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# **Enhanced detection of quantum dots labeled protein by simultaneous bismuth electrodeposition into microfluidic channel**

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## **ABSTRACT**

In this work, a disposable microfluidic platform for immunoassay application, using quantum dots (QDs) as electrochemical labels and their enhanced detection using bismuth (Bi) is proposed as an alternative of mercury-based detection platforms. CdSe@ZnS QDs are used as labels to detect human Immunoglobulin G (HlgG) as a model protein, through highly sensitive stripping voltammetry of the dissolved metallic component of the QDs. The modification of the in-chip integrated screen printed carbon electrodes (SPCEs) is done by a simple Bi electrodeposition simultaneously introduced with the sample containing QDs. A micromixer is used to carry out the bioconjugation steps. A permanent magnet is placed at the end of the mixer in order to capture the magnetic beads (MBs) used as solid immobilization support for the immunoassay. Different parameters (e.g. bismuth deposition, flow rate, incubation time, mixer

geometry, etc.), are optimized to achieve sensitive and reproducible measurements. The limit of detection (LOD) for Human Immunoglobulin G (HlgG) in presence of bismuth is very low ( $3.5 \text{ ng mL}^{-1}$ ) with a RSD of 13.2 %. This LOD is 3.5 lower than the one obtained without bismuth. Moreover, the sensitivity of the system is increased 100 folds in comparison with the batch experiments carried out with classical SPCEs (with the sample containing Bi).

**Keywords:** Bismuth, Electrochemistry, HlgG, Magnetic Beads, Microfluidic, Quantum Dots.

## 1. INTRODUCTION

Electrochemical detection exhibits great advantages in biosensing applications that include low limits of detection and high sensitivity. During the last years, the use of microfluidic platforms for electroanalysis has increased in a remarkable way. These platforms, called also lab-on-a-chip (LOC), are especially interesting for the reduction of sample and reagent volumes, reduction of the analysis time besides offering portability and integration to the whole detection system. Furthermore the use of nanomaterials as electrode modifier is a well-known strategy in biosensing applications in order to increase their sensitivity. Together, electrochemistry, nanotechnology and microfluidics could provide a really powerful biosensor platform with interest for various applications.

With the development of the nanotechnology, different kinds of nanoparticles have been used as labels to enhance the sensitivity of the electrochemical immunoassays. Among these nanoparticles, the metallic ones provide a wide range of possibilities to detect multiple analytes with a very high sensitivity through the stripping voltammetric detection of their metal components. QDs, also known as semiconductor crystals, have been

mainly used as fluorescence labels; however they can also be detected by dissolving them liberating the metal ions present in their core using the same strategies applied for heavy metals detection **(Merkoçi, et al., 2007)**.

Different materials have been used for heavy metal detection, such as mercury **(Bontidean, et al., 1998)**, bismuth (Bi) **(Rechacek, et al., 2008)**, graphene **(Wang, et al., 2013)**, etc. The high toxicity of mercury film electrodes, which have been applied for the determination of heavy metals by stripping voltammetry **(Palchetti, et al., 1999 and 2003)**, makes necessary the search for more environmental-friendly materials with similar performance. One interesting and simple alternative is bismuth, which was used for the first time by Wang et al. **(Wang, et al., 2000)** as bismuth film electrode (BiFE) on glassy-carbon or carbon-fiber substrate for the detection of microgram per liter levels of cadmium, lead, thallium and zinc by stripping voltammetry. Apart the low toxicity of BiFEs compared to mercury, they offer other advantages such as ability to form alloys with different metals, wide potential ranges and low sensitivity to dissolved oxygen. They have already been applied for the detection of several targets. Calvo-Quintana et al. coupled Bi-modified electrodes to a small portable instrument for the detection of lead at legal limits ( $20 \mu\text{g kg}^{-1}$ ) in a complex matrix such as milk **(Calvo-Quintana et al., 2011)**. Caldeira et al. applied Bi films for the determination of traces of Tl(I) separately and together with Zn(II) and Pb(II), with a limit of detection (LOD) of  $2 \text{ nmol L}^{-1}$ , showing that the sensitivity to Tl does not depend on the presence of other ions, and finally determining Tl in juice samples **(Caldeira, et al., 2011)**.

Among the biosensing applications, the detection of Zinc (Zn) in patients with problems in the functional immune system has been evaluated by Papautsky's group. Those Zn concentrations decrease in critically ill patients and could potentially benefit from Zn supplementation as a therapeutic strategy. The authors reported the Zn faster detection by anodic stripping voltammetry on bismuth microfabricated electrodes in serum as well

as in bovine serum extract (**Jothimuthu, et al., 2013, and Kang, et al., 2013**). While it is possible to find a wide range of application by using QDs as labels for protein/DNA and cell detection, however, in the literature there are few cases in which the Bismuth have been considered as an electrode modifier in order to increase the sensitivity of the QDs based in-chip biosensors. Some examples are: the work made by Chen's group, related to the development of an electrochemical biosensor for the neutravidin recognition by using QDs as labels, which were detected by using an electrodeposited bismuth film, achieving a limit of detection of 5 nM (**Dua, et al., 2008**); and a biosensor based on bismuth modification for protein and DNA detection, which was proposed by Kakabakos's group. In this work, the authors used stripping voltammetry technique for the in-batch detection of CdSe@ZnS QDs, which was used as labels for the prostate-specific antigen (PSA biomarker) and DNA quantification (**Kokkinos, et al., 2013**). Other approach by using a bismuth film was used as a sensing platform for immunoreaction assay which includes the interactions between IgE and anti-IgE molecules. IgE protein was deposited on a carbon paste electrode via bismuth (III) cations without using any membrane or functional reagent. The immobilized reagents and interaction of anti-IgE on this formation were monitored by electrochemical impedance spectroscopy (**Anik, et al., 2011**).

