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

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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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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

Table 1
Summary of optimum conditions for IgG anti-*T. canis* antibodies immunoassay.

Sequence	Condition	Time
Blocking solution	1% albumin in a 0.01 M phosphate buffer saline (PBS), pH 7.2	5 min
Washing buffer	Flow rate: 2 μL min^{-1} (PBS, pH 7.2)	3 min
Serum samples	Sample flow rate of 2 μL min^{-1}	10 min
Washing buffer	Flow rate: 2 μL min^{-1} (PBS, pH 7.2)	3 min
Enzyme conjugated	QDs-conjugated (dilution of 1:1000) 2 μL min^{-1}	5 min
Washing buffer	Flow rate: 2 μL min^{-1} (PBS, pH 7.2)	3 min
LIF detection	Excitation wavelength: 491 nm Emission wavelength: 540 nm	1 min

central and accessory channels. A silicon wafer master mold was fabricated by first spin-coating a thin layer of AZ4330 photoresist (Clariant-Sommerville, NJ, USA), on the surface of the wafer. After the prebaking, a photomask with the designed microchannel geometry was placed onto the coated silicon wafer and exposed to UV radiation. After the post-baking and developing, it was used as a mold for creating the PDMS chip. A 10:1 (w/w) mixture of PDMS prepolymer and the curing agent (Dow Corning-Midland, MI, USA) was stirred thoroughly and degassed under vacuum. Then the mixture was poured onto the master mold and cured. After curing, the PDMS replica was peeled off from the master, punched with holes to provide inlets and outlet and bonded onto a glass slide (26 × 76 × 1 mm) after oxygen plasma treatment obtaining a strong and irreversible sealing.

3.4.1. 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 [43]. Later, 100 μg mL^{-1} of TES solution was coupled to the modified AP-SNs surface via glutaraldehyde reaction

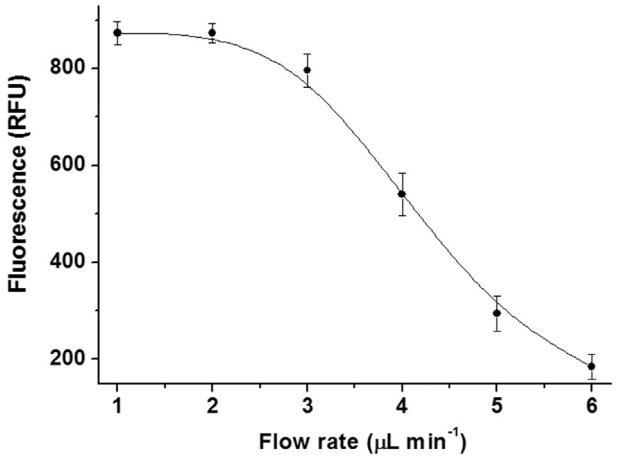

Fig. 2. Effect of flow rate analyzing a reference sera of 76 ng mL^{-1}.

[43]. 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.

3.5. 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 7.2) for 5 min and later washed with 0.01 M PBS buffer (pH 7.2) for 3 min. All solutions employed, were injected using syringe pumps at flow rate of 2 μL min^{-1} (Table 1).

