentration alone. To improve the test sensitivity, the antibody concentration must be kept low. But low concentration means low signal. Therefore, for a sensitive fluorescence immunoassay, it is important that a strong fluorophore is used. The fluorophore we applied in our assay, FEW S2314, is a Cy5 analog with some additional sulfonic acid residues for improving its water solubility (Fig. 3). With this fluorophore as label for the antibody, we can decrease the AbF concentration to 30 μg/L for the intensity measurement using LS55 spectrometer. As quencher we chose the fluorophore DY800 (excitation 777 nm/emission 791 nm; complete spectrum given at www.dyomics.com). This fluorophore–quencher combination has never been used before. The advantage of DY800 is that its emission wavelength is located at around 800 nm. The emission wavelength of S2314 is at 664 nm, so the fluorescence of the quencher does not interfere the fluorescence measurement of S2314. DY800 is indeed an appropriate quencher for S2314. In the eBSAq–AbF-complex, depending on the number of DY800 molecules linked on the BSA, the quenching can reach up to 70% of the original fluorescence of S2314 with antibody EC9 (Fig. 4). The other two monoclonal antiprogesterone antibodies reached a lower quenching rate (3% for CE7 and 20% for BF12), probably due to their weaker cross-reactivity to estradiol.

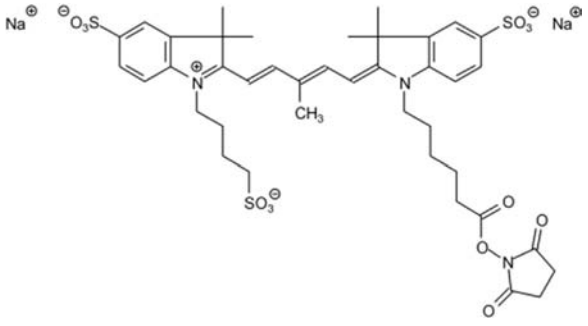

Fig. 3. Structure of fluorophore S2314-NHS-Ester. The maxima for excitation and emission are 648 nm and 663 nm, respectively. The complete spectrum is given on the company website, www.few.de.

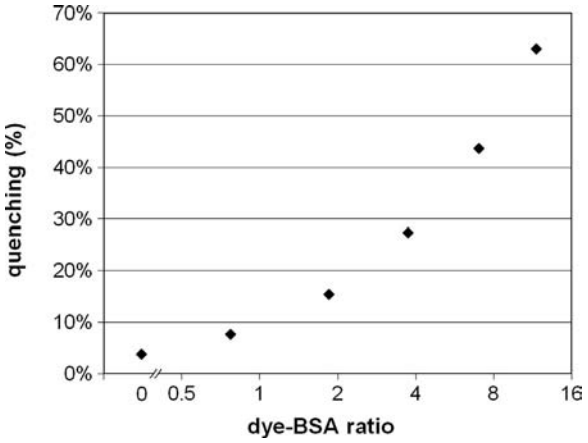

Fig. 4. Fluorescence quenching of AbF (antibody EC9) after binding to the analyte-BSA quencher (DY800) molecule according to the number of DY800 molecules per BSA.

To observe the relationship between progesterone concentrations and increased fluorescence, a calibration with eight progesterone concentrations was made. In the original measurement data (Fig. 5 small), we can see the raise of fluorescence occurs immediately after adding progesterone to the mixture. A concentration of 0.1 μg/L induces already a significant signal. The calibration curve (Fig. 5) shows that the dynamic range of measurement in PBS buffer is from 0.1 μg/L to 100 μg/L. The LOD is 0.1 μg/L. (3 time standard deviation of zero-value).

A dynamic equilibrium exists in the reagents mixture. Because the affinity between estradiol and AbF is weak, the concentration of eBSAq in the mixture must be at least 15 times higher than the antibody concentration in order to shift the equilibrium to the side of the complex. For the same reason, the dissociation rate is very high. If the antibodies encounter a binding partner with higher affinity like progesterone, the replacement occurs immediately. Therefore, we observe a very fast signal-response after adding progesterone to the mixture. When we used progesterone-BSA-DY800 instead of eBSAq as a tracer, the antibody affinity to the tracer is much higher and a replacement due to free progesterone occurred very slowly and was not significant (data not shown).

