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Development of a quantum dot-based fluorescent immunoassay for progesterone determination in bovine milk

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ABSTRACT

The use of semiconductor quantum dots (QDs) as fluorescent labels to develop a competitive immunoassay for sensitive detection and quantification of progesterone in cow's milk is described. Colloidal water-soluble CdSe/ZnS QDs are conjugated to an antigen derivative (progesterone-BSA conjugate) and a simple methodology is optimised to determine the antigen concentration in the final bioconjugate. The obtained QD-linked antigens were then employed together with unlabelled anti-progesterone monoclonal antibodies, as the biological recognition elements, in the development of the quantitative QDs-based fluorescent immunoassay for progesterone in bovine milk.

After optimization, the developed immunoassay proved to cover a progesterone concentration range from 0.3 to 14.5 ng/mL in cow milk. Milk samples were just diluted 10-fold with deionised water and directly analysed with the proposed immunoassay, without additional sample pre-treatment or analyte extraction. The minimum detectable level (IC_{10}) of the developed immunoassay turned out to be 0.1 ng/mL of progesterone in bovine milk. The sensitivity (IC_{50}) achieved was 2.2 ng/mL with a reproducibility of 3.5% RSD as obtained from the results of the analysis of the triplicate of same samples but in three different days. Applicability of the proposed methodology was evaluated by analyzing cow's milk samples enriched with known concentrations of progesterone and recoveries better than 90% were achieved.

1. Introduction

Accurate oestrus detection is crucial for timed and successful artificial insemination and early detection of the pregnancy. An early detection of a failed insemination is critical for maximising reproductive efficiency, as it could allow a meaningful elapsed time delay before a new repeated insemination. Furthermore, the poor ability to detect female animals in heat results in longer calving intervals and lower milk production (Pennington et al., 1976). Due to those reasons, reliable analytical methods to detect timely and accurately the occurrence of oestrus cycle and other reproductive states are needed (Friggens and Chagunda, 2005; Gillis et al., 2002). Several methods, including measurement of milk temperature, radiotelemetric measurement of vaginal temperature or pedometric technologies, have been used for oestrus prediction. It goes without saying that the most effective and reliable method for the purpose is the direct determination of the level of progesterone in plasma or milk (Simersky et al., 2007).

Progesterone, a steroid hormone that is secreted in milk by the female mammals, is responsible of several activities related to reproductive cycle such as breast glandular development or the preparation of the uterus for the pregnancy. Its concentration in milk has a characteristic variation along the oestrus cycle and so progesterone is accepted as an ideal biomarker to control the reproductive cow's state, to detect the animal heat and to diagnose cow's pregnancy (Käppel et al., 2007; Posthuma-Trumpie et al., 2009). It is well known that the onset of the heat is indicated by a rapid fall in the concentration of progesterone in milk to below 2-5 ng/mL. Furthermore, once the cow is pregnant the progesterone concentration remains high and constant (Carralero et al., 2007).

Different methods have been developed for accurate determination of progesterone in milk, including strategies based on thin layer, gas or liquid chromatography coupled to mass spectrometry detection (Darling et al., 1974; Díaz-Cruz et al., 2003). However, such techniques are limited by several drawbacks including substantial equipment costs and/or extensive and time-consuming sample pre-treatments, rendering progesterone routine determination an expensive analysis. Alternatively, immunochemical assays are the most popular approach nowadays for the determination of progesterone (Gillis et al., 2006). A wide variety of labels have been used in the development of progesterone immunoassays including radioisotopes, enzymes or fluorescent dyes (Byszewska and Markiewicz, 2006; Colazo et al., 2008; Käppel et al., 2007) pro-

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ducing optical or electrical signals that can be correlated with the concentration of the analyte.

In this vein, inorganic semiconductor fluorescent nanocrystallites, also known as quantum dots (QDs), have emerged in the last decade as materials with great potential in bioanalysis (Bruchez et al., 1998; Coto-García et al., 2011). Due to their innovative and highly valuable optoelectronic properties, QDs are becoming an excellent alternative to the more conventional fluorophore dyes in luminescent methodologies (Mattoussi et al., 2004). The ability to keep the functionality of biomolecules attached to QDs allowed the use of these nanomaterials as powerful luminescent labels to develop innovative fluorescent immunoassays. Since the pioneer developments in 1998 (Bruchez et al., 1998; Chan and Nie, 1998), QDs have been used to develop useful immunoassays in different formats, such as microarrays (Geho et al., 2005) or Western blotting (Bakalova et al., 2005). However, very few direct quantitative immunoassays using QDs have been developed so far.

In this article the development and analytical evaluation of a QDs-based fluorescent immunoassay for progesterone, detection and quantification in milk is discussed. The synthesis and characterization of the water soluble colloidal nanoparticles of CdSe/ZnS was carried out first. Then the QDs were bioconjugated to a derivative of the antigen, the progesterone-BSA (Pro-BSA) conjugate, following the chemistry of the carbodiimide. Considering that the antigen (progesterone) is a hapten (small molecule that only has one site of binding with the antibody), the immunoassay design was restricted to a competitive format. Thus, free antigen present in the sample and constant (and known) amounts of labelled antigen were incubated in a microtiter plate coated with a limited amount of antibody. So, the competition for the antibody binding sites is established. After that, the fluorescence signal measurement of the QD label takes place and the inhibition curve characteristic of the immunoassay is plotted.

