Research Article

Design and fabrication of a COP-based microfluidic chip: Chronoamperometric detection of Troponin T

This work demonstrates the design and fabrication of an all cyclo-olefin polymer based microfluidic device capable of capturing magnetic beads and performing electrochemical detection in a series of gold electrodes. The size of chip is of a microscope slide and features six independent measuring cells for multianalyte detection purposes. The aim of this work is to show that rapid prototyping techniques can be instrumental in the development of novel bioassays, particularly in clinical diagnosis applications. We show the successful determination of troponin-T, a cardiac disease marker, in the clinically relevant range of 0.05–1.0 ng/mL. This methodology achieves a detection limit of 0.017 ng/mL in PBS solutions, and is capable of detecting less than 1 ng/mL in a 1:50 human serum dilution.

Keywords: Amperometry / Cyclo-olefin polymer / Immunomagnetic assay / Lab-on-a-chip / Troponin

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1 Introduction

Cardiovascular diseases are among the main causes of death worldwide, followed by infectious and parasitic diseases, can-

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Abbreviations: AMI, acute myocardial infarction; COP, cyclo-olefin polymer; ImE, immobilization electrode; HRP, horseradish peroxidase; MB, magnetic bead; TMB, 3,3',5,5'-tetramethylbenzidine; WE, working electrode

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clinical presentation and electrocardiogram for the diagnosis of myocardial infarction [1, 2]. Cardiac troponins can be considered as the current biochemical “gold standard” for the diagnosis of AMI owing to their excellent specificity and sensitivity. Cardiac troponin I (cTnI) and troponin T (cTnT) are highly specific to the heart muscle and are normally not present in the blood stream. Troponin blood concentrations above a cut-off level provide 100% diagnostic sensitivity for AMI with serial sampling. Troponin complex plays an important role in the regulation of skeletal and cardiac muscle contraction. It is formed by three different subunits — TnI, troponin C (TnC), and TnT. Cardiac TnT (cTnT) and TnI are presented in different forms that the skeletal muscle and TnC does not have cardiac isoforms but forms binary complexes with TnI and ternary complexes with TnI and TnT. That is why the detection of cTnT is one of the main goals for the diagnosis of myocardial infarction. Several clinical trials and patients’ stratification studies include the monitoring of cTnT [3–5] showing the correlation between the presence of cTnT in patients arriving in emergency after chest pain.

Current point of care is that the devices for the detection of TnT are used for diagnosis, risk stratification, and guiding the management of suspected acute coronary syndrome patients. Cardiac Reader™ from Roche achieved an assay range between 0.1 and 2 ng/mL matching the current guidelines of NACB and ESC [6, 7]. The use of these systems is limited by portability issues and the need for highly trained staff. Point-of-care detection systems aim to simplify the analytical procedures and yet offer rapid and reliable diagnostic information that can help the patient or a first-aid team to take the right decisions before the heart is permanently damaged. Some key requirements of any point-of-care device are portability, ease of use, and the ability to provide rapid results at reasonable cost [8, 9]. Microfabrication allows the development of novel analytical systems that usually meet the requirements of small size and rapid results. However, these are only occasionally easy to use by a layman, and seldom fall within the range of the cost that the market is ready to accept. Bearing these constraints in mind, this work describes the design and fabrication of a miniaturized flow cell that can detect TnT chronoamperometrically. Moreover, the system is fabricated on a polymeric substrate using rapid prototyping techniques. This means that the remainder of the system may be fabricated outside a clean-room environment using more cost-effective techniques barring the fabrication of the microelectrodes. Up till now, a vast majority of lab-on-a-chip devices relied on silicon and glass substrates, combined with elastomers such as PDMS to define the microfluidic channels [10]. However, the advent of organic and printed electronics has brought new trends in the field of lab-on-a-chip devices, the use of polymeric substrates [11–14], and the sophistication of paper chromatography in the form of paper microfluidics [15, 16].

On the other hand, electrochemical detection methods are ideally suited for their integration in miniaturized systems, as clearly pointed by Nyholm [17]. Microelectrodes can be easily fabricated and the instrumentation required to carry out potentiometric and amperometric measurements is relatively simple and cheap to fabricate in comparison with optical methods, which are currently the main detection method in miniaturized systems including biosensors.