Bi can be deposited onto SPEs following different procedures, in different conditions and on different substrates depending on the target analyte or the aim of analysis. The fabrication can be done using "ex situ", "in situ" and "bulk" approaches. In the "ex situ" method, the electrodes are immersed in a solution containing  $\text{Bi}^{3+}$  and a potential is applied to reduce it to  $\text{Bi}^0$  for its deposition (**Lin, et al., 2005**). In the in situ method, both Bi and the target analyte are electrochemically deposited on the electrodes, forming alloys followed by anodic stripping of metals from the electrode (**Nie, et al., 2010**). The third option is based on the incorporation of bismuth oxide to the ink for the fabrication

of the SPE, which is then reduced to metallic bismuth for the formation of a Bi layer (Kadara, et al., 2009).

In our group, a phenol detection system by immobilization of a mushroom tissue onto plain screen printed electrode (SPE) and multiwall carbon nanotube (MWCNT) modified SPE (SPE/MWCNT) via Bi deposition was performed. This sensor achieved a LOD for phenol of 0.48 mM and 1.17 mM for SPE / Bi / Tissue and SPE / MWCNT / Bi / Tissue respectively (Merkoçi, et al., 2010).

In this work, a novel micro and nanomaterials-based platform able to operate as a sensitive immunoassay for HlgG detection within a microfluidic system, making it easily scalable to a point of care (POC) system is developed. The modification of SPE by the simultaneous electrodeposition of a thin layer of Bi together with the sample containing QDs, has been performed and evaluated so as to improve the QDs electrochemical signal, demonstrating a simple and sensitive platform. The developed system is useful not only for QDs detection with interest for immunosensing but for other applications with interest for environment control such as heavy metal detection.

## **2. MATERIALS AND METHODS**

### **2.1. Reagents**

Streptavidin-QD655, tosylactivated magnetic beads MyOne (1  $\mu\text{m}$  of diameter), Human IgG, anti-Human IgG produced in goat ( $\alpha$ -HlgG), and biotin anti-Human IgG (biotin  $\alpha$ -HlgG) produced in goat were obtained from Invitrogen (Spain). Phosphate buffer saline (PBS), Tween 20, casein, bovine serum albumin (BSA), and Bismuth (III) nitrate pentahydrate (reagent grade 98 %) were acquired from Sigma Aldrich (Spain). PBS supplemented with 5 % (w/v) of casein and 0.005 % (v/v) of Tween 20 was prepared as

blocking buffer for the channel wall treatment. PBS supplemented with 5 % (w/v) of bovine serum albumin (BSA) and 0.005 % (v/v) of Tween 20 was prepared as blocking buffer to avoid unspecific absorption onto the magnetic beads. PBS supplemented with Tween 20 at 0.05 % (v/v) was used as washing buffer (PBST). PBS was employed as immunobuffer, except for QDs conjugation. In this case, commercial buffer Borate buffer, pH: 8.3, 0.05 % sodium azide) was used. Rare earth (neodymium iron boron, 1.72 T) permanent magnets were purchased in Magnetladen, Germany.

Scanning electron microscope (SEM) analyses were performed by using an EVO (Carl Zeiss NTS GmbH, Germany). Bare and Bismuth modified SPCE were analyzed both in drop and flow configurations.

## *2.2. Immunoassay performance by using Bismuth*

Chips were produced following the same protocol previously reported by our group **(Medina-Sánchez, et al., 2014)**.

Both measurements, in batch and in-chip, were tested in order to compare the sensitivity of the systems. Commercial CdSe@ZnS QD655 (10  $\mu$ L at 5 nM of concentration) were first partially dissolved by using HCl 1M for 2 min (adding 50  $\mu$ L). After the dissolving, 200  $\mu$ L of acetate buffer (pH 4.5) was added to the solution. For the comparison between samples with and without bismuth, 40  $\mu$ L of bismuth (at different concentrations) and PBS was added respectively. The first experiment consisted in the optimization of the bismuth concentration for both platforms. Since the cadmium reduction potential (-1.1 V) is also suitable for the bismuth electrodeposition the same potential value is used. The sample containing bismuth was introduced in the same solution, ensuring the simultaneous reduction of the cadmium (II) and bismuth (III) (within 120 s).

Briefly, 40  $\mu\text{g}$  of tosylactivated magnetic particles (15  $\mu\text{L}$  from stock solution) were washed 3 times with borate buffer and suspended in 135  $\mu\text{L}$  of the same buffer. These MBs were functionalized with capture antibody by electrostatics by using a borate buffer pH 8.5 overnight under agitation (700 rpm, 37°C). The conjugated MBs were washed with PBST three times in order to remove the antibody excess, followed by a blocking step using PBS supplemented with Tween 20 at 0.05 % (v/v) and BSA at 5 % for 2 hours under agitation conditions (700 rpm, 25°C). The blocked MBs were washed again with PBST three times by using the magnetic separator. Then, the microchannel was blocked by using PBS supplemented with Tween 20 at 0.05 % (v/v) and casein at 5 %. This solution was flowed during 10 min at 5  $\mu\text{L min}^{-1}$ . After the blocking step, PBST was introduced for 10 min more at the same flow rate in order to clean the excess of the blocking solution.

