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# **Novel and highly stable strategy for the development of microfluidic enzymatic assays based on the immobilization of horseradish peroxidase (HRP) into cotton threads**

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## Abstract

The use of biological components in the development of new methods of analysis and point-of-care (POC) devices is an ever-expanding theme in analytical chemistry research, due to the immense potential for early diagnosis of diseases and monitoring of biomarkers. In the present work, the evaluation of an electrochemical microfluidic device based on the immobilization of horseradish peroxidase (HRP) enzyme into chemically treated cotton threads is described. This bioreactor was used as a channel for the build of the microfluidic device, which has allowed to use of a non-modified screen-printed electrode (SPE) as an amperometric detector. Cotton threads were treated using citric acid, and the immobilization of HRP has been performed by EDC/NHS crosslinking, connecting amine groups of the enzymes to carboxylic acids in the cellulosic structure. For the analytical evaluation, an amperometric assay for hydrogen peroxide detection was performed after the injection of H<sub>2</sub>O<sub>2</sub> and hydroquinone (HQN) concomitantly. The enzymatic reaction consumes H<sub>2</sub>O<sub>2</sub> leading to the formation of O-quinone, which is readily reducible at non-modified SPE. Several experimental parameters related to enzyme immobilization have been investigated and under the best set of conditions, a good analytical performance was obtained. In addition, the threads were freezer-stored and, after 12 weeks, 84% of hydrogen peroxide sensitivity was maintained, which is very reasonable for enzyme-based systems and still offers good analytical precision. Therefore, a simple and inexpensive microfluidic system was reported by crosslinking carboxylic groups to amine-containing macromolecules, suggesting a new platform for many other protein-based assays.

## Introduction

The miniaturization of lab equipment into handheld devices that can perform instantaneous analysis wherever needed – the point-of-care devices (POC) – is an important advancement in clinical diagnosis [1,2]. Microfluidics is concerned with handling and analyzing volumes at and below the microliter scale, which provides many benefits and possibilities to POC since they are generally miniaturized, inexpensive, easy, and quick to fabricate, and allow for a great degree of customization [3]. At the frontier level, the development of novel techniques in microfabrication for these devices can also lead to a remarkable improvement in the analyte sensitivity, fundamental for the quantification of low concentration biomarkers [4]. An important subclass of POC is biological devices that use biomolecules, such as antibodies and antigens in immunoassays, DNA strands, and enzymes to achieve a specific interaction between substrate and analyte [5]. Several applications in food safety [6,7], environmental management [8,9], agriculture [10], pharmaceuticals [11], food compositional analysis [12], and medical care have been reported since rapid and reliable sensing of human biomarkers can lead to faster diagnosis and early medical intervention [13].

Microfluidic devices for the development of POCs have been manufactured using many substrates, such as glass-based [[14], [15], [16]] and paper-based systems [17]. Paper is a particularly interesting substrate for low cost and simple microfluidic systems, since it is easily obtainable and has naturally high capillary action, which usually eliminates the need for flow induction [18]. However, low tensile strength when wet and the need for flow directing through microfabrication can be considered relevant drawbacks [19,20]. One strategy to circumvent these limitations is the use of cotton threads, which are also cellulosic-based materials, but displays superior mechanical characteristics that improve the simplicity and portability of the microfluidic devices [21,22]. The benefits of cheap and readily available analytical devices can also be highlighted by the novel Coronavirus (SARS-CoV-2) pandemic. Mass testing can greatly influence the containment of epidemic and pandemic incidents since government policies aimed towards proactive detection of infected cases can greatly reduce the economic impact [23] and loss of life [24,25]. However, even for electrochemical systems based on cotton threads, the use of electrochemical biosensors capable of selective interaction with the analyte or catalysts is still necessary. Usually, this is achieved by the surface modification of the transducer, which increases the complexity of the construction process and may decrease the accuracy and sensitivity of the measurements.

In recent years, the use of microfluidic systems based on cotton threads obtained from commercial hydrophilic gauze has been successfully reported for detection of several species [[26], [27], [28], [29]]. A chromatographic microfluid system for biomarkers separation and quantification was proposed through the modification of threads with citric acid, which increased the carboxylic acid content in cellulose [30]. The successful incorporation of more carboxyl groups in the cotton threads opens up the possibility of enzyme immobilization through crosslinking [31] using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-Hydroxysuccinimide (NHS) [32]. This fact is extremely important since the presence of immobilized biological material on microchannels allows no need for transducer superficial modification, improving the repeatability and reproducibility between different arrangements, besides the preparation of electrochemical biosensors involves many laborious steps and/or the need of other material and reagents to anchor the biomaterial on the electrode surface.