2. Materials and methods

2.1. Reagents and materials

All experiments were carried out using analytical grade reagents (see below) used as received without any further purification. Deionised ultrapure water (resistivity 18.2 MΩ/cm) was used throughout the work.

2.1.1. Reagents used for the synthesis of the fluorescent nanoparticles

The CdSe/ZnS QDs, used as fluorescent labels, were synthesized in our laboratory. The precursors used for the synthesis of the nanoparticles were selenium powder (100 mesh, 99.99%), cadmium oxide (99.99%), hexamethyldisilathiane, 1.0 M diethyl zinc solution in hexane, trioctylphosphine (TOP, 90%) and trioctylphosphine oxide (TOPO, 99%), all of them purchased from Sigma Aldrich (Milwaukee, WIS, USA) and hexylphosphonic acid (HPA) obtained from Alfa Aesar (Karlsruhe, Germany). The methanol HPLC gradient grade and chloroform anhydrous (≥99%), used to purify the synthesized QDs, were from Prolabo (Leuven, Belgium) and Sigma Aldrich, respectively.

A selenium stock solution was prepared in an Ar-filled dry-box by dissolving 1.63 mmol of selenium powder in 7.5 mL of TOP, to produce a solution of trioctylphosphine selenide (SeTOP). A zinc sulphide (Zn/S/TOP) stock solution was also prepared in Ar atmosphere by mixing 1.18 mmol of hexamethyldisilathiane, 8.34 mmol of diethyl zinc and 10.25 mL of TOP.

Water-solubilisation of QDs was achieved by coating them with an amphiphilic polymer synthesized in our laboratory (Fernández-Argüelles et al., 2007). For such purpose, poly(isobutylene-alt-maleic anhydride), dodecyl amine and tetrahydrofuran anhydrous were purchased from Fluka (Basel, Switzerland) and Sigma Aldrich, respectively. The cross linker bis(6-aminohexyl)amine (Fluka) employed for the stabilization of the polymeric layer was stored at 4 °C.

2.1.2. Immunological reagents and solutions

The antigen, 98% progesterone, was obtained from Sigma–Aldrich. The progesterone bovine serum albumin conjugate (Pro-BSA), employed in the bioconjugation to the QDs, was obtained from AbD Serotec (Dusseldorf, Germany). The mouse monoclonal anti-progesterone antibody (1 mg/mL) was purchased from AbD Serotec. The ethyl-3-(dimethylaminopropyl) carbodiimide (EDC) was purchased from Fluka and was stored at −18 °C in the freezer. For the QDs bioconjugation a solution 0.01 M of EDC was prepared in 100 mM phosphate buffered saline (PBS) pH 7.4 and used immediately.

Bovine serum albumin (BSA), casein and powder milk obtained from Merck and Sigma Aldrich were compared as blocking solutions. A 0.05% Tween 20 solution (Sigma–Aldrich) in PBS pH 7.4 buffer was used as washing solution.

A 50 mM borate buffer solution (SBB) at pH 12 was prepared to break the immunological interactions to allow dissolution of the QDs from the immunoassay well plate before fluorescence measurement.

Amicon Ultra-4 100 KDa centrifugal filters from Millipore (Madrid, Spain) were used to purify the Pro-BSA-QD bioconjugates after synthesis. A Bradford reagent from Sigma was employed in the indirect estimation of progesterone concentration in the bioconjugate based on Bradford Test.

2.2. Instrumentation

White microtiter plates (96 wells) were obtained from Varian (Madrid, Spain) and used in all immunoassays. The spectrophotometric measurements were carried out in a UV/Vis/NIR spectrophotometer (Perkin Elmer Lambda 900). The fluorescent measurements were performed in a spectrofluorimeter Cary Eclipse (Varian) equipped with a microplate reader module. A SUPRASIL quartz cuvette (model 105.254-QS of Hellma, Müllheim, Germany) with 45 μL volume was employed for spectrophotometric measurements. To process the data obtained and fit the inhibition curves a SOFTmax Pro software from Molecular Devices (Ismaning, Germany) was used.

Photoactivation of our synthesised CdSe/ZnS QDs was performed using a 365 nm UV lamp (6 W, Vilbert Lourmat, Torcy, France).

A heating mantle with energy heater control (JP Selecta, Barcelona, Spain) with simultaneous stirring was employed to carry out the nanoparticle synthesis. To keep a constant argon atmosphere inside the flask, a needle with argon flow was used. This flow was set to 25 mL/min by means of a rotameter (Serv' Instrumentation, Irigny, France).

2.3. Protocols

2.3.1. Synthesis of CdSe/ZnS quantum dots

CdSe/ZnS QDs were synthesized using CdO as precursor *via* the organometallic route described by Peng's group (Peng and Peng, 2001) with slight modifications. Briefly, 1.23 mmol of CdO, 0.6 g of HPA and 17.5 g of TOPO were loaded into a 250 mL glass three-necked flask. The mixture was heated up to 300–320 °C under argon flow for 15–20 min to allow the complete dissolution of CdO in HPA and TOPO. After cooling the temperature of the solution down to 270 °C, 6 mL of the SeTOP solution was swiftly injected. After injection, nanocrystals were left to grow for about 11 min at 250 °C. Once