The working electrode was then activated by cyclic voltammetry (10 cycles at a potential range from -0.8 V to 0.8 V and a scan rate of 100  $\text{mV s}^{-1}$  in PBS buffer). Magnetic beads already modified with antibodies were then immobilized into the mixer by location of a permanent magnet behind the mixer outlet, with the optimized volume and flow-rate (2 min at 5  $\mu\text{L min}^{-1}$ ).

7.5  $\mu\text{L}$  of QD 655 ITK Streptavidin (2  $\mu\text{M}$  from stock solution) were suspended in 492.5  $\mu\text{L}$  of QD incubation buffer (50 mM borate pH 8.3, 0.05% sodium azide), as well as 45  $\mu\text{L}$  of Anti-Human IgG (gamma-chain specific)-biotin. They were mixed and incubated for 30 min (750 rpm 25°C) in batch. Then the excess of antibody was removed by filtering using eppendorf filters (Eppendorf® Safe-Lock microcentrifuge tubes from Sigma Aldrich) and centrifuged at 15000 rpm for 5 min.

An optimized micromixer (500  $\mu\text{m}$  diameter and 50  $\mu\text{m}$  thickness) with two inlets was fabricated and used for the immunoassay performance (Figure S1). In one of the inlets,



a syringe containing the analyte (Human IgG) is located while in the second inlet, the QDs already functionalized with detection antibody were located. Both of them were simultaneously introduced at a flow-rate of  $2.5 \mu\text{L min}^{-1}$  during 15 min. After the mixing and incubation, the channel was washed with PBST at a flow-rate of  $2.5 \mu\text{L min}^{-1}$  for 15 min. Then, a dissolving solution (HCl 1M) was introduced from the outlet in order to dissolve the QDs (labels in the immunoassay). This procedure was performed at flow-rate of  $5 \mu\text{L min}^{-1}$  for 3.5 min (the time needed to fill the entire channel at this flow-rate). The dissolving solution was remained in static mode for 3 min. Finally, the mixer was connected to the measurement chip (a simple channel with integrated screen printed electrode), then, acetate buffer (pH. 4.5) was introduced from the two inlets with a flow rate of  $5 \mu\text{L min}^{-1}$  and the electrochemical measurement was performed using the following parameters (without conditional potential because for each measurement, a new chip was used, so it was no necessary to reoxidize the cadmium deposited onto the electrode after the measurement). The deposition potential was defined at -1.1 V as already reported in (Medina-Sánchez, et al., 2014), and the deposition time was set at 400 s to allow all the solution to reach the electrode surface. The stripping measurement was done from -1.1 V to -0.15 V with a step potential of  $5 \text{ mV sec}^{-1}$  and amplitude of 0.3 V. This measurement was performed in the presence or not of bismuth in acetate buffer.

### **3. RESULTS AND DISCUSSION**

#### **3.1. *Bismuth concentration optimization***

The modification of the SPCEs was done by electrodeposition of bismuth previously mixed with the sample containing QDs. Figure S2 shows the typical peaks corresponding to the cadmium present in the QDs core at -0.85 V and the bismuth peak, which appears at -0.25 V.

This optimization was performed in batch (a drop of sample onto SPE) and in-chip format. In Figure 1A, the QDs signal for different bismuth concentrations, in drop format is shown. The continuous lines show how the peak is increased when bismuth is added, while the dotted lines represent the system saturation after  $50 \mu\text{g mL}^{-1}$  bismuth concentrations. In Figure 1B, the same results are shown for in-chip measurements. In the microchannel system, the saturation is achieved with smaller bismuth concentration compared to the drop format. This could be explained by the more uniform film of bismuth obtained when the electrodeposition is done under flow condition as seen in Figure 2. Indeed, the modification of the SPEs after the deposition of bismuth in flow and static modes has been evaluated by scanning electron microscopy. Homogeneous distribution of the Bi on the electrodes surface can be observed when the deposition is in flow mode due to the continuous renovation of the Bi solution onto the electrode and the diffusion phenomena that occurs faster than in static mode.

### 3.2. *Comparison between batch and in-chip measurements*

Figure 3A, B show the comparison between QDs calibration in bare-SPE and QDs sample containing bismuth respectively. In figure 3C, the respective calibration curves are also showed. The same figure also shows the SPCE response integrated into a microchannel system.

In batch measurements, the use of bismuth causes an increase of the electrochemical signal of around 1.2 folds, with a sensitivity of  $7 \times 10^{-6} \text{ A nM}^{-1}$  and  $R^2$  of 0.997, while in chip, the increase is 5.5 fold with a sensitivity of  $5 \times 10^{-6} \text{ A nM}^{-1}$  and  $R^2$  of 0.915. In order to compare the two systems, current density was calculated. By comparing the chip with the batch measurements, the achieved sensitivity is 100 fold better in flow than in batch mode. This means that the chip format helped to improve the sensitivity due to the fact that the sample is permanently flowing onto the working electrode.

### 3.3. *Immunoassay performance*

In order to demonstrate the use of bismuth as strategy to improve the detection of QDs as labels in a magneto-immunoassay, a micromixer was integrated with a detection channel. A neodymium magnet was located at the end of the channel (see Figure 4) in order to immobilize the previously modified magnetic beads.

The conjugation parameters in flow were also optimized, in order to choose the shortest incubation time without losing efficiency. This study was performed following the same protocol previously described in Experimental section. All incubation steps were performed by changing the flow rate from 1 to 5  $\mu\text{L min}^{-1}$ . The most efficient conjugation was obtained using flow rates of up to 5  $\mu\text{L min}^{-1}$ , otherwise, the higher flow could reduce the efficiency of binding event inside the micromixer. Different incubation time intervals such as 5, 10, and 15 min were also tested. A proper electrochemical signal was observed at 15 min. Indeed the first 5 minutes were necessary to fill the entire micromixer at this flow rate, being necessary 10 min more to perform the binding between the immobilized magnetic beads and the detection antibody labeled with QDs. The effect of washing time (set at 5  $\mu\text{L min}^{-1}$ ) was also studied finding that 10 min was a sufficient time to clean the channel from antibodies excess.