This work describes the development of a cyclo-olefin polymer (COP) microfluidic chip that integrates a series of channel microband electrodes, and a capture zone in which functionalized magnetic particles are captured. COP was selected due to its low impurity levels, high glass transition temperature (Tg), and for being the plastic with the lowest water absorption, making it a perfect structural material for medical devices. The chip is the size of a microscope slide and contains six independent measuring units. This allows the simultaneous or sequential performance of multianalyte measurements on the same chip. A holder to host the chip and provide electrical and fluidic connections has been fabricated using 3D printing techniques.

2 Materials and methods

All solutions were prepared using deionized water of resistivity not less than 18 MΩ cm, and all chemicals used in this work were of analytical grade and used as received without further purification. These were potassium nitrate (≥99.0%, Sigma Aldrich), potassium hexacyanoferrate II (Reagent Plus, ≥98.5%), 3M KCl (electrolyte solution for Ag/AgCl electrode, Metrohm, Spain), and anhydrous cyclohexane, P99.5%.

Streptavidin-modified magnetic beads (strep-MBs, 2.8 μm, 10 mg/mL) (Dynabeads® M-280 Streptavidin), were purchased from Dynal Biotech ASA; human cTnT (Cat 8T13), biotinylated monoclonal mouse anti-cTnT (biotin-anti-cTnT, Cat 4T19B MAb 1C11), and peroxidase-conjugated monoclonal mouse anti-cTnT (anti-cTnT-horseradish peroxidase (HRP), Cat 4T19C MAb 9G6) were purchased from HyTest Ltd. Progesterone-deficient human sera (Cat S7394) was purchased from Sigma. 3,3′,5,5′-tetramethylbenzidine (TMB)–H2O2, K-Blue reagent solution from Neogen (Abyntek, Spain) in a ready-to-use reagent format (K-Blue enhanced activity substrate, also containing H2O2) and BSA (Type IV, Gerbu) were also used.

The following solutions, prepared in deionized water, were prepared from reagents purchased from Scharlau unless otherwise stated: B&W buffer, consisting of 0.1 M sodium phosphate buffer containing 0.01% Tween 20 (Aldrich), pH 7.5; 20 mM Tris-HCl solution containing 150 mM NaCl, pH 7.5 and phosphate buffered saline (1× PBS buffer, pH 7.5) containing 137 mM NaCl, 1.4 mM NaH2PO4, 8.1 mM Na2HPO4, and 2.7 mM KCl. Stock solutions of the capture antibody (biotin-anti-cTnT) were prepared in the 20 mM Tris-HCl buffer described above while solutions of the detection antibody (anti-cTnT-HRP) were prepared in 1× PBS buffer. A 0.005% w/v biotin solution in Tris-HCl buffer was also prepared to block the remaining groups of the strep-MBs after capturing the biotin-anti-cTnT.

All experiments (except the modification of MBs) were conducted at 24 ± 2°C. An Optic Ivmen® System

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constant temperature incubator shaker (Comecta S.A.) and a DynaMag™-2 (Dynal Biotech ASA) magnetic particle concentrator for the MBs modification and their magnetic separation during the incubation/washing steps, respectively, were also used.

Electrochemical experiments were performed using either μAutolab III (Metrohm, Spain) interfaced to a Windows XP-based PC, using software GPES (version 4.9), or Autolab PGSTAT101 potentiostat/galvanostat using the software package Nova 1.7. Optical microscopy images were obtained using a Leica DM4000 optical microscope.

Zeonor sheets (ZF14–188) were purchased from Ibidi (Germany), and were cut with a Roland GX24-CAMM-1 cutter-plottter (Arista SL, Spain). A P.O. Weber manual press (Germany) with temperature control module was used to bond Zeonor sheets as described below. A Basic-540 milling machine (Step-FOUR, Austria) controlled by XPert Mill software running on a Windows 98-based PC was used to dice out the chips after Zeonor bonding.

