Biosensors and Bioelectronics 85 (2016) 568–572

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Performance enhanced UV/vis spectroscopic microfluidic sensor for ascorbic acid quantification in human blood

Hongyan Bi [a,*], Carla M. Duarte [b], Marina Brito [a], Vânia Vilas-Boas [a,c], Susana Cardoso [b], Paulo Freitas [a]

[a] International Iberian Nanotechnology Laboratory (INL), Av. Mestre José Veiga, 4715-330 Braga, Portugal
[b] INESC Microsistemas e Nanotecnologias (INESC MN), Rua Alves Redol, 9-1, 1000-029 Lisbon, Portugal, and Physics Department, Instituto Superior Técnico, Universidade de Lisboa, 1000 Lisbon, Portugal
[c] UCIBIO-REQUIMTE, Laboratory of Toxicology, Biological Sciences Department, Faculty of Pharmacy, University of Porto, 4050-313 Porto, Portugal

ARTICLE INFO

Article history:
Received 8 March 2016
Received in revised form
8 May 2016
Accepted 17 May 2016
Available online 18 May 2016

Keywords:
Antioxidant analysis
UV/vis spectroscopic sensor
PDMS microfluidic chip
Human blood
Sol-gel

ABSTRACT

Quantitative analysis of antioxidants in a fast, simple and accurate manner is of great importance in the view of real-time monitoring the health of individuals. Recently, we have developed a UV/vis spectroscopic microfluidic sensor to specifically quantify ascorbic acid based on the immobilization of ascorbate oxidase, a relatively unstable enzyme. In this work, three different strategies for the immobilization of the unstable enzyme, including alumina sol-gel encapsulation, physisorption to PDMS channels with, and without alumina xerogel modification, were compared to build a microsensor. We found that the loading amount of the enzyme is not the determinative factor for the performance of the microfluidic biosensor but the retained activity of the enzyme and diffusion in the microfluidic channel. Taking into account of the two factors, the protocol of adsorbing enzymes to alumina (Al_2O_3) xerogel modified PDMS surface was demonstrated to be the best for preparing the microfluidic sensor among the utilized protocols. The microsensor prepared under the optimized protocol was further used to quantify ascorbic acid in human blood, where only dozens of microliters of blood (few drops) was required, demonstrating its potential application in clinical diagnosis. The developed strategy is featured with optimized enzymatic activity, simple process of microfluidic platform, low sample consumption, and straightforward spectrophotometry based detection.

1. Introduction

L-ascorbic acid (AA), also known as vitamin C, is one of the most important *in-vivo* antioxidants. The deficiency of vitamin C will manifest within weeks, and may cause death in 5–6 months (Bsoul and Terezhalmy, 2004). The importance of AA in human body fluids is revalued recently and may play more important roles than people thought. On-going studies have revaluated the role of AA in cancer treatment, in the prevention and/or therapy of asthma, allergic rhinitis, atopic dermatitis, cardiovascular diseases, obesity, neurodegenerative diseases, hypertension and autoimmune diseases (Rutkowski et al., 2012; Ashor et al., 2014; Figueroa-Mendez and Rivas-Arancibia, 2015). During clinical diagnosis, it is of great value to develop methods that consume small amount of sample. For instance, fingerprick test or single drop blood test are very good models for performing human body fluids analysis (Stothard et al., 2009). Besides, due to the heavy population that can be involved for blood test, a facile and rapid sensing system is always requested.

Microfluidic sensors based on spectrometry are simple, fast, portable and disposable for numerous assays. It can be miniaturized, and provide quick analysis without the need of well-trained users. Among the materials for the production of microfluidic devices, such as silicon, glass, polymer, paper, and cotton thread (Becker and Locascio, 2002; Ren et al., 2013; Wang et al., 2014; Yetisen et al., 2013), polydimethylsiloxane (PDMS) can provide rapid fabrication of complex microstructures, and relies on low investment of infrastructure (Tu et al., 2012). PDMS therefore has been widely used, and dominates the microfluidic fabrication *via* casting-based soft lithography (Liu, 2007).