Herein, we propose the use of cotton thread as microchannels, which act as a bioreactor, as an alternative to the use of biosensors as electrochemical detector cells. This approach allows the use of commercial non-modified electrodes since the bioreaction is carried out in the microchannels. The modification of cotton threads can be an interesting alternative since they are a low-cost material and easily modifiable by feasible routes. The strategy of having the biological component separated from the electrode can be advantageous as it allows for storage in separate conditions, which may help to preserve the lifetime of both enzymes and electrodes. In addition, the more expensive and complex electrodes are preserved and would not need to be discarded when enzymes lose function, allowing for their long-term use.

## Experimental

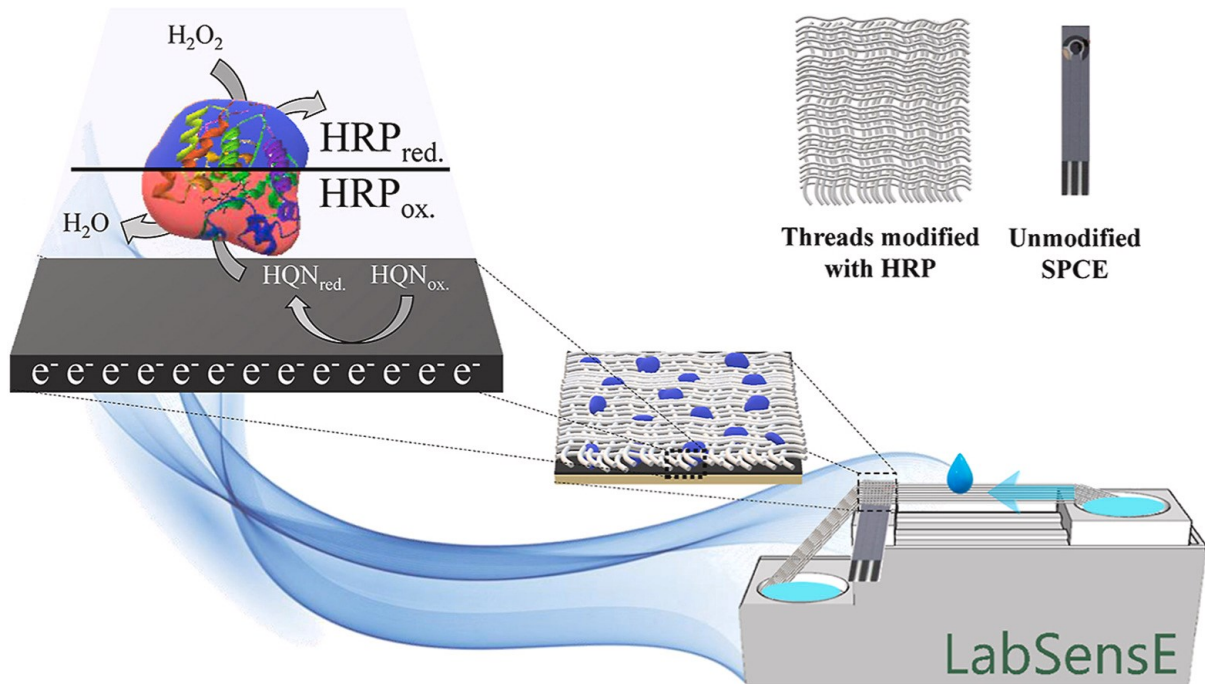
### Chemicals and materials

All chemicals were of analytical grade used without further purification and obtained commercially from Merck (Darmstadt, Germany). Deionized water (specific resistivity higher than 18.2 MΩ cm), obtained by a Millipore Direct-Q3 water purification system (Bedford, MA, USA) was used to prepare all solutions. Sodium phosphates, both dibasic and monobasic monohydrate, were used to prepare a phosphate buffer (PB) at pH 7.40. Other chemicals used were citric acid for gauze treatment; EDC and NHS for crosslinking in a 2-ethanesulfonic acid (MES) buffer; horseradish peroxidase (HRP) enzyme (250 units mg<sup>-1</sup>); hydrogen peroxide (30%) and HQN for amperometric analyses. Materials used in the manufacturing of the microfluidic system were obtained from local markets (Curitiba, PR, Brazil).