In Figure 4 all the needed steps for the immunoassay performance and detection are represented.

### 3.4. *Electrochemical measurements*

SWASV was applied for QDs detection used as labels that in turn are related with the protein concentration. The measurements were carried out in 0.1 M acetate buffer (pH 4.5) after the partial dissolving by using 1M HCl. As it was mentioned before, the reduction potential was -1.1 V. The duration of the measurement was 400 s. In order to confirm the advantages of the use of bismuth in the improvement of immunoassay

performance, a magneto-immunoassay with three different concentrations of analyte (0, 50 and 100 ng mL<sup>-1</sup>) was performed. After the cleaning and dissolving steps, the measurements were performed in acetate buffer in presence or absence of bismuth (see results in Figure 5). It can be observed that the sensitivity in the case of immunocomplex (labeled with QDs) is lower than in the case of QDs alone due to the fact that the biological sample inhibits the electrochemical signal and affects the electrode performance along the time; however the increasing of the signal for the measurement with the presence of bismuth was around 3 times with a RSD of 13.2 %.

The increase of the sensitivity for an immunoassay based on QDs as electrochemical labels was demonstrated as a proof of concept with the performance of a complete magneto-immunoassay for human IgG determination (optimized protocol in the previous sections) into a microfluidic system, by addition of bismuth in the acetate buffer at the last step. It is an easy and promising strategy to reduce the LOD with a simple addition in the measurement buffer without the addition of more steps in a magneto-immunoassay.

#### **4. CONCLUSIONS**

A microfluidic device based on enhancement via bismuth for protein detection achieving very low LOD (3.5 ng mL<sup>-1</sup>) as well as an increase in the sensitivity up to 100 fold respects to the batch format is successfully developed. The device takes advantage of the speed and low cost of the conventional immunoassay technologies. Under optimal conditions, the device was capable of detecting three times less concentration than the conventional system, in 6.6 min by using the sample containing the bismuth solution at the optimized concentration.

The exposed devices coupled with a portable electrochemical analyzer are promising for in-field quantitative testing for disease-related protein biomarkers. The developed device thus provides an efficient tool for rapid and quantitative detection of protein that can also be extended to DNA analysis.

## **ACKNOWLEDGMENTS**

MICINN through project MAT2011-25870 and E.U. through FP7 “NADINE” project (contract number 246513) have funded this research.

## FIGURE LEGENDS

**Fig. 1.** Bismuth concentration optimization in (A) drop and (B) in-chip formats. The Figure shows the typical peak of QDs at -0.85 V and its increasing due to the bismuth addition at different concentrations.

**Fig. 2.** Bismuth electrodeposition onto screen printed carbon electrode (SPCE). (A) Bare SPCE, (B) Electrodeposited bismuth in static mode, and (C) Thin layer of bismuth onto the SPE when the bismuth is deposited in flow mode.

**Fig. 3.** Calibration curves corresponding to (A) drop measurements, (B) In chip measurement. Both of them with and without bismuth (the higher slope for the curve related with the sample containing bismuth), and (C) The comparison between drop and chip formats. Measurements (a) with Bi, and (b) without Bi.

**Fig. 4.** Schematic of the steps to perform the immunoassay including the detection. 1) the introduction of blocking step, 2) the immobilization of magnetic beads already modified with the capture antibody and blocking, 3) the conjugation step of the model protein and detection antibody already modified with QDs. Between each step a washing step is performed by using PBST. After the last washing step, in 4) HCl 1M is introduced from the outlet to dissolve the external layer of the QDs and liberate the Cd ions from the QDs core to be detected in step 5) After the introduction of acetate buffer.

**Fig. 5.** Electrochemical response of QDs used as labels in a magnetimmunoassay for HlgG determination. (A) Effect of the bismuth presence in the electrochemical measurement for an immunoassay with a fixed concentration of HlgG (50 ng mL<sup>-1</sup>), and

(B) Comparison between three different concentrations of HIgG. Measurements (a) with Bi, and (b) without Bi.