We have recently developed a microfluidic biosensor with serpentine PDMS channel for AA determination in food matrix like dietary supplement products. The biosensor was fabricated by adsorbing one layer of enzymes, ascorbate oxidase, to the inner surface of the PDMS microfluidic channel. The process of AA

* Corresponding author.
E-mail address: hongyan.bi@inl.int (H. Bi).

http://dx.doi.org/10.1016/j.bios.2016.05.054
0956-5663/

oxidation under the catalysis of the enzyme was monitored by end-channel UV/vis spectroscopic detection. The developed strategy is economical, specific, and accurate in the quantification of AA with good chip-to-chip reproducibility (RSD = 7.7% for n = 3) and a detection limit (LOD) of 1 μM (Bi et al., 2016). However, human body fluids are usually more complicated and contain high concentration of biomolecules, such as proteins, that can be non-specifically adsorbed onto PDMS surface and influence the sensitivity and reproducibility of PDMS microfluidic sensors. For specifically sensing AA in human body fluids, the performance of the ascorbate oxidase based spectroscopic microfluidic sensor should be further enhanced by taking biofouling effect into account. To the best of the authors' knowledge, there is no reported microfluidic sensors for human body fluids analysis based on UV/vis spectroscopy. Besides, no research was performed by adsorbing proteins to the surface of xerogel surface for the fabrication of enzymatic biosensor.

Indeed, the performance of microfluidic biosensors is always an important consideration for their real application. Some recently published studies focus on improving the performance of enzymes utilized in biosensors (Tang et al., 2014; Wang et al., 2015). Oldenziel et al. found that the performance of glutamate microsensors can be improved by purifying different batch of ascorbate oxidase, where 3 types of enzymes were coated to electrodes for glutamate analysis in brain tissue (Oldenziel et al., 2006; Oldenziel and Westerink, 2005). It has been broadly reported that sol-gel encapsulation strategy can enhance the performance of immobilized enzymes due to sol-gel's properties such as high surface area and good biocompatibility (Dulay et al., 2005; Liu et al., 2000; Wu et al., 2004). However, the understanding for the immobilization of relatively unstable enzymes is still very limited. We noticed that ascorbate oxidase that we obtained, with 80% (w/w) sucrose as stabilizer, could completely lose its activity in 5 h when dissolved in water at 2 μg/mL and stored at room temperature and ambient condition. It may be a slow process to form dried sol-gel (xerogel) with encapsulated enzymes in a microfluidic device. Days of incubation time are usually needed (Wu et al., 2004). The activity and stability of enzymes can vary with pH fluctuation that obviously exists during the drying of sol-gel (Talley and Alexov, 2010). Thus, when unstable enzymes are considered for immobilization, a long time of drying procedure can dramatically affect the enzymes' activity, and thereby the performance of biosensors.

In the present work, we endeavoured to optimize the immobilization of ascorbate oxidase in PDMS microfluidic sensors before handling them to quantify AA in human body fluids. Three different methods were compared to load the enzyme, including direct adsorption by PDMS surface, encapsulation by alumina (Al_2O_3) sol-gel, and adsorption by Al_2O_3 xerogel modified PDMS channel. It was found that the protocol of enzyme adsorption to Al_2O_3 xerogel modified PDMS surface is the best by considering the catalysis effect to the oxidation of AA in the obtained microfluidic sensor. With the optimized protocol, an UV/vis spectroscopic microfluidic sensor was further developed to quantify AA in human blood, where only dozens of microliters of sample amount is required.

2. Materials and methods

2.1. Fabrication of microfluidic chips

PDMS microfluidic straight channels with cross section of 100 μm × 100 μm and length of 4 cm, fabricated by a typical soft lithography method, were used in the present study. Reservoirs with diameter of 1 mm were integrated in both ends of the channels. The channels were bonded with a glass substrate (Corning® microscope slides, L × W, 75 mm × 50 mm, Sigma-Aldrich, MO, USA).