### HRP incorporation and microfluidic system assembly

Fig. 1 shows the complete treatment procedure. To increase carboxyl groups available for crosslinking, the cotton threads were treated (24 h at 50 °C and 1 h at 120 °C) with citric acid (0.50 mol L<sup>-1</sup>) at a proportion of 0.5 g of threads to 6.0 mL of solution [33], which has been shown to substantially increase carboxylic acids in cotton threads [30] (step 1, Fig. 1). Then, microfluidic channels were formed and treated individually. An EDC/NHS solution was prepared at a proportion of 1.0 mg EDC/NHS for 1.0 mL MES buffer (pH 5.50), into which a section of 10 mm of the microfluidic channel (previously delimited by clamps) was submerged for 90 min (step 2, Fig. 1); and in the sequence, the same delimited section was left in contact with 200 μL of HRP solution (0.3 mg of enzyme for 5.0 mL of PB buffer) for 90 min (step 3, Fig. 1). When added to the microfluidic device, the treated section immediately preceded the electrode position. All threads were prepared on the same day of use (unless otherwise stated) and added to the microfluidic system (step 4, Fig. 1).

Fig. 1. Illustration of the complete treatment procedure for HRP immobilization and microfluidic device assembly. Hydrophilic cotton threads, previously treated with citric acid (1), were treated with EDC/NHS in a 10 mm segment (2), and then an enzyme solution was dropped, with a micropipette, into the same segment of the microfluidic channel (3). After the immobilization period, threads were fixed onto the inlet and outlet reservoirs with double-sided tape (4). The screen-printed electrode was placed below the microfluidic channel, with the detection zone directly beneath the threads (5).



The microfluidic device was assembled (step 5, Fig. 1) on a 3D printed platform previously developed [34] and using a screen-printed electrode (SPE). The microfluidic channel, consisting of five cotton threads twisted together (15 cm long), were obtained from hydrophilic gauze and fixed with adhesive tape between two reservoirs (inlet and outlet) 12 cm apart, with the outlet set 5.0 cm lower than the inlet to enhance flow by gravitational pull. For a more in-depth physical and chemical characterization of the microfluidic channel, we refer the reader to Agustini et al., 2016 and Agustini et al., 2018 [[26], [30]].

#### Colorimetric assay

Untreated (I) and treated threads were prepared by different procedures: (II) only EDC/NHS; (III) only citric acid; (IV) only HRP; (V) citric acid + EDC/NHS; (VI) citric acid + HRP; (VII) EDC/NHS + HRP; and (VIII) citric acid + EDC/NHS + HRP. To evaluate the effectiveness of the treatments, a colorimetric assay was performed on all threads (Fig. S1). The chromogenic substrate used was 3,3',5,5'-tetramethylbenzidine (TMB), which is oxidized by the hydroxyl radical formed in the decomposition of H<sub>2</sub>O<sub>2</sub> by HRP, forming a blue product [35,36]. A solution of TMB and hydrogen peroxide was prepared in sodium citrate and dropped on to the threads using a micropipette.

#### Electrochemical assays

All electrochemical measurements were performed with a potentiostat/galvanostat ( $\mu$ Autolab type III; Metrohm Autolab B.V., Utrecht, Netherlands). The amperometric assays were performed by micro flow injection analysis ( $\mu$ FIA) and an illustration of the procedure is provided in Fig. S2, as well as the redox reactions that occur in the proposed system. Threads (I), (III), (V) and (VIII) were tested on their electrochemical response to solutions: excess of HQN ( $1.4 \text{ mmol L}^{-1}$ ),  $1.0 \text{ mmol L}^{-1} \text{ H}_2\text{O}_2$  and their mixture by amperometry, to demonstrate an increase in H<sub>2</sub>O<sub>2</sub> signal due to HRP activity in the microfluidic channels. The applied potential was chosen according to a cyclic voltammetry (CV) of the mixture and was also applied to all subsequent assays. Then, a standard analytical curve was obtained with thread (VIII) to evaluate the analytical performance of the proposed system. Finally, for evaluations of treatment stability over time, four separate threads (VIII) were briefly air-dried and stored free of buffer solution in a freezer (below  $-1.0 \text{ }^\circ\text{C}$ ). After 0, 4, 8 and 12 weeks, three concentrations of mixture solutions were injected ( $n = 3$ ), and the respective sensitivities were compared.



