LIF based fluorescent immunosensor using AP-SNs and QDs for quantitation of IgG anti *Toxocara canis* in human serum samples

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ABSTRACT

The aim of this work was to develop a microfluidic immunosensor for determination of IgG antibodies to *Toxocara canis* (IgG anti-*T.canis*), causal agent of toxocariasis. This disease is caused by accidental ingestion of infective eggs that hatch into the first portion of the intestine. Subsequently, the juvenile stages are distributed throughout the body, generating symptoms from mild to severe manifestations. IgG anti-*T. canis* antibodies detection was carried out using a non-competitive immunoassay, in which excretory-secretory antigens from *T. canis* second-stage larvae (TES) were covalently immobilized on 3-aminopropyl-functionalized silica-nanoparticles (AP-SNs). Antibodies present in serum samples immunologically reacted with TES and then were quantified by using a second antibody labeled with cadmium selenide zinc sulfide quantum dots (CdSe-ZnS QDs). The concentration of IgG anti-*T. canis* antibodies present in the serum sample was measured by LIF detector, using excitation lambda at 491 nm and emission at 540 nm.

Keywords: Toxocara; microfluidic immunosensor; laser-induced fluorescence; quantum dot; silica particles.

1. INTRODUCTION

Toxocariasis, one of the most common zoonotic infections worldwide, is caused by *T. canis* [1,2]. In humans, the infection is acquired by oral route through accidental ingestion of infective eggs from soil-contaminated hands, consumption of poorly sanitized vegetables and raw or undercooked meats. Toxocara eggs hatch in the intestine and release larvae into the lumen, where they can penetrate the small intestine, reach the circulation and then spread by the systemic route. The larvae migrate throughout the body but cannot mature, and instead encyst as second-stage larvae [3,4]. The possibility of early diagnosis is of great importance, allowing proper management and treatment of patients suffering from toxocariasis.

The enzyme-linked immunosorbent assay (ELISA) using excretory-secretory antigens from *T. canis* second-stage larvae (TES) is the most widely used test to detect anti-Toxocara antibodies. Alternatively, immunosensors represent an interesting choice to achieve the diagnostic of human parasitosis.

In last years, nanotechnology has contributed to the development of miniaturized immunosensor-based devices with high-throughput analytical properties [5,6]. Different nanomaterials such as quantum dots (QDs), silica nanoparticles (SNs), and other nanoparticles have emerged as promising alternatives for a wide range of immunosensors applications. SNs have attracted significant interest because of their unique properties such as, versatile silane chemistry for surface functionalization, excellent biocompatibility, high thermal stability, ease of large-scale synthesis, and low cost of SNs production. QDs are characterized by their unique size-dependent optical and electronic properties, which favor their use for biomedical diagnostics. Recently, the progress in controlled synthesis of high quality QDs, as well as the effective surface modifications make them excellent optical labels for sensing and biosensing events.

The aim of this work was to develop a microfluidic immunosensor that include the use of nanomaterials for the quantitative determination of IgG antibodies to IgG anti-*T. canis*. The IgG anti-*T. canis* antibodies detection in serum samples were carried out using a non-competitive format immunoassay. TES immobilized on 3-aminopropyl-functionalized silica-nanoparticles (AP-SNs) covalently incorporated in the central channel of the microfluidic device are recognized specifically by the anti-*T. canis* antibodies in the sample. The subsequent detection was achieved by adding a second antibody conjugated with cadmium selenide zinc sulfide quantum dots (CdSe-ZnS QDs) specific to human IgG.

2. MATERIALS AND METHODS

2.1. CdSe-ZnS QDs antibody conjugation

Covalent QDs antibody conjugation is commonly based on crosslinking reactions between amine and carboxylic groups. QDs activation was performed with N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrocloride (EDC)/N-hydroxysuccinimide (NHS) in methanol. Briefly, 15 µL QDs (8 µM) were mixed with 3 µL EDC (2.2 mM in methanol) and 3 µL NHS (4 mM in methanol), followed by the addition of another 9 µL methanol yielding a total volume of 30 µL [7].

2.2. Design and fabrication of microfluidic chip

The microfluidic chip design was created using CorelDraw software version 11.0 (Corel) and made by following the standard soft lithography protocol. The microfluidic chip consists in a T format design with central and accessory channels.

2.3. Immobilization of TES

To carry out the modification process, AP-SNs were immobilized on the 3-aminopropyl triethoxysilane (APTES) modified surface glass cheap, according to reference [8]. Later, 100 μg mL^{-1} of TES solution was coupled to the modified AP-SNs surface via glutaraldehyde reaction. The immobilized antigens were finally washed three times with phosphate buffer (pH 7.00) and stored in the same buffer at 5 °C. The immobilized antigen preparation was perfectly stable for at least 1 month.

2.4. LIF detection

The procedure for the quantification of anti-*T. canis* IgG antibodies involves the following steps: firstly, in order to avoid the unspecific bindings, a blocking treatment was performed through injecting 1% of bovine serum albumin (BSA) in 0.01 M PBS (pH 7.20) for 5 min and later washed with 0.01 M PBS buffer (pH 7.20) for 3 min. All solutions employed, were injected using syringe pumps at flow rate of 2 μL min^{-1}.

