Microchemical Journal 130 (2017) 436–441

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Fluorescent immunosensor using AP-SNs and QDs for quantitation of IgG anti-*Toxocara canis*

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ARTICLE INFO

Article history:
Received 29 March 2016
Received in revised form 18 August 2016
Accepted 27 October 2016
Available online 29 October 2016

Keywords:
Toxocara
Microfluidic immunosensor
Laser-induced fluorescence
Quantum dot
Silica particles

ABSTRACT

The objective of this work was to develop a microfluidic immunosensor for the quantitative 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. The possibility of early diagnosis is of great importance, allowing proper management and treatment of patients suffering from toxocariasis.

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. The total assay time was 30 min, having made LIF detection in less than 1 min. The detection limit calculated for the proposed methodology was 0.12 ng mL^{-1} and the coefficients of intra- and inter-assay variation were less than 6%. The results show the usefulness of the developed immunosensor for the fast determination of IgG antibodies anti-*T. canis*.

1. Introduction

Toxocariasis, one of the most common zoonotic infection worldwide, is caused by *Toxocara canis* (*T. canis*), or less commonly, *Toxocara cati* [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 [3,4]. Toxocara eggs hatch in the intestine and release larvae into the lumen [5–8], 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 [9]. The inflammatory process, caused by the larvae stage, is attributed to a large amount of secretion and excretion products (lectins, mucins, enzymes) which interact and modulate the host immune response [10,11]. In summary, clinical manifestations of toxocariasis are related to the larval migration and the host immune response. The clinical forms of toxocariasis are systemic (visceral larva migrans), localized (ocular and neurological) and asymptomatic. [12,13].

A definitive diagnosis of human toxocariasis is often a challenge. It is based on clinical, epidemiological and serological data. Whilst chronic eosinophilia is generally considered a reliable indicator of tissue helminthiases, serological testing using immunological techniques is recognized as the most effective approach to the laboratory diagnosis of human toxocariasis [14,15]. 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 [16].

Alternatively, immunosensors represent an interesting choice to achieve the diagnostic of human parasitosis. The miniaturization of immunoassays using microfluidic technology represents an attractive strategy, due to its advantages, such as high degree of integration and low regent consumption among them [17,18].

Fluorescence is one of the most sensitive detection method widely used for immunosensor design [19–21]. Several different setups are employed with fluorescence detection, such as a microscope focused on the microchannel and connected to a charge coupled device camera or a photomultiplier tube. Since other parts of the fluorescence setup, such as the excitation source and detection device, can be miniaturized, the entire device can be made easily portable [22,23]. Laser-induced fluorescence (LIF) applied for analyte detection is one of the most sensitive detection techniques, which is capable of reaching concentration detection below 10–13 mol L^{-1} and a mass detection of less than 10 molecules. Radiation from a laser source can be focused, making it a useful tool for detection in very small volumes [24,25]. This property makes LIF detection a method of choice for detecting analytes on microfluidic

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http://dx.doi.org/10.1016/j.microc.2016.10.027
0026-265X/

devices, where the characteristic length scales are of the order of micrometers or even smaller [26–28].

In last years, nanotechnology has contributed to the development of miniaturized immunosensor-based devices with high-throughput analytical properties [29,30]. 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 [31]. QDs are characterized by their unique size-dependent optical and electronic properties, which favor their use for biomedical diagnostics [32–33]. Recently, the progress in controlled synthesis of high quality QDs, as well as the effective surface modifications [34,35] make them excellent optical labels for sensing and biosensing events [36].

The objective of this work was to develop a microfluidic immunosensor with LIF detection, incorporating AP-SNs as support for TES immobilization and cadmium selenide zinc sulfide quantum dots (CdSe-ZnS QDs) as fluorescent labels for anti-*T. canis* antibodies quantification. TES immobilized on AP-SNs covalently incorporated in the central channel of the device are recognized specifically by the anti-*T. canis* antibodies in the sample. The subsequent detection was achieved by adding QDs-conjugated second antibodies specific to human IgG. The measurement was carried out by LIF using excitation at 491 nm and emission at 540 nm.

2. Materials

2.1. Reagents and solutions

Soda lime glass wafers (26 × 76 × 1 mm) were purchased from Glass Técnica (São Paulo, Brazil). Sylgard 184 and AZ4330 photoresist (PR) were obtained from Dow Corning (Midland, MI, USA) and Clariant (Sommerville, NJ, USA), respectively. 3-aminopropyl-functionalized silica-nanoparticles, (AP-SNs) 100 nm particle size, DLS), 3-aminopropyl triethoxysilane (APTES), *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrocloride (EDC), *N*-hydroxysuccinimide (98%) (NHS), were purchased from Sigma–Aldrich. Glutaraldehyde (25% aqueous solution) was purchased from Merck. The enzyme immunoassay for the qualitative determination of IgG antibodies against *Toxocara canis* in human serum, RIDASCREEN® Toxocara IgG test, was purchased from R-Biopharm AG-Darmstadt Germany and was used according to the manufacturer's instructions [37]. Anti-human γ-chain, was purchased from Abcam (USA). All buffer solutions were prepared with Milli-Q water.