2.2. Immobilization of proteins in PDMS channels

Al_2O_3 sol-gel was synthesized according to a previously published method (Liu et al., 2000). The microfluidic channels were filled with the sol-gel and incubated to obtain Al_2O_3 xerogel modified surface, or used directly for protein adsorption. Otherwise, the sol-gel was mixed with proteins and then injected into the microchannel to immobilize proteins.

2.3. Activity study of ascorbate oxidase in microfluidic biosensor

100 μM AA in 50 mM KH_2PO_4-NaOH (pH 6.2) was injected into the microfluidic channel immobilized with or without ascorbate oxidase. The reaction time was adjusted by controlling the substrate to flow through the microfluidic channel at different flow rates. The decrease of AA concentration during the reaction was online monitored by UV/vis spectroscopic analysis of the output solution from the microchip. All the measurements were performed at least in triplicate.

2.4. Serum analysis

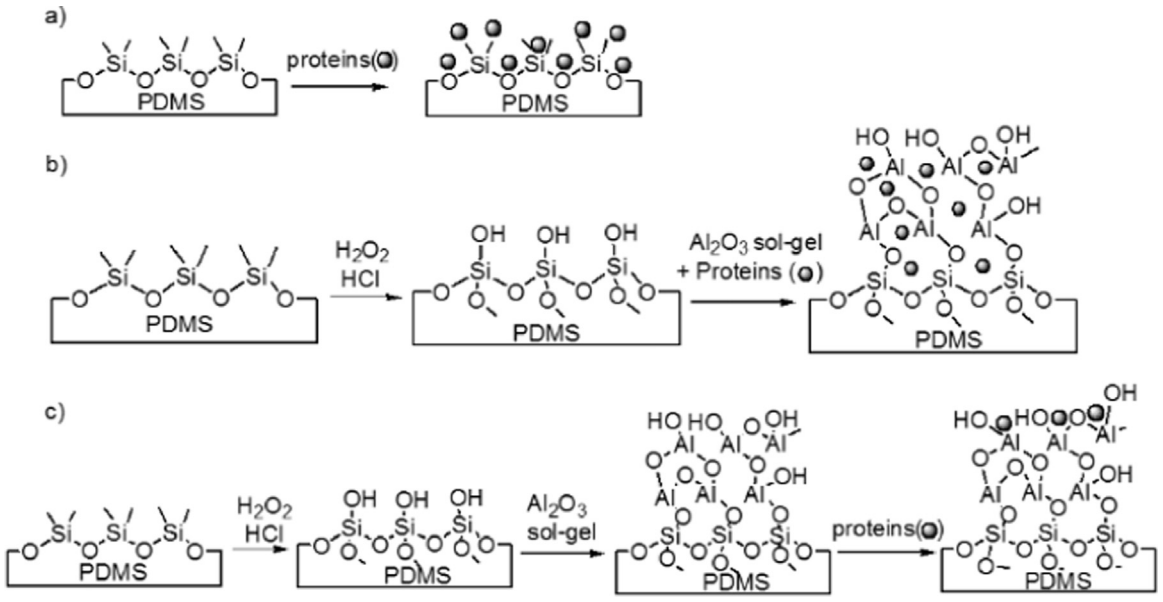
Non-fasting human blood was provided by a healthy volunteer in her 30 s, collected by the Laboratório de Patologia Clinica Hilário de Lima (Unilabs, Braga, Portugal). The serum was treated to remove partially proteins, and stored at −20 °C. After 28 h of storage, AA in the serum was analyzed by an ascorbate oxidase loaded microfluidic sensor. The sensor was formed by physisorption of 0.1 mg/mL ascorbate oxidase in a xerogel modified PDMS channel for 1 h. During the analysis, serum was diluted (1:1, v/v) by 20 mM phosphate buffer (pH 7.4) with 0.15 M NaCl, and then pumped through the microfluidic channel. Each measurement used around 20 μl of sample, which corresponded to 10 μl of serum or 20–25 μl of whole blood sample.