## Results and discussion

### Colorimetric test

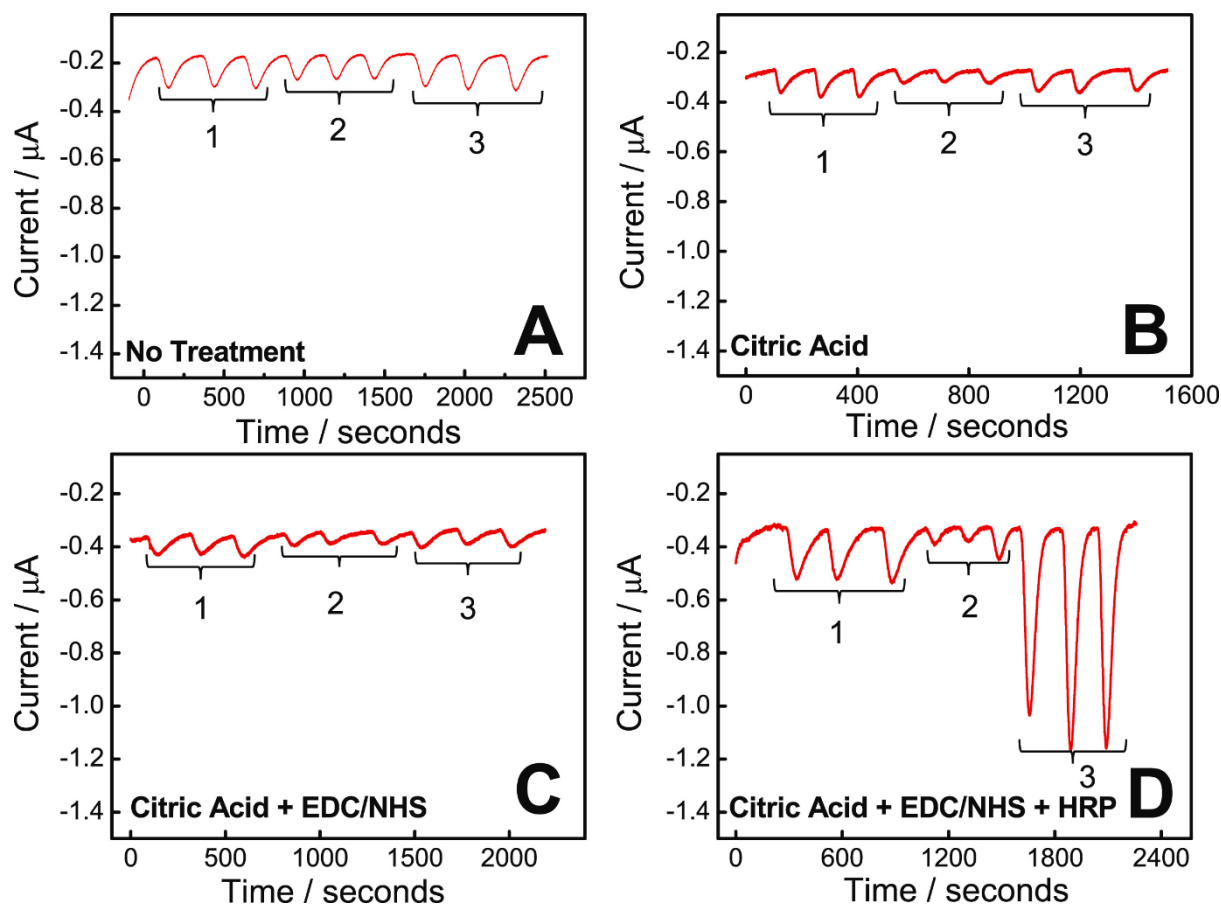
Fig. S1 shows a side-by-side comparison of threads (A) pre- and (B) post-colorimetric assay, where the blue color is due to the formation of a TMB radical cation (and subsequent formation of a charge-transfer complex between oxidized and neutral TMB molecules), while the yellow color is due to the formation of a stable diimine (Fig. S1C) [37]. As expected, threads untreated with HRP did not respond to the colorimetric substrate, whereas threads that we attempted to incorporate enzymes showed a positive result. It is noteworthy that stronger reactions were observed in threads (VI) and (VIII) pre-treated with citric acid, indicating that an increase in carboxyl groups can improve enzyme attachment. The presence of these groups on the cotton threads surface was characterized and discussed in a previous work developed by Agustini et al. [30]. Thread (VIII), also subjected to the EDC/NHS solution, displayed the strongest colorimetric effect, and thus justifies the employment of all treatment steps.