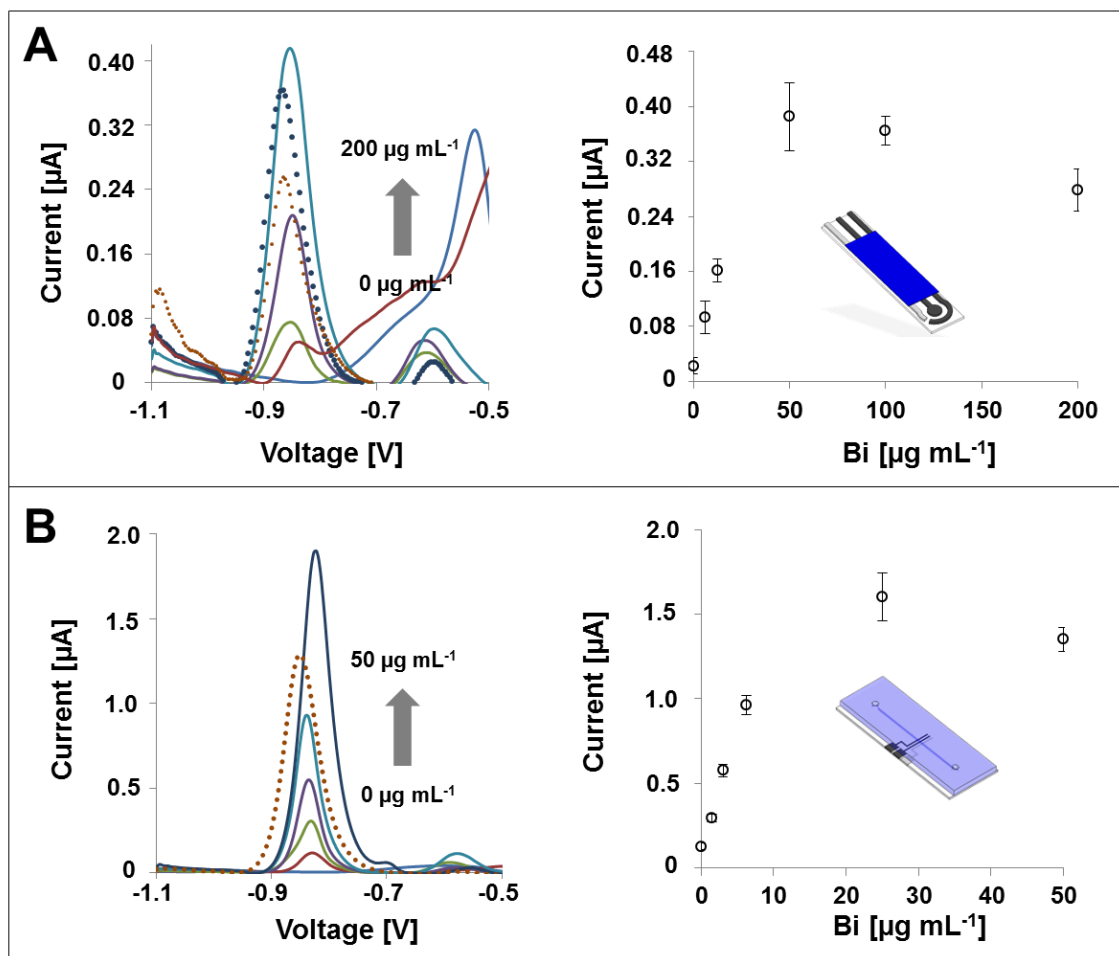
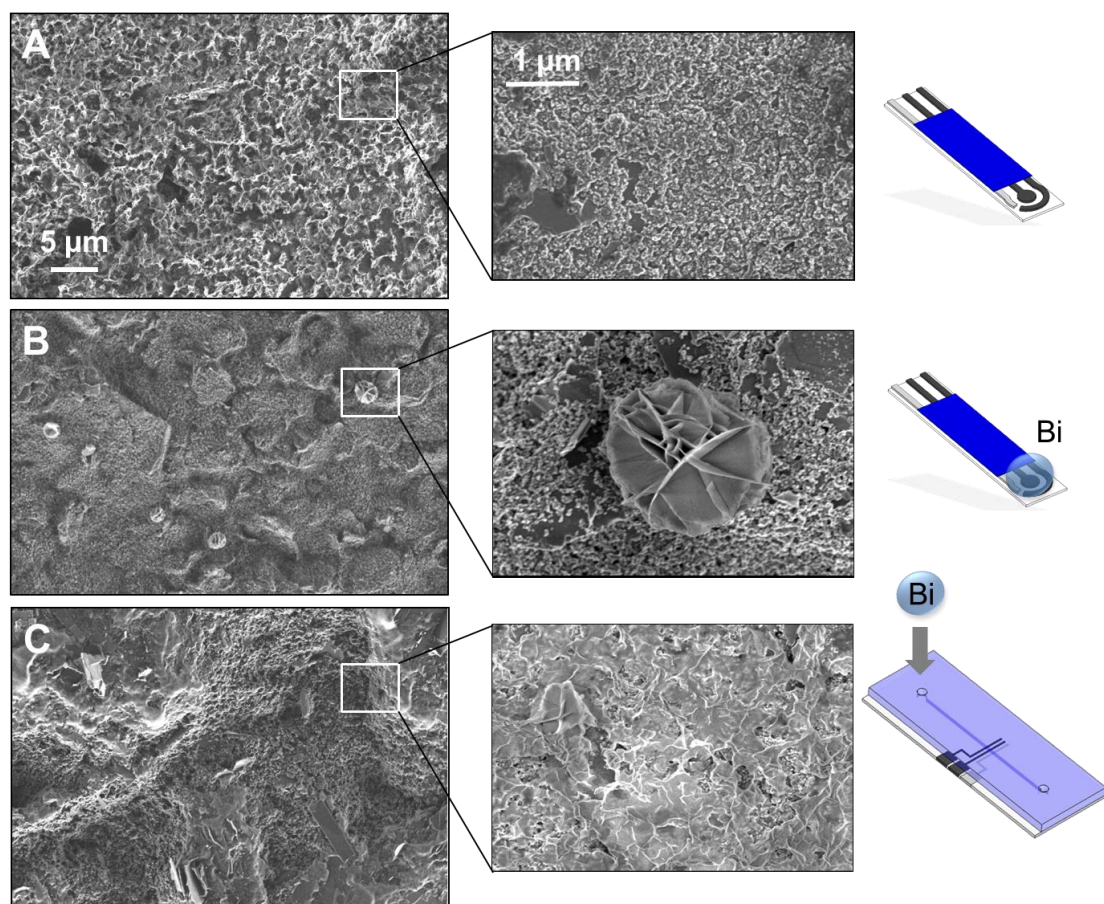