2.1 Design and fabrication

Our aim was to develop a disposable microfluidic cartridge that allowed the performance of several independent analyses. The cartridge consisted of six independent sensing groups with their corresponding microfluidics features. It was completely fabricated using rapid prototyping techniques and low-cost materials. Special care was taken during the design phase of the cartridge to allow a smooth transition from rapid prototyping to mass production based on injection molding and standard plastic bonding techniques. Standard microscope slides dimensions, 25 × 75 mm, were chosen to facilitate manipulation and compatibility. The cartridge could be easily inserted on a specifically designed holder for fluidic and electrical connections.

As shown in Fig. 1A, each sensing group consisted of a four-electrode configuration, when the usual electrochemical cell for chronoamperometric detection comprises three electrodes: working, counter, and reference electrodes. Our fourth electrode, located between the pseudoreference electrode and the working electrode (WE), is an immobilization electrode (ImE) where MBs were captured by magnets placed under each of these ImEs. Separating immobilization from detection, both improved the stability and reliability of the WEs.

The whole device consisted on a microfluidic cartridge, containing the sensing microelectrodes and an external holder for fluidic and electrical connections. The microfluidic cartridge was completely fabricated in 188-μm-thick Zeonor sheets (ZF14–188, Zeonex Gmbh), which is a commercial COP [11]. It was assembled bonding three stacked 188-μm-thick layers. As shown in Fig. 1A, layer 3 contains the patterned gold microelectrodes and layers 1 and 2 were used to define the microelectrodes width as well as the microfluidic channel, and the fluidic inlets and outlets. Defining the microelectrodes width with the microfluidic channel avoided further passivation steps during fabrication of the gold microelectrodes, reducing cost, and fabrication time. The microfluidics features (layers 1 and 2 in Fig. 1A) were patterned using a cutter-plottter.

The gold microelectrodes were fabricated using standard photolithographic techniques. First, a Zeonor wafer (100-mm diameter) was metallized by e-beam evaporation (15 nm Ti + 15 nm Ni + 150 nm Au). The metallized Zeonor wafer was then coated with positive photoresist S1813 (Fujifilm), and subsequently exposed to UV light through a bright-field high-resolution patterned transparency (Leicrom, ES). After developing the resist, the metal layer was removed from the undesired areas in different etching baths. Thus, gold was etched in an I$_2$/I$^-$ solution, and Ni was etched in a 4:1 dilution of concentrated HNO$_3$ (70%). Finally, the titanium layer was etched in a propylene glycol:HF mixture. Excess resist was removed in an aceton bath, and the wafer was rinsed with isopropyl alcohol and water. Finally, a drop of Ag/AgCl paste was deposited on the corresponding reference electrode (Fig. 1A).

Afterwards, the microfluidic cartridge was assembled bonding the three Zeonor layers together following a solvent vapor procedure described by Morgan et al. [18]. After cutting the wafers, the protective liners were removed and the sides to be bonded were exposed to cyclohexane vapor at 40°C for 4 min in a custom-made holder. Next, the parts were aligned using pins and pressed against each other under 700 kPa and 60°C for 30 min, and left to cool down to 35–40°C still under pressure.

A specific packaging was designed and fabricated for a proper encapsulation of the cartridge. The package was required to allow both fluidic and electrical access to the
cartridge in a reliable and simple manner, and the possibility to place and remove small magnets at the desired places when required during the protocol.

Regarding the fluidic and electrical connections, silicone o-rings guarantee watertight sealing to the cartridge inlets and outlets, and spring-loaded pins provide electrical contact to the electrode pads on the chip. Once the cartridge is inserted on the bottom part of the packaging, the latter is closed using a hinge, allowing electrical interconnection between packaging and cartridge.

Small holes were pierced on the bottom piece of the package at the places where the magnets were required. A magnet holder system consisting of a platform with small pillars and magnets at the top was designed to match the holes of the bottom packaging piece, in such a way that when both pieces are put together, the magnets are inserted near the bottom of the chip. When both pieces (packaging and magnet holder) are placed together, the magnets are close enough to trap the MBs traveling through the microchannels. Once the magnets are not required any more, both pieces are separated, and the MBs will be free to move inside the chip. The different packaging pieces were made in an Objet Eden 3D printer using Fullcure720 material.

