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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).

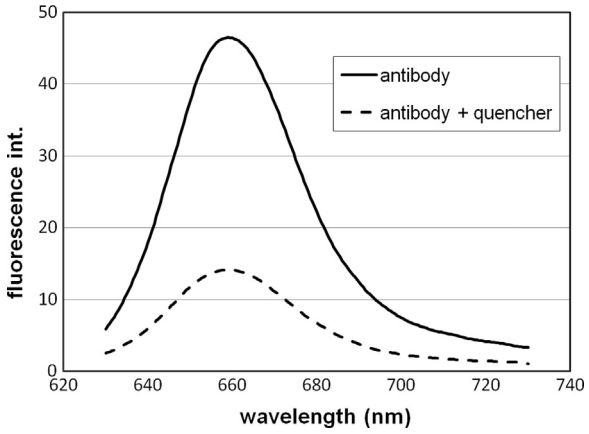

Fig. 2. Fluorescence spectrum of AbF before and after adding eBSAq. After binding of the dye labeled antibody to the quencher labeled antigen complex, the fluorescence intensity is reduced significantly.

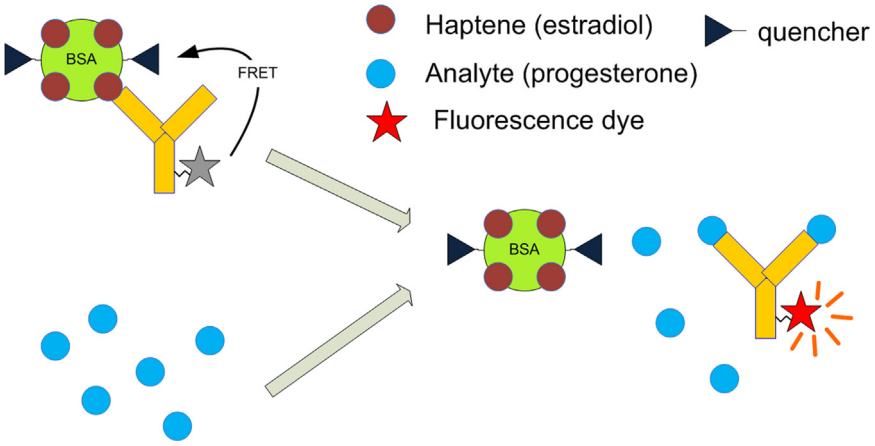

Fig. 1. Schematic representation of the new homogeneous immunoassay: The equilibrium of the complex of dye-labeled antibody and quencher labeled antigen-BSA conjugate is disturbed by free antigen from the sample. Then molecules of the dye containing antigen–antibody complex are destroyed and the fluorescence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