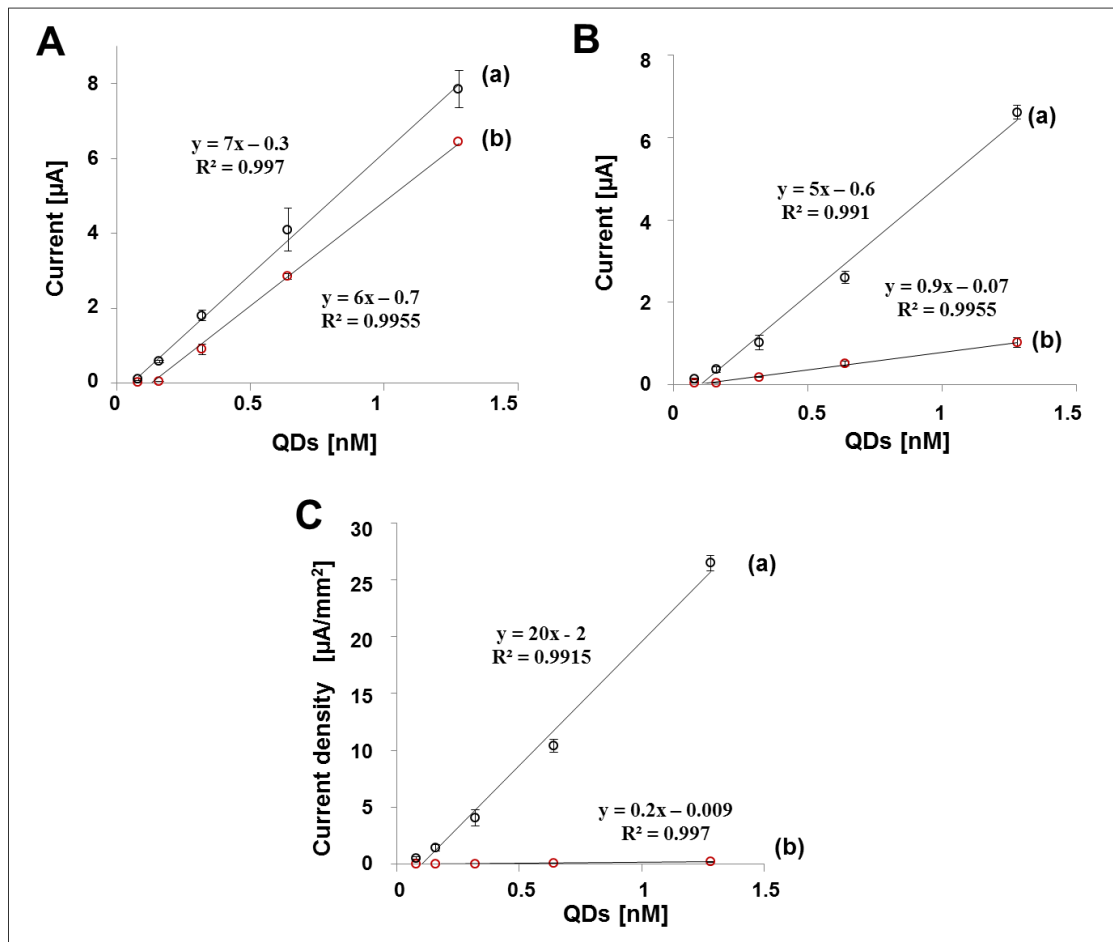