### Electrochemical measurements

Electrochemical evaluation of the threads was carried out by amperometry using HQN as electrochemical probe. Hence, a CV measurement of  $1.4 \text{ mmol L}^{-1}$  HQN and  $1.0 \text{ mmol L}^{-1}$   $\text{H}_2\text{O}_2$  in solution was performed against an Ag/AgCl reference electrode (Fig. S3), which helped to define the potential of detection ( $-0.50 \text{ V}$ ) applied in all subsequent amperometric analyses. In order to evaluate enzymatic activity,  $2.0 \text{ }\mu\text{L}$  of three solutions ( $1.4 \text{ mmol L}^{-1}$  HQN (1),  $1.0 \text{ mmol L}^{-1}$   $\text{H}_2\text{O}_2$  (2) and the mixture of both (3)) were injected (in triplicate) into different microfluid systems built using cotton threads non-treated (I) and chemically treated (III), (V) and (VIII). Fig. 2A–D shows all transient signals recorded. Poor amperometric responses associated with HQN and/or of  $\text{H}_2\text{O}_2$  electrochemical reduction directly in the SPE [38] were observed for microfluidics devices built with cotton threads (I), (III) and (V). In addition, using same systems (Fig. 2A–C, injection 3) similar transient signals were also observed for mixture of solution HQN and  $\text{H}_2\text{O}_2$ . Due to the presence of HRP in the complete treatment procedure, an increase of approximately 952% in signal was observed in presence of  $\text{H}_2\text{O}_2$  when paired with HQN (Fig. 2D, injection 3). Hydrogen peroxide injected on the microchannels is reduced by HRP covalently bound on cotton threads in the analytical path [[48], [49]]. In presence of HQN excess, it is oxidized by the enzymatic process forming the correspondent quinone. Electrochemical reduction of quinone can be performed on electrode surface leading to the enhancement of amperometric signals (Fig. S1B). Comparing transient signals recorded using non-treated (I) and enzyme-modified cotton

thread (VIII) is possible to observe an increase of 466% for injections of  $\text{H}_2\text{O}_2$ /HQN. This result shows the potential of the proposed strategy of immobilization for development of enzyme-based assays using a quick and cheap microfluidic device.

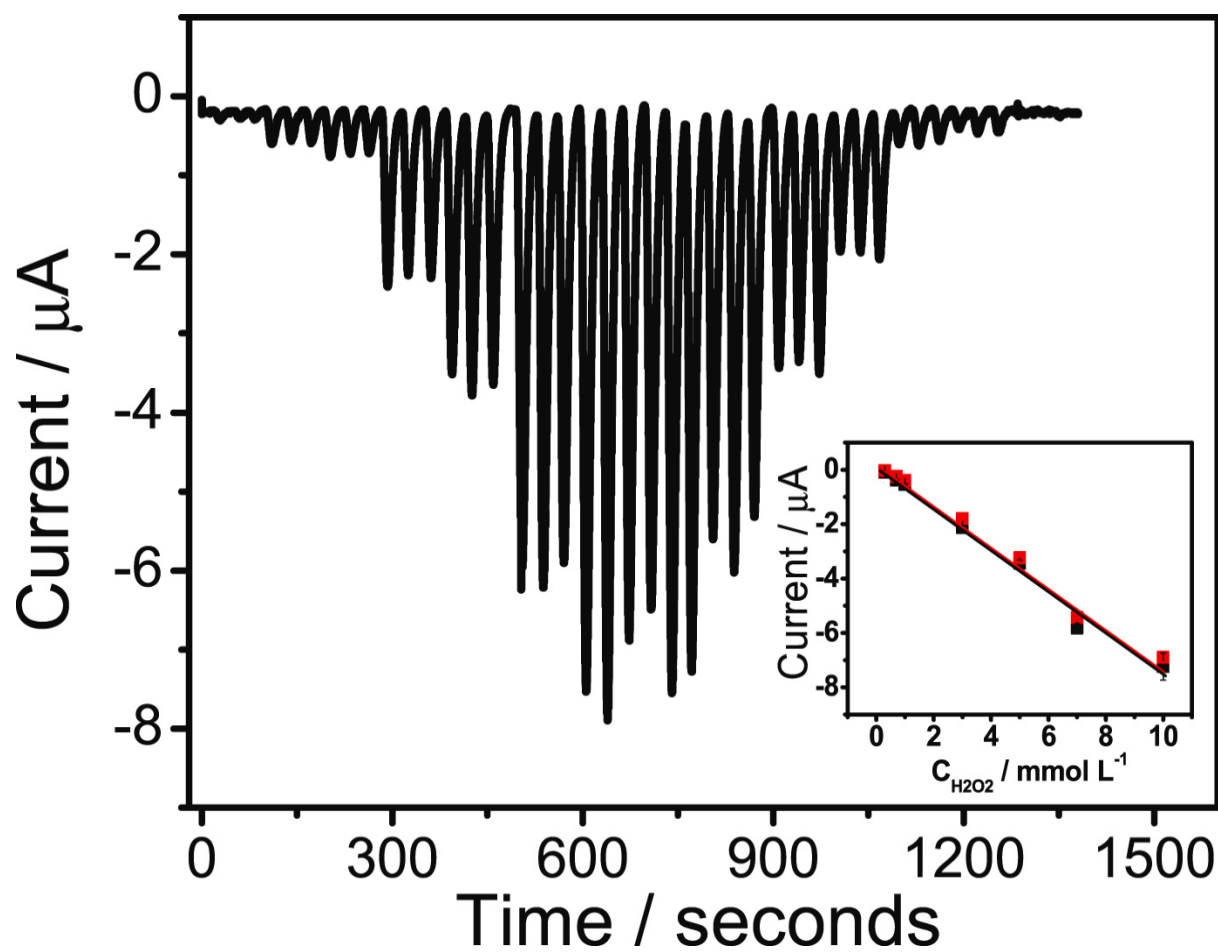
Fig. 2. Amperometric signals obtained for cotton threads (A) no treatment; (B) citric acid treatment; (C) citric acid + EDC/NHS treatment; and (D) citric acid + EDC/NHS + HRP treatment; from injections (n = 3) of (1) 1.4 mmol L<sup>-1</sup> HQN, (2) 1.0 mmol L<sup>-1</sup> H<sub>2</sub>O<sub>2</sub>, and (3) a mixture of the previous two solutions, for the evaluation of enzymatic activity in the microfluidic channels. Supporting electrolyte: Phosphate buffer, pH = 7.40. Volume injected: 2.0 μL. Applied potential: -0.50 V.