In view of molecular structure, sexual hormones are steroid derivatives which do not distinguish from each other very much. Therefore, antibodies against one hormone always have weak cross reactivity with the others. In real samples like sera, urine or saliva, there are always many sexual hormones co-existing in different concentrations. In our assay, we utilized the cross reactivity to generate the quenching signal and the test should work in serum samples. Therefore, it was necessary to check, if other sexual hormones would interfere the progesterone antibody interaction. Three common sexual hormones were tested: testosterone, estradiol and dehydroepiandrosterone (DHEA) at different concentrations were added to the assay reagent-mixture to see if they also induce a change of the fluorescence. The results are shown in Fig. 6. These three hormones only at very high concentrations (1 mg/L in cuvette) lead to a slight fluorescence-raising in the assay. The physiological concentrations of the hormones in serum are much lower than 1 mg/L. Considering the dilution factor (1:20 for serum samples), the concentration of these hormones in real tests with serum samples would not be higher than 50 μg/L in the cuvette and this concentrations would not affect the measurement of progesterone. There are many other sexual hormones and synthetic steroids. We only tested these 4 hormones because they are common hormones in serum-sample. In special case that other hormones and steroids could be present in the sample, the cross-

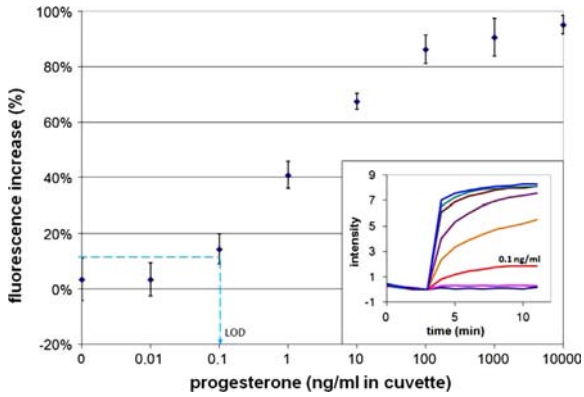

Fig. 5. Calibration curve of the homogeneous assay using antibody EC9 and various progesterone concentrations in PBS, three times replication, with three times standard deviation. Small figure: raw measurement data.

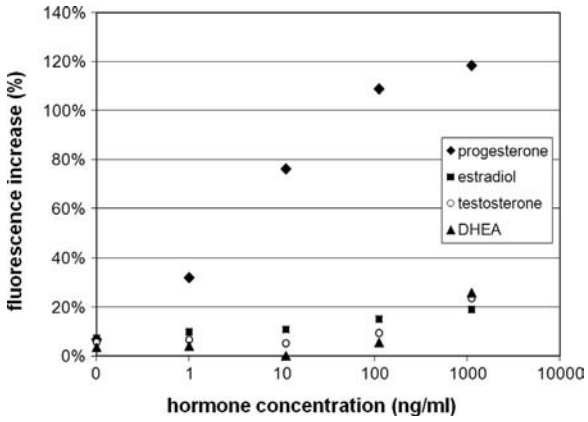

Fig. 6. Study of the specificity of the novel homogeneous assay: no significant cross reactivity with other steroid hormones could be detected.

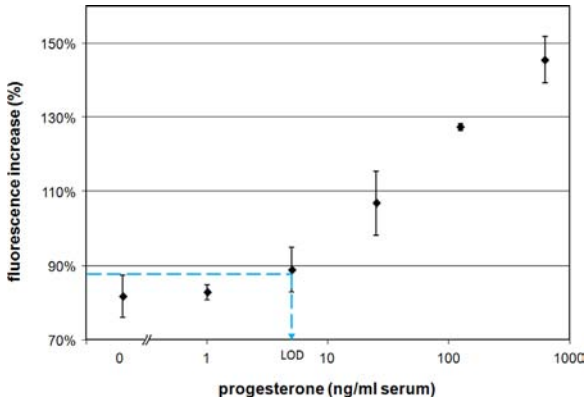

Fig. 7. Application of the assay to human blood serum samples. Progesterone could be detected from about 5 μg/L serum (three times replication, with three time standard deviation).

reactivity of these hormones and steroids could be tested specifically.