Note: More details on the technical points during the experiments can refer to Section A, Appendices.

3. Results and discussion

3.1. Protocols for enzyme immobilization

Different from enzymes that can keep stable for months in solutions, e.g. α-trypsin that can maintain 95% of its initial activity under pH 3 and 4 °C for 10 months (Santos et al., 2008), ascorbate oxidase can only keep its specific activity at 4 °C for one month (Reinhammar et al., 1997). The activity and stability of this enzyme can be even worse according to our results (Section D, Appendices). We endeavoured here to optimize the immobilization methods of ascorbate oxidase to build a sensitive microfluidic sensor for the quantification of AA in human body fluids, and meanwhile to figure out a performance enhanced method for fabricating relatively unstable enzyme based microsensor. As shown in Scheme 1, three types of ascorbate oxidase immobilized microfluidic sensor were prepared based on physisorption to PDMS channels with or without alumina xerogel modification, and alumina sol-gel encapsulation. The utilized amount of ascorbate oxidase was kept constant to develop the different biosensors in order to compare the effectiveness of the immobilization protocols.



Scheme 1. Schematic representation of the different protein-loading strategies. a) Proteins were directly adsorbed to the surface of PDMS microfluidic channel; b) sol-gel encapsulation of proteins onto the inner surface of PDMS microfluidic channel; c) the inner surface of microfluidic channel was sinanolized and then deposited with one layer of Al_2O_3 xerogel, and then used for the physisorption of proteins.

3.2. Activity of enzyme immobilized in microfluidic channels: theory

The activity of ascorbate oxidase immobilized in microfluidic channel can be characterized based on its catalysis ability to AA oxidation. However, the reaction kinetics in a microchannel with enzymes immobilized on the channel surface is different from bulk solution reaction due to the diffusional effects (Section C, Appendices) (Koyayash and Laidler, 1974). In a microchannel, there is a concentration gradient of substrate from the middle to the inner surface of the channel. The theory was studied in detail by Koyayash and Laidler (1974), where they defined a utilization factor, η, as the ratio of the real reaction rate (r_d) in microchannel with immobilized enzyme to that without the diffusional effects,

$$r_d = \eta r = \eta \frac{V_{\max}\,[S]}{[S] + K_M} \tag{1}$$

η is a constant determined by the Michaelis constant (K_M) of the enzyme, the initial substrate concentration $[S]_0$, and the Damköhler number (D_a) (Koyayash and Laidler, 1974). [S] is the averaged substrate concentration when neglecting the diffusional effect. When the microchannel is a cylinder and the enzyme is immobilized on the channel surface, D_a can be expressed as,

$$D_a = 0.7801\left(\frac{RL}{D_s^2 v_m}\right)^{1/3} \frac{V_{\max}}{K_M + [S]_0} \tag{2}$$

where R is the radius of the microchannel, L is the length of the channel, D_s is the diffusion coefficient of the substrate in the solution, and ν_m is the mean sample infusion rate into the microfluidic channel. It should be mentioned that the (Eqs. (1) and 2) are valid under a laminar flow condition. In our experiments, we chose a 4-cm straight channel and low flow rates (< 1 μL/min) to guarantee laminar flow during the experiments.