### Analytical performance

Fig. 3 shows the analytical curve obtained by the injection (in triplicate) of hydrogen peroxide in varying concentrations (0.30–10.0 mmol L<sup>-1</sup>); a solution of 1.4 mmol L<sup>-1</sup> HQN in PBS was used as mobile phase by placing it in the inlet reservoir, providing a constant stream of redox intermediate. H<sub>2</sub>O<sub>2</sub> concentrations displayed a linear response with sensitivities of 0.758 μA L mmol<sup>-1</sup> and 0.679 μA L mmol<sup>-1</sup> in ascending and descending concentration curves (Inset), respectively. Then, by averaging all peak current intensities for each concentration, a general calibration curve was obtained, described by the equation  $I (\mu\text{A}) = -0.181 (\mu\text{A}) + 0.730 C_{\text{H}_2\text{O}_2} (\text{mmol L}^{-1})$  ( $R^2 = 0.9914$ ).

Fig. 3. Amperometric signals obtained from injections ( $n = 3$ ) of  $\text{H}_2\text{O}_2$  solutions in concentrations between  $0.300$  and  $10.0 \text{ mmol}^{-1}$  (Inset) Calibration curves. Supporting electrolyte:  $1.4 \text{ mmol L}^{-1}$  HQN in phosphate buffer,  $\text{pH} = 7.40$ . Volume injected:  $2.0 \mu\text{L}$ . Applied potential:  $-0.50 \text{ V}$ .



The limit of detection (LOD) was attributed as the first concentration of analytical curve,  $300 \mu\text{mol L}^{-1}$ . A comparison between our work and some recent enzyme-based electrochemical  $\text{H}_2\text{O}_2$  detection systems is provided in Table 1.

Table 1. Analytical performance comparison of some enzyme-based systems for hydrogen peroxide detection.

Electrode	Linear Dynamic Range ( $\mu\text{mol L}^{-1}$ )	LOD ( $\mu\text{molL}^{-1}$ )	Residual Activity/ Time Elapsed	Ref.
HRP/HAuDE	3.0–400	1.5	"	[39]
HRP/TH/ERGO/GCE	30–350	4.65	97%/2 weeks	[40]
HRP–MoS <sub>2</sub> –Gr/GCE	0.20–103	0.049	84%/4 weeks	[41]
Poly(styrene-g-oleic amide)/Rh/HRP/GCE	50–120,000	44	80%/4 weeks	[42]
AuNPs-HRP	0.05–500; 1000–5000	0.8	90%/3 weeks	[43]
MoS <sub>2</sub> /Graphite	0.10–90	0.03	90%/3 weeks	[44]
HPC-Fc/HRP	0.10–0.80	0.1	90%/2 weeks	[45]
Cotton/HRP/SPE	300–10,000	300	84%/12 weeks	This Work