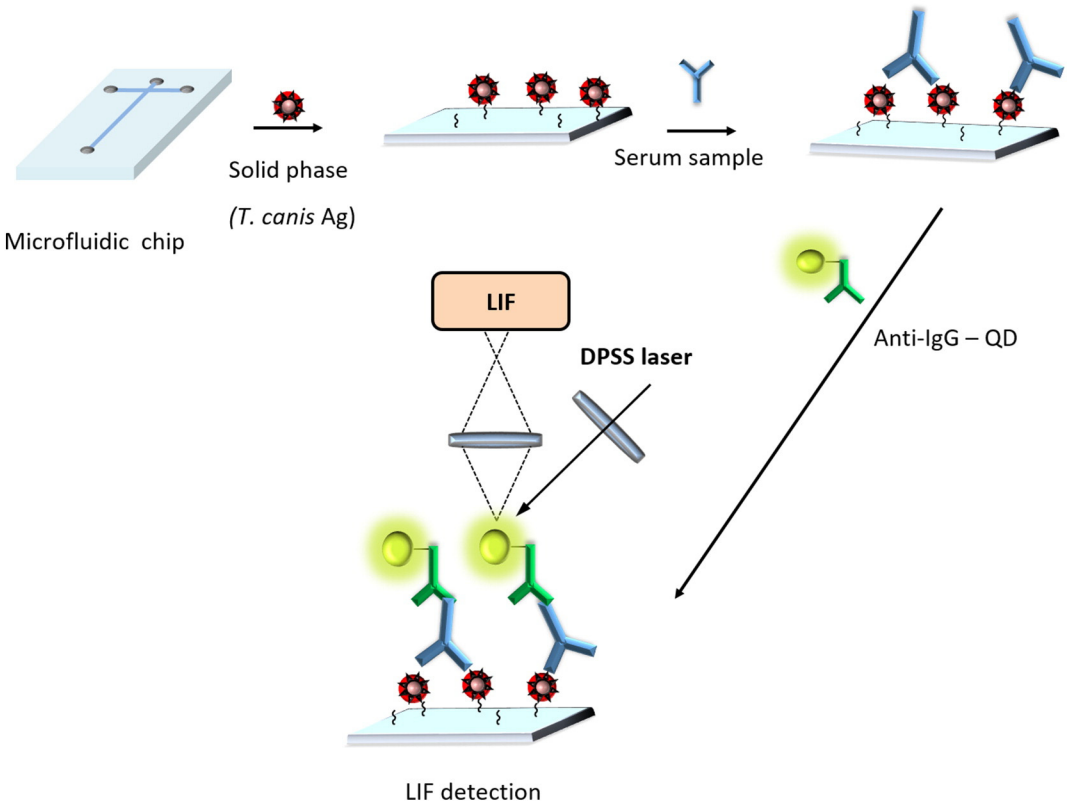

Fig. 1. Schematic representation of the immunoassay on microfluidic. IgG anti-*T. canis* antibodies present in the human serum sample reacted immunologically with TES immobilized on AP-SNs. Bound IgG anti-*T. canis* were quantified by QDs-conjugated second antibodies specific to human IgG.

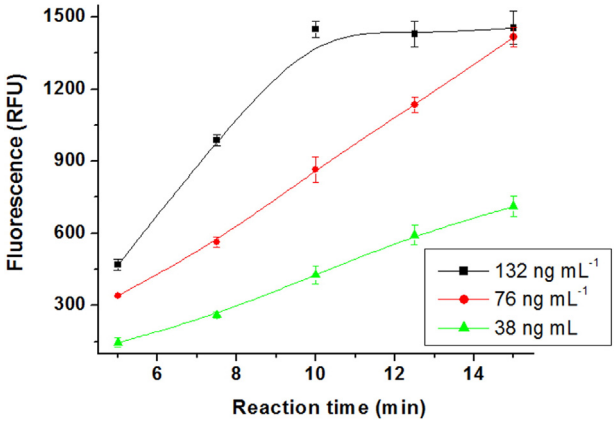

Fig. 3. Fluorescence intensity as a function of reaction time for 38, 76 and 132 ng mL^{-1} of IgG anti-*T. canis* control serum concentrations. The flow rate was 2 μL min^{-1}.

In the second place, serum samples, firstly diluted 100-fold with 0.01 M PBS buffer (pH 7.2), were injected for 10 min and rinsed for 3 min with 0.01 M PBS buffer (pH 7.2). IgG specific antibodies to *T. canis* present in the samples, reacted with TES immobilized on AP-SNs surface, after, microfluidic device was washed with 0.01 M PBS (pH 7.2) 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 7.2) injected for 5 min. The relative fluorescence was measured by using excitation at 491 nm and emission at 540 nm. Before each sample analysis, the immunosensor was exposed to a flow of desorption buffer (0.1 M glycine–HCl, pH 2) at a flow rate of 2.0 μL min^{-1} for 5 min and then was washed with PBS, pH 7.2. 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 0.01 M PBS (pH 7.2) at 4 °C (Fig. 1).

