Smartphone–based enzymatic biosensor for oral fluid L-lactate detection in one minute using confined multilayer paper reflectometry

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Abstract

The development of smartphone–based biosensors for point-of-care testing (POCT) applications allows realizing “all in one” instruments, with large potential distribution among the general population. With this respect, paper color-based detection performed by reflectance measurement is the most popular, simple, inexpensive and straightforward method. Despite the large number of scientific publications related to these biosensors, they still suffer from a poor detectability and reproducibility related to inhomogeneity of color development, which leads to low assay reproducibility. To overcome these problems, we propose a smartphone paper-based biosensor, in which all the reagents necessary to complete the analysis are co-entrapped on paper in a “wafer”-like bilayer film of polyelectrolytes (Poly (allyl amine hydrochloride/poly(sodium 4-styrene sulfonate)). Using a 3D printing low-cost technology we fabricated the smartphone-based device that consists in a cover accessory attached to the smartphone and incorporating a light diffuser over the flash to improve the image quality, a mini dark box and a disposable analytical cartridge containing all the reagents necessary for the complete analysis. The biosensor was developed exploiting coupled enzyme reactions for quantifying L-lactate in oral fluid, which is considered a biomarker of poor tissue perfusion, a key element in the management of severe sepsis, septic shock and in sports performance evaluation. The developed method is sensitive, rapid, and it allows detecting L-lactate in oral fluid in the relevant physiological range, with a limit of detection of 0.1 mmol L−1. The extreme simplicity of assay execution (no reagents need to be added) and flexibility of fabrication of the device, together with the high assay versatility (any oxidase can be coupled with HRP-based color change reaction) make our approach suitable for the realization of smartphone-based biosensors able to non-invasively detect a large variety of analytes of clinical interest.

Keywords:

Smartphone; Enzymatic biosensor; Reagentless; Colorimetric paper-based assay; Salivary L-lactate; Point-of-care diagnostics.

1. Introduction

The continuous improvements of smartphones technology have unexpectedly expanded their range of applications, enabling their use as analytical chemistry instrumentation. In particular, the advancements of complementary metal oxide semiconductor (CMOS) photocamera technology enabled its use as an imaging device and photons detector in optical biosensors. The use of smartphones for the development of biosensors and point-of-care (POC) devices allows realizing “all in one” analytical instruments, potentially enabling anyone for the execution of chemical analyses exploiting their personal smartphone to perform multiple functions, such as detection, computational on line data analysis, and remote connection to send the results of the tests by messaging (SMS, MMS, video or images) or e-mail (Roda et al., 2016). In recent years, many examples of smartphone devices based on different optical (e.g., surface plasmon resonance, absorbance, reflectance, fluorescence, bioluminescence, chemiluminescence, and electrochemiluminescence) and electrochemical principles have been reported (Zhang and Qingjun, 2016; Roda et al., 2016). In most of these devices, the smartphone has been equipped with relatively complex accessories providing additional functionalities, such as light sources for photoluminescence excitation or electrodes for electrochemical measurements. Various applications were proposed for smartphone-based devices, exploiting specific enzymatic reactions, gene probe and immunoassays with different analytical formats. Among all the developed systems, color-based detection, based on reflectance measurements is the most popular, simple, inexpensive and straightforward method for producing signals on paper-based biosensors.

Back-illuminated complementary metal oxide semiconductors (BICMOS) integrated into smartphones are suitable for detecting color changes through reflectance measurements, because the sensor use the Bayer color filter array (CFA) to capture the color image. The CFA is an array of alternating color filters (RGB filters) that samples only one color band at

each pixel location. Furthermore BI-CMOS architecture allows reducing the pixel pitch and increasing the optical efficiency even in low light conditions, thus reducing digital noise and allowing more accurate colours acquisition and a better quality of the image.