In our experiments, K_M (782.5 μM) is always much larger than [S] (≤ 100 μM) (Section D, Appendices), therefore a pseudo-first order reaction kinetics is also applicable to the microchannel-based enzymatic reaction, where the kinetics Eq. (1) is simplified as

$$r_d = \eta \frac{V_{\max}}{K_M}[S] \tag{3}$$

and the effective maximum rate is $\eta V_{\max}$, which is the key factor to evaluate the microfluidic sensors prepared under different methods. The microsensor with larger $\eta V_{\max}$ can convert a higher percentage of AA to its oxidized product in unit time, thereby a higher sensitivity in sensing AA. From Eq. (3), the reaction rate constant K' of a microfluidic sensor is equal to $\eta V_{\max}/K_M$, where $K_M = 782.5$ μM. Therefore, the effective maximum rate ($\eta V_{\max}$) was characterized for each microfluidic sensor by measuring the K' of AA oxidation in the microfluidic sensors.

3.3. Activity study of enzyme immobilized in PDMS microfluidic channels

All the three protein immobilization methods can load proteins to the inner surface of PDMS microfluidic channel. The untreated PDMS and Al_2O_3 xerogel were characterized by scanning electron microscopy (SEM) analysis and energy dispersive X-ray microanalysis (EDX) (Section B, Appendices). In the case of physisorption to PDMS surface, 0.1 mg/mL ascorbate oxidase in 10 mM HEPES buffer was filled into the PDMS microfluidic channel for 1 h of adsorption, and then washed away. In the case of sol-gel encapsulation, the channel was filled with the mixture of sol-gel suspension and ascorbate oxidase solution (final concentration = 0.1 mg/mL), either for 8.5 days at 4 °C to completely dry the sol-gel, or just overnight at 4 °C before the unfixed sol-gel and enzymes were flushed away by nitrogen gas. In the case of physisorption to alumina xerogel modified PDMS surface, the microchannel modified with alumina xerogel was filled with 0.1 mg/mL ascorbate oxidase in 10 mM HEPES buffer for 1 h of adsorption. Indeed, we have observed that the amount of ascorbate oxidase by physisorption to alumina xerogel modified microchannel can reach a plateau value after 60 min of incubation (Fig. G1, Appendices).

With the method illustrated in Section E Appendices, reaction rate constant K' and effective maximum rate $\eta V_{\max}$ were measured for AA oxidation in the different microfluidic sensors and summarized in Table 1. It is clear that the microfluidic biosensor built

Table 1.
Measured reaction rate constant and effective maximum rate with the biosensors prepared by different enzyme loading methods.

Enzymes were immobilized by		Reaction constant rate	Effective maximum rate
Physisorption to PDMS surface		$0.025 \pm 0.002\ s^{-1}$	$19.6 \pm 1.6\ \mu M/s$
Sol-gel encapsulation	8.5 days of drying	$0.047 \pm 0.005\ s^{-1}$	$36.8 \pm 3.9\ \mu M/s$
	Overnight drying	$0.017 \pm 0.001\ s^{-1}$	$13.3 \pm 0.8\ \mu M/s$
Physisorption to Al_2O_3 xerogel surface		$0.080 \pm 0.001\ s^{-1}$	$62.6 \pm 0.8\ \mu M/s$

by enzyme physisorption to Al_2O_3 xerogel modified PDMS microchannel shows the highest enzymatic activity, thereby microsensor sensitivity.

Under the impact of diffusional effect, the performance of the biosensor without sol-gel modification is not as good as the other microfluidic biosensors. We have estimated the Damköhler number of the microfluidic biosensor without xerogel modification, and concluded that the utilization factor is very low ($< 10\%$) for unmodified PDMS microchannel, indicating that the diffusional effect in the microfluidic channel mainly limits the reaction kinetics (Section F, Appendices).

One advantage of sol-gel modification is to increase the specific surface area inside microfluidic channels, thereby decrease the diffusional limitation. However, the sol-gel encapsulation method is not suitable for the immobilization of unstable enzymes, which could lose their activities during the very long time of immobilization procedure. The biosensor, formed by physisorbing enzymes to the Al_2O_3 xerogel deposited PDMS surface, provides the highest catalysis effect among the employed protocols. With the protocol, the long time of enzyme immobilization procedure can be avoided. While the advantage in reducing diffusional effect during enzymatic reaction of the xerogel filled microchannel is kept thanks to the large specific surface area of the xerogel layer (Section B, Appendices).