4. Results and discussion

4.1. Optimization of experimental variables

Relevant studies of experimental variables that affect the performance of microfluidic immunosensor for IgG anti-*T. canis* antibodies determination were done. For this purpose, a reference serum of 76 ng mL^{-1} was employed. One of the parameters evaluated was the optimal flow rate, which was determined by employing different flow

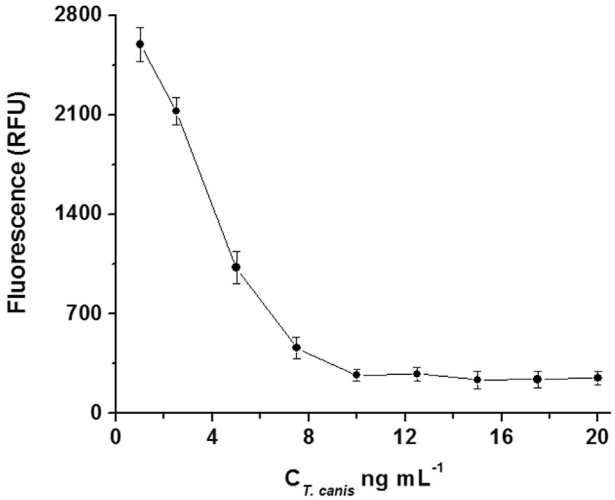

Fig. 4. Effect of the variation of TES concentration in the immobilization procedure.

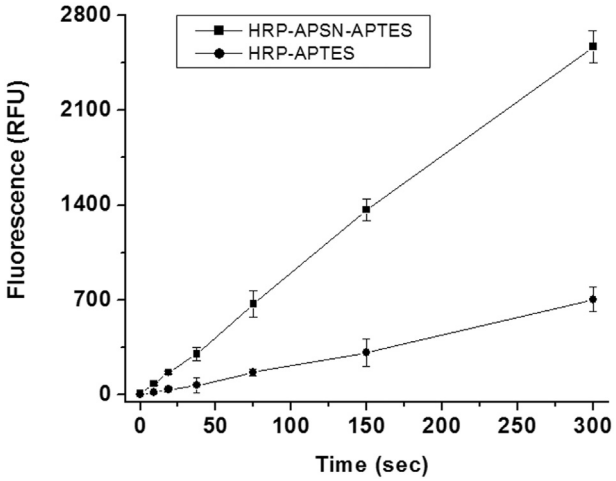

Fig. 5. Obtained signal intensity using HRP as indicator model. This figure compares the signal of the sensor incorporating HRP-AP-SNs and the sensor with a central channel modified with HRP-APTES. For this study, 0.1 M phosphate–citrate buffer, pH 5.0, containing 0.001 M H_2O_2 and ADHP as enzimatic mediator were injected at 2 μL min^{-1}, and the enzymatic product was measured using excitation at 561 nm and emission at 585 nm.

rates and evaluating the relative fluorescence obtained during the immune reaction. As shown in the Fig. 2, flow rates from 1 to 2 μL min^{-1} had little effect over immune response and over signals obtained, whereas when the flow rate exceeded 3 μL min^{-1} the signal was dramatically reduced. Therefore, a flow rate of 2 μL min^{-1} was used for injections of samples, reagents and washing buffer.

Regarding incubation time, the minimum time required for IgG anti-*T. canis* binding is also a critical assay factor, especially when the use of a minimum total analysis time is desired. Fig. 3 shows the measured fluorescence for 38, 76 and 132 ng mL^{-1} IgG anti-*T. canis* reference serum concentrations. The fluorescence intensity increased when the IgG anti-*T. canis* concentration grew. As expected, the intensity of the fluorescence increased with the reaction time. The intensity of the fluorescence, however, did not increase considerably until 10 min had passed, which was likely due to saturation of the specific antigen sites in the microfluidic immunosensor. Therefore, the optimal reaction time was 10 min.

The optimal concentration of TES to be immobilized on AP-SNs surface was evaluated using different concentrations of TES (1.0–20.0 μg mL^{-1}), due to the fact that the amount of these affect the sensitivity of the immunoassay (Fig. 4). Then a constant and saturating concentration of HRP (1 mg mL^{-1} of enzyme prepared in PBS) was adsorbed in unoccupied sites (saturation method with peroxidase). The enzymatic activity was studied using 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) as enzymatic mediator. The HRP catalyzed the oxidation of non-fluorescent ADHP to highly fluorescent resorufin, which was measured by LIF, using excitation at 561 nm and emission at 585 nm. The generated signal was inversely proportional to the amount of immobilized TES. The optimal TES concentration to AP-SNs modification was 10 μg mL^{-1}.