2.2. Instrumentation

The optical system was constructed using the procedure of Ref. [38] according to the following modification. A 491-nm monochromatic DPSS laser (Cobolt, USA) operated at 25 mW served as the fluorescence excitation source. It was focused on the detection channel at 45° to the surface using a lens with a focal distance of 30 cm. The relative fluorescence signal of CdSe-Zns QDs was measured using excitation at 491 nm and emission at 540 nm. The paths of the reflected beams were arranged so that they did not strike the capillary channels elsewhere and to avoid photobleaching. The fluorescent radiation was detected with the optical axis of the assembly perpendicular to the plane of the device. Light was collected with a microscope objective (10:1, NA 0.30, working distance 6 mm, PZO, Poland) mounted on a microscope body (BIOLAR L, PZO). A fiber-optic collection bundle was mounted on a sealed housing at the end of the lens of the microscope, which was connected to a QE65000-FL scientific-grade spectrometer (Ocean Optics, USA). The entire assembly was covered with a large box to eliminate ambient light.

A syringe pumps system (Baby Bee Syringe Pump, Bioanalytical Systems) was used for pumping, sample introduction, and stopping flow. All solutions and reagent temperatures were conditioned before the experiment using a Vicking Masson II laboratory water bath (Vicking SRL, Buenos Aires, Argentina). Absorbance was detected by Bio-Rad Benchmark microplate reader (Japan) and Beckman DU 520 general UV–vis spectrophotometer. All pH measurements were made with an Orion Expandable Ion Analyzer (Orion Research Inc., Cambridge, MA, USA) Model EA 940 equipped with a glass combination electrode (Orion Research Inc.)

3. Methods

3.1. SeCd-ZnS QDs conjugation

Covalent QDs conjugation is commonly based on crosslinking reactions between amine and carboxylic acid groups. QDs activation was performed with *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrocloride (EDC)/*N*-hydroxysuccinimide (NHS) in methanol to 15 μL QDs (8 μM), add 3 μL EDC (2.2 mM in methanol) and 3 μL NHS (4 mM in methanol), followed by another 9 μL methanol, yielding a total volume of 30 μL. Leave this at RT for 30 min [39].

3.2. Immunoadsorption procedures

To obtain the human anti-*T. canis* reference sera, the procedure proposed by Sanchez-Sus et al. [40] was followed, with the following modifications: eight serum samples obtained from patients with were confirmed for toxocariasis disease were employed. These samples showed a marked reactivity against *T. canis*. In the first step for the obtaining *T. canis* reference serum, an immunoadsorption procedure was made. The TES were coupled to AP-SNs which previously reacted with an aqueous solution of glutaraldehyde, then; this preparation was packed into a column in which the serum sample was added. After the elimination of non-adsorbed serum proteins, we get a final eluted solution containing specific antibodies anti-*T. canis*. In the second stage, obtaining of the reference IgG antibodies anti-*T. canis* sera was carried out, using a new immunoadsorption procedure. In this case, we coupled AP-SNs with anti-human IgG antibodies, and this modified support was packed into of a new column, in which this immunoadsorbent was put in contact with the eluted obtained in the first immunoadsorption procedure. As consequence of this second procedure, the eluted contained only IgG antibodies anti-*T. canis*. These antibodies were quantified using Quantitative Human IgG ELISA. According to this determination, the concentration of IgG antibodies anti-*T. canis* in our reference serum, obtained by double affinity chromatography procedure was 132 ng mL^{-1}, the same, was later employed for the construction of the calibration curve for the proposed method.

3.3. TES antigen preparation

The TES antigens were obtained according to the technique described by Gillespie [41]. TES were maintained at 37 °C with 5% CO_2 atmosphere and an adjusted pH of 6.4–6.5 in Iscove's modified Dulbecco's culture medium supplemented with HEPES buffer and a Penicillin-Streptomycin solution. The culture supernatant was removed weekly; the supernatant pool was kept at −70 °C. This was concentrated by filtration through polyethersulphone membranes and dialysed; the protein content was then estimated by the Bradford method with bovine albumin as the standard protein [42].

3.4. Design and fabrication of microfluidic chip

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