Progesterone concentration in blood-sera is an indicator of ovulating, pregnancy and placenta development [21]. It is important for us to know if the new homogeneous assay is capable to measure the progesterone concentration in serum. Because of various components in serum, this is not an optimal matrix for fluorescence measurement. The protein particles and impurities in serum can scatter the excitation light and induces background raising. In this assay, only 50 μL of serum was added to the reagent-mixture (950 μL). In this diluted serum (dilution factor 20) fluorescence could be measured with raising of the background signal for about five units, which does not influence the calculation of progesterone concentration. Another challenge for the progesterone assay was that many carrier proteins like human serum albumin (HSA) exist in serum. These proteins associate strongly with hydrophobic molecules like progesterone. There is equilibrium of free progesterone and carrier-bound progesterone in serum. Before an antibody binds a progesterone molecule, it must be set free from the carrier protein first. Therefore, after adding the serum sample, the fluorescence could be only slowly released. Therefore, we used the signal after 10 min incubation for the calculation.

The result (Fig. 7) shows that the assay could also estimate progesterone concentration in serum with the test range from 5 μg/L to 500 μg/L (concentration in serum). The LOD was at 5 μg/L (3 time standard deviation of zero-value). Within a menstruation cycle, the value of progesterone in female blood sera varies between 1 to 40 μg/L. During pregnancy the value can raise up to 150 μg/L. The dynamic range of measurement matches to the range of progesterone concentration in female serum. The increased fluorescence signal was between 70% and 150% of original (blank) signal, which was higher than the measured signal in buffer, the reason of this higher signal was the raised background due to the scattered excitation light. But the signal of released fluorescence induced by free progesterone in the samples was significant, the change of background does not influence the specific fluorescence increase.

In many competitive immunoassays, labeled antigens are used to generate signals. Free antigens or haptens in samples compete against labeled antigens for antibody binding sites. However, because the antibodies have almost the same affinity to labeled and free antigens, the displacement by free antigens is not easy. To solve this problem, the reactions could be separated: the antibodies react with free antigen in the sample first, then the labeled antigens are added to the system. Therefore, two steps are needed to accomplish the test. For an immunoassay, each additional assay-step means more work, more time, more cost and a larger error. That is one reason why we wanted to simplify the assay to a one-step test. Another reason was to reduce the test duration. There are a few progesterone immunoassays published in the recent years. For example, one assay utilizes luminescence [22], which had a linear range of detection from 8 to 800 nM (2.5–250 μg/L) in serum; and another immunoassay with quantum dots as labeling [23], which had a sensitivity of 2.2 μg/L in bovine milk. But because the assay processes of both assays are complicated, these tests lasted hours. There are also lateral flow assays developed for progesterone [24,25], but the duration of the tests was still 15 min at least. In the last years a novel homogenous electrochemical assay was developed in our institute [26], which could detect various analytes like progesterone, human chorionic gonadotropin and the diabetes marker HbA1c in serum with very short test duration (1.5 min) and high sensitivity. However, this two step assay needs two different antibodies and is, therefore, a bit more complicated.

Most immunoassays need several reagents which are working together or consecutively during the test. In this assay all we need is to prepare the assay-mixture. The mixture could be prepared in sufficient volume and stored for days. The relationship between functionality of the assay mixture and the length of the storage was not determined yet. We know the absolute fluorescence would decrease during the storage, but the quenching effect remains. Therefore, after days of storage the mixture could still be used for progesterone determination despite the weaker signal.

4. Conclusion

In this study, a one-step homogeneous fluorescence immunoassay was developed. A new fluorophore–quencher combination was used and proven to be effective. To our knowledge, this is the first application described, where the novel fluorophore S2314 was used. The assay duration was between 3 and 10 min (depending on samples). With this assay the concentration of progesterone could be measured in a large dynamic range in buffer and serum. It is conceivable to apply this assay to other analytes and samples.