Table 2
Within-assay precision (five measurements in the same run for each reference serum) and between-assay precision (five measurements for each reference serum, repeated for three consecutive days).

[a]Reference serum	Within-assay		Between-assay	
	Mean	CV %	Mean	CV %
5	4.9	3.9	5.2	4.8
38	38.0	5.1	37.3	5.6
132	133.1	4.7	133.2	6.0

[a] ng mL^{-1} IgG anti-*T. canis* antibody.

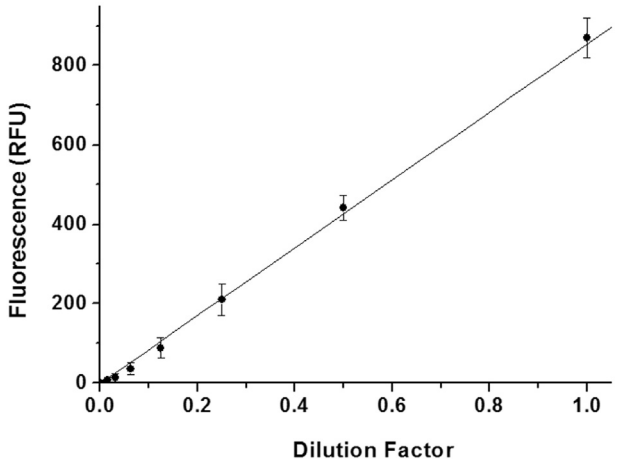

Fig. 6. Dilution test was performed using 76 ng mL^{-1} IgG anti-*T. canis*-specific antibody control serum in 0.01 M PBS, pH 7.2. Flow rate of 2 μL min^{-1}. Each value of relative fluorescence is based on five determinations.

4.2. Amplification effect of the obtained signal

With the purpose of evaluate the amplification effect resulting from the incorporation of the AP-SNs, the signal intensity obtained using the sensor was compared with a signal obtained from a similar sensor in which the immunoreagents were incorporated by a direct modification of the central channel with APTES (Supplementary data). The Fig. 5 shows the signal amplification corresponding to the incorporation of AP-SNs into the central cannel.

4.3. Quantitative determination of IgG anti-T. canis antibodies in the microfluidic immunosensor

Our proposed device, which was applied to the quantification of IgG anti-*T. canis* antibody concentration, was developed as an alternative tool to provide fast and automated results. Also, we envision that our device improves the diagnosis and management of this disease.

The IgG anti-*T. canis* antibodies calibration plot was obtained by plotting relative fluorescence units (RFU) versus IgG anti-*T. canis* antibody concentration. A linear relation, RFU = 4.74 + 11.03 × CIgG 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). The detection limit (DL) was considered to be the concentration that gives a signal three times the standard deviation (SD) of the blank. For LIF detection procedures the DL 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 within-assay precision was tested with five measurements in the same run for each serum. These series of analyses were repeated for 3 consecutive days in order to estimate the between-assay precision. The IgG anti-*T. canis* assay showed good precision; the CV within-assay values were below 5.1% and the between-assay values were below 6% (Table 2).

The accuracy of the LIF microfluidic immunosensor was tested with a dilution test which was performed with 76 ng mL^{-1} IgG anti-*T. canis*-specific antibody reference serum which was serially diluted in 0.01 M PBS, pH 7.2 (Fig. 6). The linear regression equation was RFU = 2.83 + 5.26 × $_{C\ IgG\ antibodies}$, with the linear regression coefficient r = 0.997 (Fig. 5).

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. The positive samples were later analyzed by our proposed quantitative method, which revealed high concentrations of IgG-specific antibodies in all of them. The negative sera were also negative for our proposed method.

According to our detailed search, no articles intended to the development of a sensor for the quantitative determination of IgG anti-*T. canis* antibodies have been reported.

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 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 article 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 sensitivity. Besides, to date, no one method for the quantification of anti-*T. canis* IgG antibodies has been reported. Among the most relevant advantages offered by our system, we can mention the possibility of antibody quantification, less sample volume required and the lower detection limit. 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.microc.2016.10.027.

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