In the second step, the serum samples, firstly diluted 100-fold with 0.01 M PBS buffer (pH 7.20), were injected for 10 min and rinsed for 3 min with 0.01 M PBS buffer (pH 7.20). IgG specific antibodies to *T. canis* present in the samples, reacted with TES immobilized on AP-SNs surface, then, the microfluidic device was washed with 0.01M PBS (pH 7.20) to remove excess of sample. Bound antibodies were quantified using QDs-conjugated second antibodies specific to human IgG (dilution of 1:1000 in 0.01 M PBS, pH 7.20) injected for 5 min. The relative fluorescence was measured by LIF detector, using excitation lambda at 491 nm and emission at 540 nm (Fig. 1).

Before each sample analysis, the immunosensor was exposed to a flow of desorption buffer (0.1 M glycine–HCl, pH 2.00) at a flow rate of 2.0 μL min^{-1} for 5 min and then was washed with PBS, pH 7.20. With this treatment, anti-*T. canis* antibodies bound to immobilized antigen, were desorbed, allowing to start with a next determination. The storage of the device was made in 0.01M PBS (pH 7.20) at 4º C.

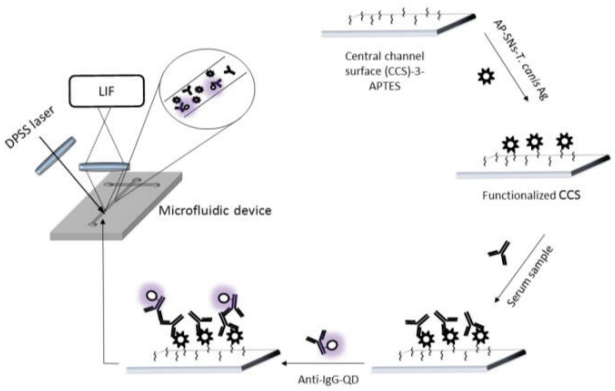

Figure 1. Schematic representation of the immunoassay on microfluidic device.

3. RESULTS

Relevant studies of experimental variables that affect the performance of microfluidic immunosensor for IgG anti-*T. canis* antibodies determination were optimized. Between them, the optimal flow rate, incubation time, concentration of TES to be immobilized, enzymatic activity and the amplification effect resulting from the incorporation of the AP-SNs, were studied.

3.1. Determination of IgG anti-*T. canis* antibodies in the microfluidic immunosensor

The proposed device was developed as an alternative tool for quantification of IgG anti-*T. canis* antibody concentration.

The IgG anti-*T. canis* antibodies calibration curve was obtained by plotting relative fluorescence units (RFU) versus IgG anti-*T. canis* antibody concentration. A linear relation, $RFU = 4.74 + 11.03 \times C_{IgG\ antibodies}$, was observed between the RFU and the IgG concentration in the range of 0.1 and 132 ng mL^{-1}. The correlation coefficient (r) for this plot was 0.998. The coefficient of variation (CV) for the determination of 76 ng mL^{-1} IgG anti-*T. canis* antibodies was 4.56 % (five replicates). For LIF detection procedures the LOD was 0.12 ng mL^{-1}. The precision of the microfluidic immunosensor assay was checked with control serum at 5, 38, and 132 ng mL^{-1} IgG anti-*T. canis*-specific antibody concentrations. The IgG anti-*T. canis* assay exhibited good precision; the CV within-assay values were below 5.1% and the between-assay values were below 6%.

In this work, 8 positive and 8 negative human serum samples were analyzed. These were previously confirmed for toxocariosis disease using a commercial test, which is currently used in clinical diagnostics.

It is relevant to emphasize, that the proposed method combines LIF detection using CdSe-ZnS QDs as labels with AP-SNs-APTES biorecognition support in a microfluidic platform. The incorporation of AP-SNs increases the active area improving the achieved LOD and consequently the analytical sensitivity. The covalent

attachment of specific antibodies against *T. canis* antigen provided the specificity to the device. No clean-up of the detection system was needed between analyses in comparison to electrochemical detectors, which make an improvement in time consuming analysis and lifetime of the immunosensor. Moreover, our device has inherent benefits such as miniaturization, integration, portability and the possibility to perform *on-site* analysis. Due to the previously mentioned features, our proposed immunosensor has potential application in clinical diagnosis.

5. CONCLUSIONS

The present work describes a microfluidic immunosensor coupled to LIF detection applied to the diagnostic of human toxocariasis. The combination of two different nanomaterials; AP-SNs as bioaffinity supports and QDs as fluorescent labels, enabled us to achieve a useful alternative tool for *T. canis* diagnostic. SNs proved to be an excellent choice for optical sensing, increasing the active area and consequently the analytical sensitivity. Among the most relevant advantages offered by our system, we can mention the possibility of antibody quantification, less sample volume required and the lower LOD. Moreover, our device can obtain diagnostic results in 30 min, much less than conventional clinical immunoassays. Finally, our analytical proposed method proved to have a strong potential for the alternative immunodiagnostic of toxocariasis.

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