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References

[1] E. Ullman, Homogeneous immunoassays, in: D. Wild (Ed.), The Immunoassay Handbook, Elsevier Ltd, UK, 2005, pp. 212–230.
[2] H. Päkkilä, E. Malmi, S. Lahtinen, T. Soukka, Biosens. Bioelectron. 62 (2014) 201–207.
[3] T. Pulli, M. Höyhtyä, H. Söderlund, K. Takkinen, Anal. Chem. 77 (2005) 2637–2642.
[4] K. Enomoto, T. Nagasaki, et al., Anal. Biochem. 351 (2006) 229–240.
[5] J. Qian, C. Wang, X. Pan, S. Liu, Anal. Chim. Acta 763 (2013) 43–49.
[6] R. Drost, T. Plomp, A. Teunissen, A. Maes, Clin. Chim. Acta 79 (1977) 557–567.
[7] M.L. Sánchez-Martínez, M.P. Aguilar-Caballos, A. Gómez-Hens, Talanta 78 (2009) 305–309.
[8] D.R. Henderson, S.B. Friedman, J.D. Harris, W.B. Manning, M.A. Zoccoli, Clin. Chem. 32 (1986) 1637–1641.
[9] B. Du, Z. Li, Y. Cheng, Talanta 75 (2008) 959–964.
[10] H. Cai, P. Yang, J. Feng, J. Cai, Sens. Actuators, B 135 (2009) 603–609.
[11] F. Sellrie, A. Warsinke, B. Micheel, Anal. Bioanal. Chem. 386 (2006) 206–210.
[12] C. Tan, N. Gajovic-Eichelmann, R. Polzius, N. Hildebrand, F.F. Bier, Anal. Bioanal. Chem. 398 (2010) 2133–2140.
[13] C. Tan, N. Gajovic-Eichelmann, W.F. Stöcklein, R. Polzius, Anal. Chim. Acta 658 (2010) 187–192.
[14] J. Tian, L. Zhou, Y. Zhao, Y. Wang, Y. Peng, S. Zhao, Talanta 92 (2012) 72–77.
[15] F. Sellrie, M. Beck, N. Hildebrandt, B. Micheel, Anal. Methods 2 (2010) 1298–1301.
[16] F. Sellrie, E. Graser, C. Lenz, T. Hillebrand, J.A. Schenk, Biosens. Bioelectron. 42 (2013) 512–515.
[17] C.A. Swindlehurst, E.W. Voss jr, Biophys. J. 59 (1991) 619–628.
[18] G. Köhler, C. Milstein, Nature 256 (1975) 495–497.
[19] J.A. Schenk, F. Matyssek, B. Micheel, In Vivo 18 (2004) 649–652.
[20] J.A. Schenk, J. Fettke, C. Lenz, K. Albers, F. Mallwitz, N. Gajovic-Eichelmann, E. Ehrentreich-Förster, E. Kusch, F. Sellrie, J. Biotechnol. 158 (2012) 34–35.
[21] N. Laufer, D. Navot, J.G. Schenker, Am. J. Obstet. Gynecol. 143 (1982) 808–813.
[22] F. Ye, J. Liu, Y. Huang, S. Li, S. Zhao, J. Chromatogr. B 936 (2013) 74–79.
[23] L. Trapiella-Alfonso, J.M. Costa-Fernandez, R. Pereiro, A. Sanz-Medel, Biosens. Bioelectron. 26 (2011) 4753–4759.
[24] V.A. Safronova, J.V. Samsonova, V.G. Grigorenko, A.P. Osipov, Moscow Univ. Chem. Bull 67 (2012) 241–248.
[25] G.A. Posthuma-Trumpie, J. Korf, A. van Amerongen, Anal. Bioanal. Chem. 392 (2008) 1215–1223.
[26] J. Ettlinger, J.A. Schenk, B. Micheel, E. Ehrentreich-Förster, N. Gajovic-Eichelmann, Electroanalysis 24 (2012) 1567–1575.
[27] S.A. Marras, F.R. Russell, S. Tyagi, Nucleic Acids Res. 30 (2002) e122.
[28] U. Schobel, H.J. Egelhaaf, A. Brecht, D. Oelkrug, G. Gauglitz, Bioconjugate Chem. 10 (1999) 1107–1114.
[29] M. Seidel, D.M. Dankbar, G. Gauglitz, Anal. Bioanal. Chem. 379 (2004) 904–912.
[30] B. Dubertret, M. Calame, A.J. Libchaber, Nat. Biotechnol 19 (2001) 365–370.