2.2 Experimental setup and procedure

The main steps in the assay were (i) the specific capture and HRP labeling of cTnT on MBs outside the chip, as depicted in Fig. 2, (ii) the capture of the beads on a reaction chamber inside the chip, and (iii) the electrochemical detection of TMB at a gold microband WE downstream. This section describes each of these steps in more detail, as well as the electrochemical characterization of the electrodes.

2.2.1 Electrochemical characterization

Before the detection of troponin, the chips need to be calibrated to ensure the working conditions of the WEs as well as to check the channel geometry. After blocking the entire system with BSA to prevent the nonspecific adsorption of sample proteins and other reagents on the system surfaces, the WE was activated electrochemically using a series of eight potential steps of 10 s each, alternating between –2.0 and 0 V in a 0.1 M KNO$_3$ solution.

Following the activation, an equimolar solution of ferro/ferricyanide (2 mM) in 0.1 M KNO$_3$ is passed through the system, and cyclic voltammograms at 50 mV/s are recorded at different flow rates as described previously in [19, 20]. Figure 2 shows typical hydrodynamic cyclic voltammograms. As Fig. 2B shows the steady-state currents depend linearly on the cube root of the flow rate, in excellent agreement with Levich's equation for a microband channel electrode, in international system units [21]:

\[
I = 0.925nFcw(x_e D)^{2/3}(Q/h^2d)^{1/3}
\]

where \(n\) is the number of electrons involved, \(F\) is Faraday’s constant, \(c\) and \(D\) are the concentration and the diffusion coefficient of the electroactive species, \(x_e\) and \(w\) are the length and width of the electrode, \(d\) and \(h\) are the width and the half-height of the channel, respectively, and \(Q\) is the volumetric flow rate.

2.2.2 Modification of MBs

The procedure to modify the MBs was optimized previously [22]. Briefly, a 5 \(\mu\)L drop of the strep-MBs commercial suspension was transferred into a 1.5-mL tube where the MBs were washed twice with 100 \(\mu\)L B&W buffer solution. Each washing step entailed the resuspension of the beads, followed by removal of the supernatant with the aid of magnetic separation. The MBs were then resuspended in 100 \(\mu\)L of a 2 \(\mu\)g/mL biotinylated anti-cTnT solution (in Tris-HCl buffer).
Beads and antibody were incubated for 30 min at 37°C under orbital shaking at 600 rpm. Next, the anti-cTnT-modified MBs were washed twice with 100 μL of PBS buffer solution (pH 7.5), and the unreacted streptavidin groups of the MBs were blocked with biotin (100 μL of the 0.005% biotin solution) and incubating the suspension under continuous stirring (600 rpm) for 15 min at 37°C.

After two washing steps with 100 μL of 1× PBS buffer (pH 7.5), the anti-cTnT-coated MBs were resuspended in 100 μL of the antigen solution (cTnT) prepared in 1× PBS buffer (pH 7.5). After 30-min incubation at 37°C under stirring (600 rpm), the cTnT-anti-cTnT-attached MBs were washed twice with 100 μL of 1× PBS buffer solution (pH 7.5).

The last incubation step with the anti-cTnT-HRP was accomplished by resuspending the modified MBs in 100 μL of 1 μg/mL anti-cTnT-HRP solution prepared in 1× PBS (pH 7.5). This last recognition reaction was left to proceed for 45 min at 37°C with continuous stirring (600 rpm). The resulting modified MBs were then washed five times for 2 min with 100 μL of 0.1 M sodium phosphate buffer solution (pH 7.0) under the same conditions (37°C, 600 rpm). The modified MBs were subsequently used for cTnT determination or resuspended in 50 μL of NaCl PBS and stored at 4°C prior to the analysis either in a magneto-ELISA assay or on the COP-based microfluidic chip (see Section 2.2.3).

2.2.3 cTnT magneto-ELISA

The assay was developed using standard ELISA microtiter plates before it was transferred to the chip. The procedure was as follows: selected concentrations of cTnT (0, 0.2, 0.6, and 1.0 ng/mL) in the whole complex anti-cTnT-HRP/cTnT/anti-cTnT-biotin to MBs were evaluated in a nonbinding microwell plate coupled to a magnet. Five microliters from the stock suspension for each concentration were diluted 20 times in PBS. After three washes with PBS using the magnet the substrate solution was added (100 μL per well). The plates were incubated for 30 min and protected from light, at room temperature, before the enzymatic reaction was stopped by addition of 4 N H2SO4 (50 μL per well). Absorbance was read at 450 nm.