Data not informed. HRP/HAuDE: HRP immobilized on a Heated Au Disk Electrode; HRP/TH/ERGO/GCE: HRP immobilized on Thionine layered over an Electrochemically Reduced Graphene Oxide modified GCE; HRP–MoS<sub>2</sub>–Gr/GCE: GCE modified with a Molybdenum Disulfide-Graphene HRP composite; Poly(styrene-g-oleic amide)/Rh/HRP/GCE: Poly(styrene-g-oleic amide) film coated on a Rhodium nanoparticle modified GCE; AuNPs-HRP: Tungsten microwire modified with gold nanoparticles and HRP; MoS<sub>2</sub>/Graphite: MoS<sub>2</sub> nanosheet synthesized on a graphite nanofiber and incorporated with HRP; HPC-Fc/HRP: HRP immobilized on a ferrocene functionalized hydroxypropyl cellulose matrix coated on a carbon electrode.

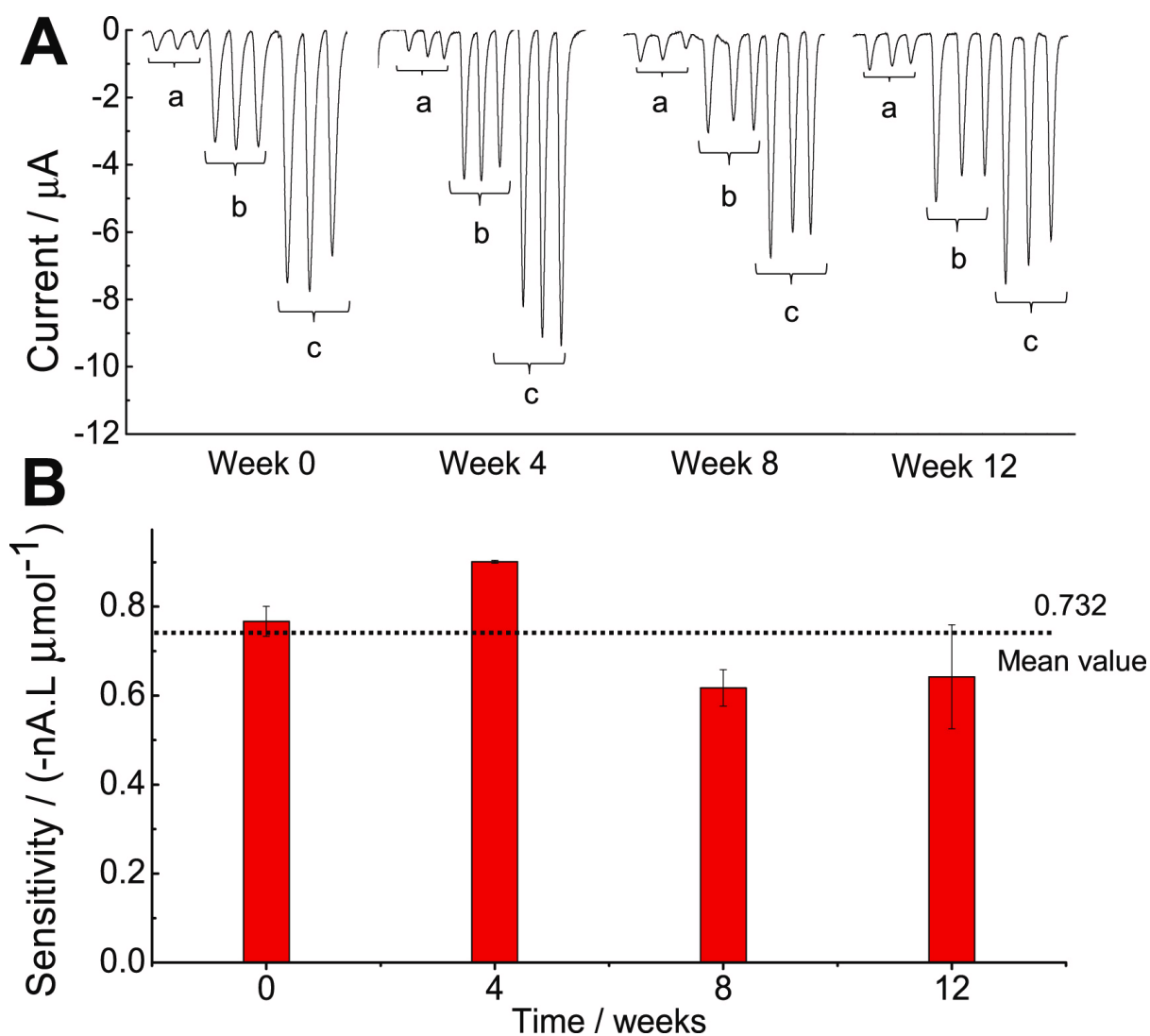
In general, more complex electroanalytical methodologies presented better performance considering linear dynamic ranges and limits of detection. However, the main approach of these methods is based on biosensors, which are prepared by using of laborious procedures. The proposed immobilization strategy shows a little loss of analytical performance, but it might be a valid trade-off since the system shows excellent figures of merits. In addition, it is manufacture using simple materials, feasible methods of and allows the use of non-modified electrodes. This result represents a great advantage where enzymes are concerned, since they tend to lose their activity over time, and the possibility of easily producing on a large scale at a very low cost is a more sustainable approach to performing analyses in the long-term. Furthermore, enzyme-based electrochemical sensors face the challenge of conciliating enzyme stability with electrode performance, since cold storage of these electrodes may promote a change of physical characteristics, and the alternative is to risk loss of enzymatic activity through storage in ambient conditions. This is circumvented here, as the enzymes are immobilized away from the electrodes and storage strategies can differ.