Fig. 1

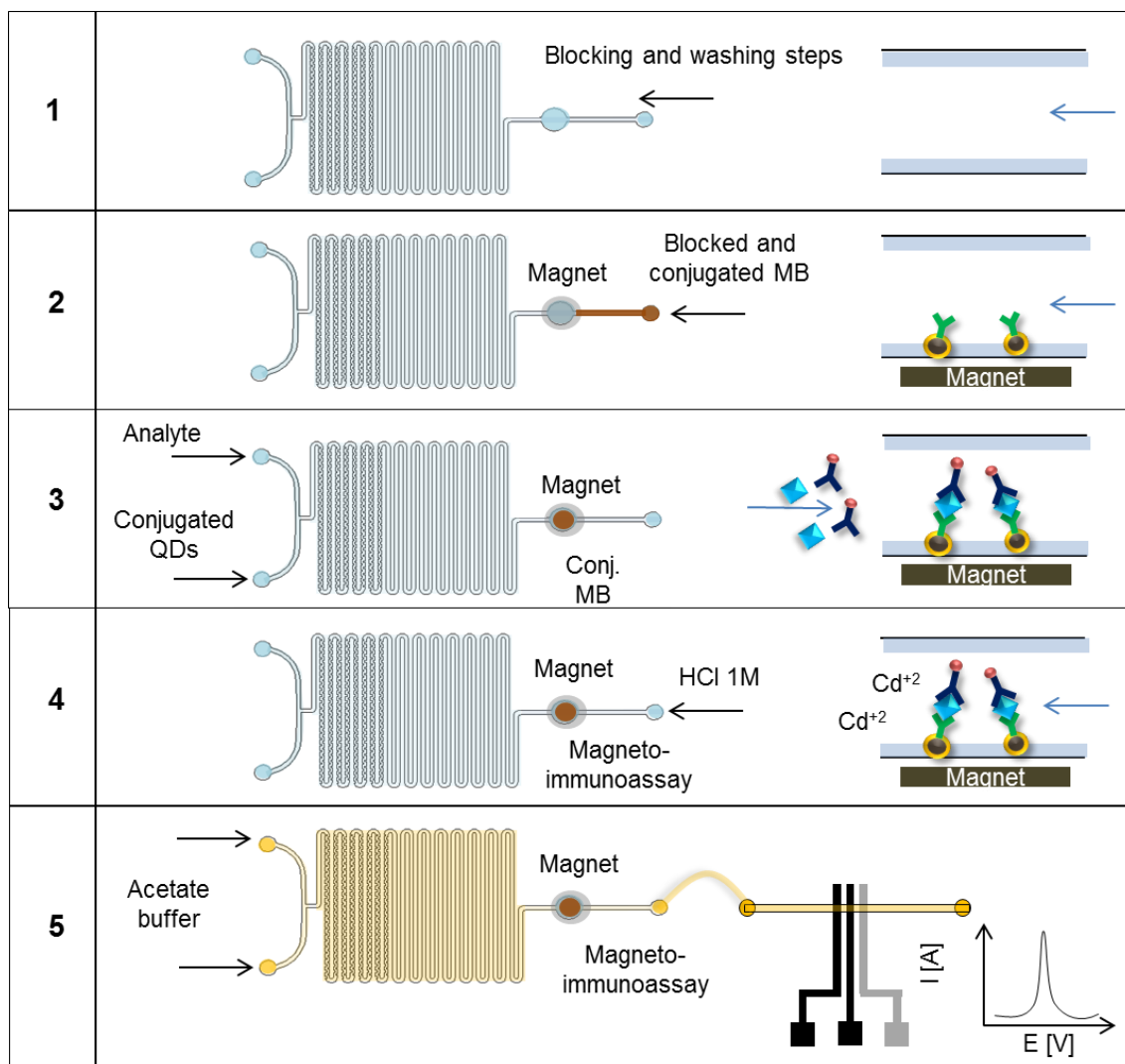




**Fig. 2**



**Fig. 3.**



**Fig. 4.**

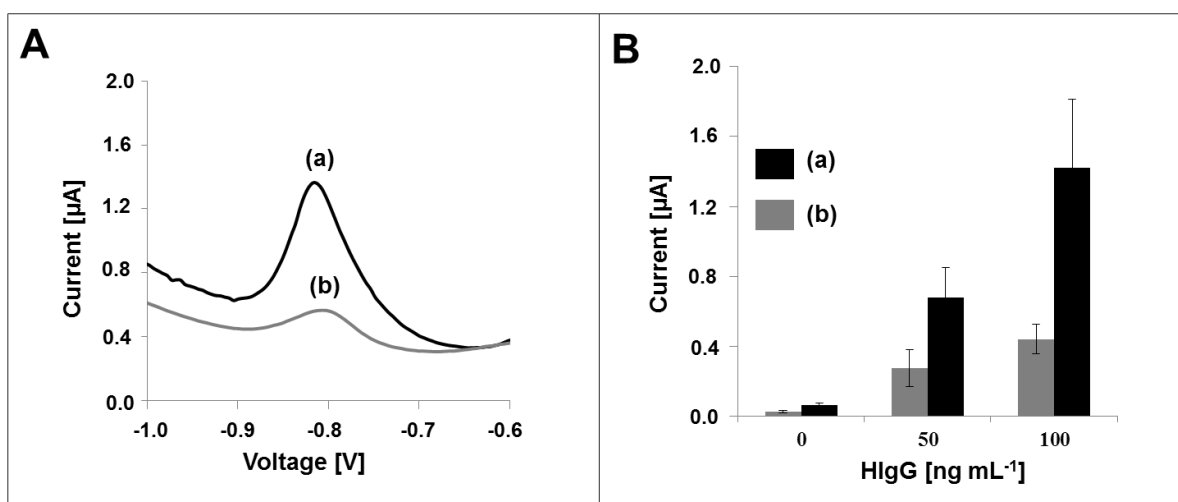


Fig. 5.

## Supporting Information

# **Enhanced detection of quantum dots labeled protein by simultaneous bismuth electrodeposition into microfluidic channel**

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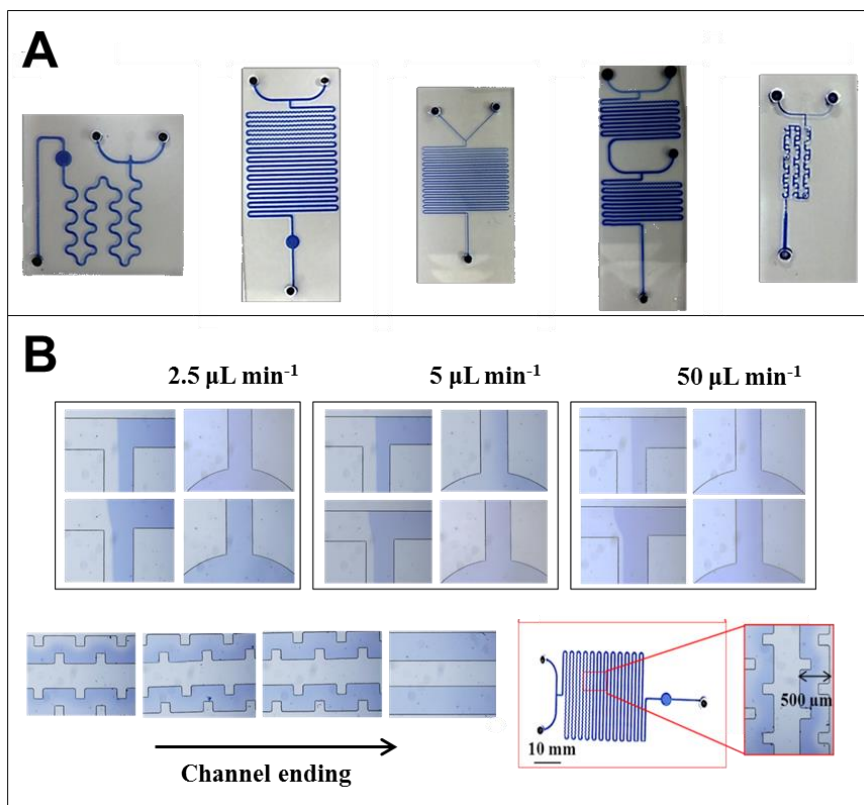
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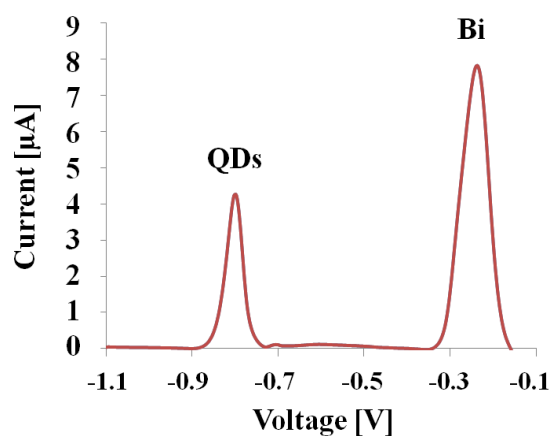
<sup>2</sup>ICREA, Institució Catalana de Recerca i Estudis Avançats.