3.4. Quantification of AA in human blood by the UV colorimetric microfluidic sensor

Microfluidic sensor prepared by the optimized enzyme immobilization method was utilized to quantify AA in human blood to test its feasibility in the analysis of bio-samples with complicated matrices. When a complex biosample is considered, microfluidic sensors with higher sensitivity and anti-biofouling property must be developed. Serum contains large amounts of proteins that also have absorbance around 266 nm. Biomolecules like proteins can also be adsorbed by PDMS. As shown in Fig. H1 (Section H, Appendices), even when serum was pretreated to remove proteins with molecular weight (MW) higher than 10 kDa, there was still strong absorbance at 280 nm which could be from proteins with MW < 10 kDa, or other small molecules that have absorbance near 280 nm. Therefore, the absorbance of pretreated human serum at 266 nm is summed from AA and other substances with absorbance near this wavelength, *e.g.* caffeine and proteins. Indeed, our volunteer consumed one cup of coffee just before donating blood. Our recently published work has shown that the ascorbate oxidase immobilized bio-sensors have specific response to AA but "blind" to the interference (Bi et al., 2016).

Table 2 lists the absorbance change of the pretreated and diluted serum at 266 nm before and after passing through different microfluidic channels. The results show that Al_2O_3 xerogel modified channel can dramatically suppress the non-specific adsorption of biomolecules in the serum. This phenomenon keeps consistent with our previously published work on the anti-biofouling effect of alumina xerogel (Bi et al., 2005). The drop of absorbance at 266 nm after passing the serum through the ascorbate oxidase loaded Al_2O_3 xerogel-PDMS channel could thus be attributed to the consumption of AA under the catalysis of ascorbate oxidase (Section I, Appendices).

The AA concentration in serum can be calculated by using an external calibration method. Fig. J1 (Section J, Appendices) shows the plot of the absorbance change ($-\Delta A$) at 266 nm of AA solution after passing through (flow rate = 1 μL/min) the ascorbate oxidase loaded Al_2O_3 xerogel-PDMS channel *versus* the initial concentration of AA, resulting in a linear dependence ($r^2 = 0.9893$). The sensitivity of the bio-sensor prepared under the optimized enzyme loading strategy to AA is then $(1.14 \pm 0.07) \times 10^{-2}$ a.u./μM at sample infusion flow rate of 1 μL/min. A higher sensitivity can of course be achieved by increasing the channel length or decreasing the sample infusion rate. With the data shown in Table 2 and the external calibration curve, AA concentration in the used serum can be calculated as $2 \times (22.9 \pm 1.4) = 45.8 \pm 2.8\ \mu M$, which is reasonable according to the reference range of AA concentration in blood, 23–85 μM. Considering the noise level of the UV/vis spectrophotometer, the limit of detection (LOD) can be 3δ, where $\delta = 0.009$ and is the standard deviation of the baseline during UV/vis absorption analysis at 266 nm. Limit of quantification (LOQ) can be 10δ. Therefore, LOD is expected as $2.4 \pm 0.1\ \mu M$, and LOQ as $7.9 \pm 0.5\ \mu M$.

From the strategy showed here, it could conclude that there are many advantages when applying this optimized biosensor strategy for AA test in human body fluid. To detect AA in a sample with complicated matrices like human body fluid, separation of substrate samples is usually necessary, *e.g.* by ion-pair liquid chromatography (Hernandez et al., 2006), reverse phase high performance liquid chromatography (Emadi-Konjin et al., 2005), and capillary electrophoresis (Law et al., 2005), followed with detection methods such as fluorometric assay, UV/vis spectrophotometry, and electrochemical techniques. For the separation-based methods, pretreatment of sample, optimization of chromatographic conditions, and choice of proper columns must be carefully considered, as well as the ascription of peaks in chromatograms or electropherograms. The main advantage of the newly developed biosensing method here is that it can omit the separation procedure and with a LOD in the level of few μM. In addition, the biosensor can be directly used for samples that include proteins or other biomolecules since alumina xerogel in the biosensor can suppress the adsorption of these biomolecules. The low amount of sample consumption is another advantage. Using