#### Treatment stability over time

Stability of enzymatic activity of modified cotton threads has been investigated using injections ( $n = 3$ ) of solutions of hydrogen peroxide (1.0, 5.0 and 10.0 mmol L<sup>-1</sup>) in microfluidic devices built using freezer-stored treated cotton threads (0, 4, 8 and 12 weeks). The recorded transient signals as well as the obtained sensitivities are shown in Fig. 4A and B, respectively. Signals displayed for week 0 were extracted from the ascending part of the analytical curve.



Fig. 4. (A) Amperometric signals obtained in the microfluidic device by injection ( $n = 3$ ) of hydrogen peroxide, in concentrations of 1.0 (a), 5.0 (b) and 10.0  $\text{mmol L}^{-1}$  (c) performed in fully treated threads after 0, 4, 8 and 12 weeks of freezer storage (below  $-1.0\text{ }^{\circ}\text{C}$ ). (B) Sensitivities calculated for each week by the respective calibration curves. Supporting electrolyte: 1.4  $\text{mmol L}^{-1}$  HQN in phosphate buffer,  $\text{pH} = 7.40$ . Volume injected: 2.0  $\mu\text{L}$ . Applied potential:  $-0.50\text{ V}$ .



It was observed a similar behavior in transient signals for all performed experiments, where a mean sensitivity value of  $0.732 \mu\text{A L mmol}^{-1}$  has been achieved. Although standard deviation (SD) can be seen as elevated, it can be reasonably attributed to a normal experimental variation and considered a good result. Besides, prior to use the system needs to be calibrated using standard solutions, which can provide an accurate value of sensitivity. At the 12-week mark, there is a 16% drop in sensitivity with relation to the initial response which is consistent with other attempts at improving enzymatic stability through immobilization [46,47]. Table 1 also provides some stability data for the other electroanalytical methods found in the literature, which shows that proposed system is capable of maintaining a comparable level of enzymatic activity for a much longer period. This result to the fact that the proposed threads – and, by extension, the enzymes – were safely stored in optimal conditions, with no need for any concern with electrode integrity.

It is noteworthy that the loss in sensitivity at week 12 was of approximately the same magnitude as the week 4 increase (16% from week 0), which may also suggest that there was no actual loss of reactivity, and the variations observed may be due simply to experimental errors. Further studies with bigger weekly samples are needed for a more concrete conclusion; nevertheless, a linear response with good sensitivity was still obtained at the end of the 12-week period, and thus it is possible to treat a large batch of threads ahead of time.

## Conclusions

The incorporation of horseradish peroxidase (HRP) to highly functionalized cotton gauze threads through EDC/NHS coupling reaction were successful achieved. Through a chromogenic assay using 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide was demonstrated the efficiency of pretreatment of threads with citric acid, which provided more sites able to promote enzyme immobilization. Good analytical parameters, such as linear dynamic range (LDR), limit of detection (LOD) and sensitivity were achieved using modified cotton threads as bioreactor and microchannel in the same device. The results reported in the present work show a stable, low-cost and feasible way to build analytical platforms using biological components immobilized on cotton threads. The simplicity of the device construction represents an advantage when a large volume of analyses is needed and when enzymatic degradation can complicate their use outside of a laboratory. Furthermore, amperometric assays were performed in enzyme-treated threads stored for up to 12 weeks, showing an unsurprising loss of relative sensitivity, but nonetheless maintaining good analytical performance.

Based on present results similar approaches can be investigated to anchor other recognition elements as those commonly used in lateral flow, ELISA and biosensors assays.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Data availability

No data was used for the research described in the article.

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