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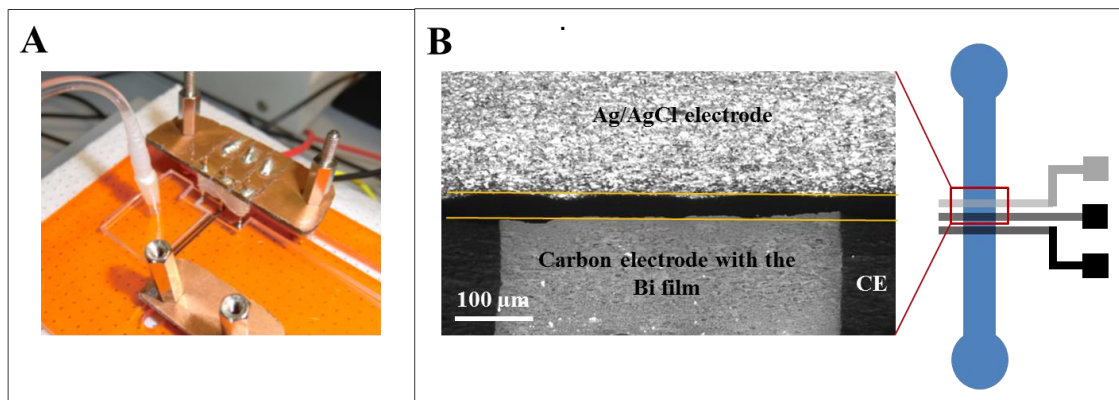
In order to check the mixing performance, trypan blue dispersion in water (obtained from Sigma Aldrich) were studied in different mixers geometry in order to mimic the experimental conditions. In Figure S1A a higher dispersion rate of trypan blue is observed after the mixing process (at the end of the channel) in the chosen mixer in comparison with the others. In Figure S1B it is also possible to observe the diffusion interphase between trypan blue suspended in water compared with water only. This diffusion interphase is less defined when the flow rate increases from 2.5 to 50  $\mu\text{L min}^{-1}$ , even that, the complete mixing is achieved at the end of the channel for the tested range of flow-rate.



**Figure S1.** (A) Different mixer performances, and (B) chosen mixer, its performance at different flow rates, as well as its respective simulation



**Fig. S2.** Typical peaks corresponding to the QDs and Bismuth in chip measurements. Deposition time of 120 s at -1.1 V and stripping between -1.1 to -0.1 V.



**Fig. S3.** (A) Set-up of the detection microfluidic channel, and (B) SEM Image of the SPCE after the Bi electrodeposition in flow format.

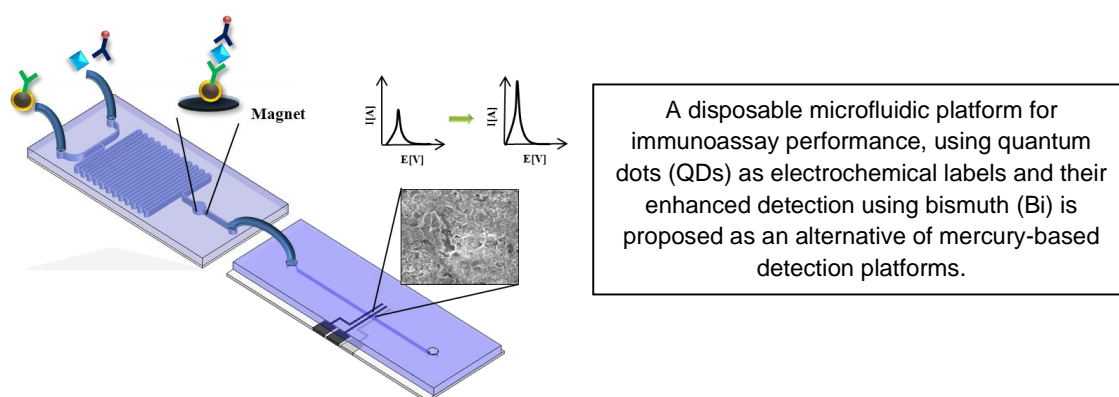
## TOC

### Enhanced detection of quantum dots labeled protein by simultaneous bismuth electrodeposition into microfluidic channel

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