Table 2.
Measured absorbance of serum at 266 nm before and after passing through microfluidic channels with different modifications. The serum was pretreated by centrifugal filter to remove proteins with MW ≥ 10 K Da and diluted by 20 mM phosphate buffered (pH 7.4) saline (0.15 M NaCl) (1:1, v/v). *A′* is the absorbance of the serum at 266 nm after passing through various microfluidic channels. The flow rate for sample infusion was 1 μL/min.

*A*0 at 266 nm of pretreated and diluted serum/a.u.	After passing the pretreated and diluted serum through	*A′*/a.u.	$-\Delta A$/a.u.	$-\Delta A/A0$
1.058 ± 0.010	Un-treated PDMS channel	0.936 ± 0.013	0.122 ± 0.016	(11.5 ± 1.6) %
	Al_2O_3 xerogel modified channel	1.027 ± 0.012	0.031 ± 0.015	(2.9 ± 1.5) %
	ascorbate oxidase loaded Al_2O_3 xerogel-PDMS channel	0.797 ± 0.008	0.261 ± 0.013	(24.7 ± 1.2) %

the developed microfluidic biosensor for AA detection in blood, the consumption of sample can be dramatically reduced for single-drop blood test. Furthermore, the fabrication of the biosensor is greatly simplified, and shows advantage in the term of cost. The design of the biosensor is simple with only one straight channel that can greatly save production cost. The procedure to prepare the biosensor is simplified comparing to enzyme encapsulation method since the xerogel can be formed in the channel in advance.

4. Conclusion

Several different protocols for enzyme immobilization on PDMS microfluidic chip were compared to get an optimized method to prepare the UV/vis spectroscopic microfluidic sensor of AA. The performance of the established enzymatic sensors was supervised by their catalysis effect to substrate reaction. The results show that physisorption of enzymes to the alumina xerogel functionalized PDMS chip is the best choice among the tested protocols. The optimized strategy was successfully used to AA analysis in human serum. This study can also provide practical guidance to build a PDMS microfluidic channel based biosensor for other unstable enzymes.

Notes

The authors declare no competing financial interests.

Live subject statement

The blood sample was collected under the consent of the donor. No research on genetic material was carried out. And these samples were in agreement with the WHO's rules for “the clinical use of blood”.

Acknowledgment

The authors acknowledge the Laboratório de Patologia Clinica Hilário de Lima (Unilabs, Braga, PT) for blood collection. This work was supported by European Commission's 7th RTD Framework Programme People – Marie Curie actions and NanoTRAINforGrowth COFUND Scheme (No. 600375). Work partially supported by the projects FP7-ICT-2011-8-318372 (Nanodem) and FCT – EXCL/CTM-NAN/0441/2012. INESC-MN acknowledges FCT funding through the IN Associated Laboratory (Pest-OE/CTM/LA0024/2011). VVB acknowledges FCT (Portugal) for her PhD fellowship (Grant FRH/BD/82556/2011).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.05.054.