2.2.4 Determination of cTnT using the COP-based microfluidic chip

The sensor chip was placed on the packaged microfluidic cartridge and the set was tested for fluid leaks with deionized water. Next, the microfluidic system was rinsed with a solution of 1× PBS at a flow rate of 10 μL/min and immediately after a 2% w/v BSA solution was passed through the system (1 μL/min, t = 15 min) in order to minimize the unspecific adsorption of substrate. Following this blocking step, the magnets were brought in close proximity to the chip, under the ImE, and the modified MBs, resuspended in 500 μL of 1× PBS pH 7.5, were introduced into the system at a flow rate of 20 μL/min to ensure the capture of the modified MBs by the neodymium magnet. On completion of this step, the MBs were visible by the naked eye as an orangeish cloud over the ImE surface.

The biorecognition event was monitored using the HRP/TMB system and hence the measured signal corresponded to the chronoamperometric detection of the enzymatic reduction of H2O2 mediated by TMB.

To perform the chronoamperometric measurements, buffer or enzymatic substrate solutions were passed through the system at 5 μL/min and the current was sampled for 200 s at −100 mV. After carrying out the chronoamperometric measurements, the magnets were taken away and the system was ready for subsequent measurements after washing with PBS to remove all the magnetically captured beads on the ImE.

2.2.5 Analysis of serum samples

Human serum samples were tested unspiked, and spiked with 50 ng/mL of cTnT. After diluting 50 times (in 1× PBS, pH 7.5) these serum samples, both biotin-anti-cTnT (2 μg/mL) and anti-cTnT-HRP (1 μg/mL) were added, and the immunological reactions were allowed to proceed for 90 min (37°C, 600 rpm). After that, 100 μL of this mixture were added to 5 μL of strep-MBs and the suspension was left under stirring (600 rpm) for 30 min at 37°C [22]. The resulting MBs were washed, resuspended, and measured at the COP-based microfluidic chips following exactly the same methodology described above for the standard solutions.

3 Results and discussion

3.1 System calibration

Prior to the detection of troponin, the system is calibrated using an equimolar solution of ferro/ferricyanide, as described in Section 2.2. The need for this initial characterization is twofold. First, we need to ensure that the WE responds properly after its electrochemical activation [23], and that the channel geometry is known. Fabrication using rapid prototyping techniques is not as accurate and reproducible as when standard microfabrication techniques are used. In addition, the cyclohexane treatment used is very aggressive on the Zeonor surface and occasionally too long exposures to the vapors lead to deformation of the structures. Channel height and width were determined fitting cyclic voltammetry data to Eq. (1). Second, the currents recorded during calibration allow the normalization of detection currents so that data from different chips and electrodes may be directly compared. Figure 2 shows typical voltammetric data obtained in a 2 mM equimolar ferro/ferricyanide solution in 0.1 M KNO3, and the fit of the steady-state currents to the Levich expression.

The results suggested that the cyclohexane treatment did not affect the channel height significantly as the voltammetric data led to an estimated height between 180 and 195 μm, in
very good agreement with the thickness of the Zeonor layer (188 µm). On the other hand, channel width was far from the nominal dimension in the original design (500 µm), and voltammetric data showed that the channels were between 620 and 635 µm wide. This can be explained by a combination of two factors. First, the way in which the channels have been cut (by a blade), and second by the bonding process under high pressure and temperature after exposure to cyclohexane. Despite these minor fabrication issues, an important finding was that the electrodes were not passivated during the cyclohexane-based bonding of the Zeonor layers.

It is also worth noting that no electrode passivation was observed even after carrying out several troponin detection assays on the same WE. Calibrations using ferro/ferricyanide were done between assays to check the condition of the WE, and no activity losses were found at the end of each experiment.