References

Ashor, A.W., Lara, J., Mathers, J.C., Siervo, M., 2014. Atherosclerosis 235 (1), 9–20.
Becker, H., Locascio, L.E., 2002. Talanta 56 (2), 267–287.
Bi, H., Fernandes, A.C., Cardoso, S., Freitas, P., 2016. Sens. Actuators B 224, 668–675.
Bi, H.Y., Weng, X.X., Qu, H.Y., Kong, J.L., Yang, P.Y., Liu, B.H., 2005. J. Proteome Res. 4 (6), 2154–2160.
Bsoul, S.A., Terezhalmy, G.T., 2004. J. Contemp. Dent. Pract. 5 (2), 1–13.
Dulay, M.T., Baca, Q.J., Zare, R.N., 2005. Anal. Chem. 77 (14), 4604–4610.
Emadi-Konjin, P., Verjee, Z., Levin, A.V., Adeli, K., 2005. Clin. Biochem. 38 (5), 450–456.
Figueroa-Mendez, R., Rivas-Arancibia, S., 2015. Front. Physiol. 6, 397.
Hernandez, Y., Lobo, M.G., Gonzalez, M., 2006. Food Chem. 96 (4), 654–664.
Koyayash, T., Laidler, K.J., 1974. Biotechnol. Bioeng. 16 (1), 99–118.
Law, W.S., Kuban, P., Zha, J.H., Li, S.F.Y., Hauser, P.C., 2005. Electrophoresis 26 (24), 4648–4655.
Liu, C., 2007. Adv. Mater. 19 (22), 3783–3790.
Liu, Z.J., Liu, B.H., Kong, J.L., Deng, J.Q., 2000. Anal. Chem. 72 (19), 4707–4712.
Oldenziel, W.H., Westerink, B.H.C., 2005. Anal. Chem. 77 (17), 5520–5528.
Oldenziel, W.H., de Jong, L.A.A., Dijkstra, G., Cremers, T., Westerink, B.H.C., 2006. Anal. Chem. 78 (7), 2456–2460.
Reinhammar, B., Aasa, R., Vanngard, T., Maritano, S., Marchesini, A., 1997. Biochim. Biophys. Acta-Protein Struct. Mol. Enzym. 1337 (2), 191–197.
Ren, K.N., Zhou, J.H., Wu, H.K., 2013. Acc. Chem. Res. 46 (11), 2396–2406.
Rutkowski, R., Rutkowski, K., Rutkowska-Talipska, J., Sowa, P., Sulkowski, S., 2012. Postepy Dermatol. Alergol. 29 (6), 456–460.
Santos, A.M.C., Santana, M.A., Gomide, F.T.F., Miranda, A.A.C., Oliveira, J.S., Boas, F.A. S.V., Vasconcelos, A.B., Bernquerer, M.P., Santoro, M.M., 2008. Int. J. Biol. Macromol. 42 (3), 278–284.
Stothard, J.R., Sousa-Figueiredo, J.C., Standley, C., Van Dam, G.J., Knopp, S., Utzinger, J., Ameri, H., Khamis, A.N., Khamis, I.S., Deelder, A.M., Mohammed, K.A., Rollinson, D., 2009. Acta Trop. 111 (1), 64–70.
Talley, K., Alexov, E., 2010. Proteins-Struct. Funct. Bioinform. 78 (12), 2699–2706.
Tang, C., Saquing, C.D., Morton, S.W., Glatz, B.N., Kelly, R.M., Khan, S.A., 2014. ACS Appl. Mater. Interfaces 6 (15), 11899–11906.
Tu, Q., Wang, J.C., Zhang, Y.R., Liu, R., Liu, W.M., Ren, L., Shen, S.F., Xu, J., Zhao, L., Wang, J.Y., 2012. Rev. Anal. Chem. 31 (3–4), 177–192.
Wang, L., Shi, Y.-r., Yan, H.-t., 2014. Spectrosc. Spectr. Anal. 34 (8), 2234–2237.
Wang, W., Zhou, W., Li, J., Hao, D., Su, Z., Ma, G., 2015. Bioprocess Biosyst. Eng. 38 (11), 2107–2115.
Wu, H.L., Tian, Y.P., Liu, B.H., Lu, H.J., Wang, X.Y., Zhai, J.J., Jin, H., Yang, P.Y., Xu, Y.M., Wang, H.H., 2004. J. Proteome Res. 3 (6), 1201–1209.
Yetisen, A.K., Akram, M.S., Lowe, C.R., 2013. Lab. Chip 13 (12), 2210–2251.