### 3.2 Electrochemical detection of cTnT

This paper describes the design and application of a COP-based microfluidic chip for sensitive cTnT determination, aiming at the diagnosis of AMI. To demonstrate the applicability of the microfluidic chip, MBs modified with sandwich immunocomplexes were injected in it after electrode activation and characterization. As described earlier, the modification of the MBs involved a biotinylated capture antibody, the target cardiac protein and a HRP labeled antibody. After magnetic capture of the modified MBs on the ImE surface in the encapsulated sensor chip, the electrochemical detection of the affinity reaction was carried out at the WE of the sensor chip using TMB as electron transfer mediator and H_2O_2 as the enzyme substrate under hydrodynamic conditions. The process is briefly outlined in Fig. 3.

The optimization of several variables involved in the immunoassay on the MBs, such as the concentration of the capture and detection antibodies and the time elapsed for the different incubation steps, was performed previously for disposable electrochemical magneto-immunosensors for cTnT on commercially available screen-printed gold electrodes [22]. We believe that this is a valid assumption because all the steps before the detection are performed outside the chip in both cases.

An important drawback found in the development of electrochemical immunosensors is the presence of nonspecific adsorption on the electrode surface, thus making necessary the use of blocking agents. Nonspecific adsorption can be avoided if the immunological reactions are performed outside the electrodes, such as on the surface of MBs [20]. We observed that no amperometric signals were recorded when the anti-cTnT-MBs were incubated in an anti-cTnT-HRP solution (without cTnT), thus demonstrating that nonspecific adsorption of the detection antibody was not significant.

Under the optimized experimental conditions (summarized in Table 1), the chronoamperometric signals obtained in the Zeonor chips for different cTnT concentrations were recorded, and a linear calibration plot (Fig. 4A) was constructed in the 0.05–1.0 ng/mL concentration range. The slope of this calibration curve was 38 ± 3 nA/ng mL, and the intercept was 2.0 ± 1.5 nA (r = 0.986). Very low detection and determination limits of 0.03 and 0.11 ng/mL, respectively, were achieved. These values were estimated according to the 3 s/m and 10 s/m criteria, where m is the slope of the calibration plot and s was estimated as the standard deviation (n = 10) of the amperometric signals obtained with 0.05 ng/mL of cTnT.

Also, the biofunctionality of the immunoassay using MBs was tested by magneto-ELISA using a protocol well

#### Table 1. Optimization of experimental variables involved in the MBs modification to perform the electrochemical detection at the COP-based microfluidic chip

<table>
<thead>
<tr>
<th>Experimental variable</th>
<th>Selected value</th>
</tr>
</thead>
<tbody>
<tr>
<td>V_strep-MBs (µL)</td>
<td>5</td>
</tr>
<tr>
<td>Biotin-anti-cTnT (µg/mL)</td>
<td>2</td>
</tr>
<tr>
<td>t_{biotin-antiTnT} (min)</td>
<td>30</td>
</tr>
<tr>
<td>Biotin% (w/v)</td>
<td>0.005</td>
</tr>
<tr>
<td>t_{biots} (min)</td>
<td>15</td>
</tr>
<tr>
<td>t_{cTnT} (min)</td>
<td>30</td>
</tr>
<tr>
<td>Anti-cTnT–HRP (µg/mL)</td>
<td>1</td>
</tr>
<tr>
<td>t_{anti-cTnT–HRP} (min)</td>
<td>45</td>
</tr>
<tr>
<td>Washing time (min)</td>
<td>2</td>
</tr>
</tbody>
</table>
established in the laboratory [24]. The results showed a clear parallelism observed using the electrochemical chip microfluidic platform obtaining a linear calibration plot (Fig. 4B) with a slope value of 0.15 U.A./ng mL and an intercept of 0.36 U.A. (r = 0.964).

The reproducibility of the measurements carried out with the COP-based microfluidic chip was evaluated using different batches of modified MBs prepared in the same manner. Results for five different modified MBs batches prepared on different days and using a buffer solution containing 0.4 ng/mL cTnT yielded a RSD value of 10.3% for the chronoamperometric responses obtained. This result demonstrated that the procedure used for MBs modification was reliable, and that reproducible chronoamperometric responses are obtained with different modified MBs batches prepared in the same manner using the COP-based microfluidic chip. It should be noted that although bead-loading variability may affect the magnitude of the measured signal, these results demonstrated also a reproducible and efficient magnetic capture of the modified MBs.

As a proof of concept of the developed methodology, its usefulness for the analysis of real samples was demonstrated by analyzing spiked human serum samples. Various studies have demonstrated that cardiac troponin levels increase drastically during the 24 h following an AMI episode up to levels of around 50 μg/L [25].

A previous work with disposable electrochemical magneto-immunosensors for cTnT using commercially available screen-printed gold electrodes [22] demonstrated that the strong matrix effect observed in undiluted serum samples could be largely mitigated upon a 50-time sample dilution. Preliminary assays using the protocol described in Section 2.2 for cTnT standards using a three-step format led to poor results. This was attributed to the possible interference from endogenous constituents of human serum, such as the human antimouse antibodies [25]. This problem was avoided using the one-step format described in detail in Section 2.2. In brief, this protocol involved a first step where the formation of the sandwich immunocomplexes in solution was allowed, followed by a second step implying the subsequent immobilization on MBs. This procedure was less susceptible to interfering serum constituents than that observed with the three-step procedure. Note that the current measured in dilute human serum samples containing 1 ng/mL doubled that measured in unspiked diluted serum (data not shown).

These preliminary results strongly suggest the potential of this microfluidic chip for the determination of cTnT concentrations in serum samples after a very simple sample treatment consisting in a dilution with the working buffer solution.

It is also important to point out that although the serum samples had to be diluted in order to detect cTnT without any matrix effect, the developed method is able to confirm an AMI episode when cardiac troponin concentration can increase drastically up to levels around 50 μg/L. It is worth mentioning that there is still no agreement on the clinical relevance of detecting the presence of low levels of cardiac troponins in blood and serum samples. This is because concentrations just over the decision limit are not necessarily connected with AMI episodes, but they may be due to other conditions resulting from myocardial cell damage, such as myocarditis, cardiac surgery, and sepsis [25].

Consequently, to give reliable results and troubleshoot questionable findings, it is important not to use a method with very low LODs but, instead, to have a specific and reliable methodology, such as the one presented here, able to detect large increases in cardiac troponin levels in a short period of time.

4 Concluding remarks

We have demonstrated the fabrication of a microfluidic chip with magnetic capture and electrochemical detection using polymeric substrates (Zeonor) using rapid prototyping techniques. The chips, which are disposable, allow for the performance of up to six independent assays. Moreover, we have
successfully applied these devices to the detection of cTnT, a reference cardiac disease biomarker, to concentration levels down to 0.05 ng/mL. This range is well within the clinically relevant range and compares very positively with similar experiments carried out at screen-printed microelectrodes [22].

Troponin has been chosen to demonstrate that devices made using rapid prototyping techniques can be used to develop novel biosensors and assays, in this case of clinical relevance. However, these fabrication techniques are limited to the initial stages of development, and are not intended for mass production of commercial devices.

In this work, we used a PBS solution of cTnT, but it is reasonable to think that the procedure may also be successful when dealing with real samples. This is because troponin is extracted from the sample matrix using magnetic beads outside the chip, and the beads are thoroughly washed afterwards, so that the chip should not, in principle, be in touch with blood at any time. It is worth mentioning here that the successful preliminary results achieved using diluted serum samples demonstrated that the developed methodology meets the criteria required to diagnose unequivocally an AMI.

Despite the success of the system in determining troponin down to clinically relevant levels, improvements may come from at least three directions: better design, reliability in fabrication, and assay time (that due to the multiple steps involved takes about 4 h, although the detection itself is done in a matter of minutes). Better design will have the biggest impact of the three directions. Redesigning the chip may lower the fabrication costs (mainly driven by the microelectrodes) and increase its value by integrating additional functions such as some of the sample pretreatment steps currently done outside the chip. Also, chip reproducibility may be further improved by controlling the fabrication more closely, especially the Zeonor bonding step. Other techniques such as mould injection and chiseling or punch cutting, better suited for mass production, are likely to reduce chip-to-chip differences and thus improve reproducibility. Finally, increasing the flow rates used in some process steps, particularly MB loading and washing and blocking steps, may shorten assay time.

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The authors have declared no conflict of interest.

5 References