Nevertheless, smartphone-based biosensors need improvements to increase signal detectability and assay accuracy, precision and robustness, thus enabling the quantitative detection of analytes present at sub-micromolar levels, as required for convenient POC tests. The most remarkable drawbacks associated with paper-based bioassays relying on reflectance measurements are the requirement for multiple reagents addition steps and the heterogeneity and poor quality of the color distribution in the detection area. This is mainly caused by uncontrolled flow of the solution containing enzymes and reagents towards the edge of detection in the paper surface area upon sample addition (Costa et al., 2014; Evans et al., 2014; Roda et al., 2016). Only the lateral flow immunoassay format offers the possibility to perform the assay without any reagent addition and moving the reagents simply by capillary force, but it is not a suitable format for enzyme-based assays and it still suffers of poor quantitative information in the color-based detection format (Zangheri et al., 2015, 2016).

Several strategies have been adopted to improve the analytical performance of paper-based methods, such as different bioconjugation reactions used to immobilize the enzymes via carbonyl, aldehyde, or amino groups formed on the cellulose substrate (Cate et al., 2014; Parolo and Merkoçi, 2013; Xu et al., 2016), or modification of the paper support using ceria, gold, silver nanoparticles and carbon nanotubes to improve the detection quality (Ahmed et al., 2016; Ge et al., 2014; Nery and Kubota, 2013; Ngo et al., 2011). Although each of these strategies has its own advantages, they are not universally applicable and require multiple experimental procedure steps to be performed.

Poly (allyl amine hydrochloride/poly(sodium 4-styrene sulfonate) (PAH/PSS) coating multilayer is currently used to functionalize nanoparticles or even cells, for proteins, enzymes or antibodies immobilization on surfaces that act as inert supports (Feldötö et al., 2011; Moraes et al., 2009; Ram et al., 2001). Thanks to these films, it is possible to fabricate multilayers with different functionalized surfaces and to selectively load different compounds, from small organic molecules to proteins and even nanoparticles, according to their physico-chemical and surface properties.

In this work, we present an innovative and simple procedure based on paper chemistry applicable to the most common enzymatic bioassays exploiting analyte-specific oxidases. Hydrogen peroxide (H_2O_2) produced by any of these oxidases reacts, in the presence of the enzyme horseradish peroxidase (HRP), with the chromogenic reagent 3,3′,5,5′-tetramethylbenzidine (TMB) to form a colored product. Both enzymes (oxidase and HRP) and TMB have been immobilized on different layers of the modified paper, using the formation of a “functional wafer-like” layered film of PAH/PSS polyelectrolytes system coating.

A simple smartphone accessory device has been fabricated using 3D printing technology. The device consists of a mini cartridge, a mini dark-box and a cover-like accessory: this assembly enables positioning the functionalized paper surface at the correct distance from the smartphone photocamera, to obtain consistent illumination during the measurement and to avoid interference from ambient light during the measurement. The color produced on paper is imaged by reflectance measurements using the flash function of the smartphone as a controlled light source. To obtain homogeneous illumination of the paper surface, the cover-like accessory has been designed to position a light diffuser over the flash, thus reducing the variations in the image quality.

This method has been applied to develop a simple and rapid paperbased bioassay to directly measure L-lactate concentration in oral fluid in one minute, using lactate oxidase (LOx)

coupled with HRP and TMB and smartphone imaging for color quantification by reflectance, as above proposed.

The rapid and non-invasive measurement of L-lactate in oral fluid is highly demanded by people or athletes doing endurance activity to properly and early evaluate their threshold anaerobic levels and therefore acting on their performance (Allen et al., 1985; Oliveira et al., 2015; Palacios et al., 2015; Tékus et al., 2012; Zagatto et al., 2004; Roda et al., 2014) and it may also be useful for non-invasive monitoring critical care patients and diabetics.

Since the assay is based on the detection of H2O2 produced by an analyte-specific oxidase, it can be generally applied for all oxidases, thus enabling the development of other specific bioassays to quantify several biomarkers such as glucose, cholesterol, ethanol, cortisol and bilirubin.

2. Materials and methods

2.1. Chemicals

Peroxidase (type VI-A from horseradish, 1080 U/mg protein), Llactate oxidase (from Pediococcus sp., 50 U/mg protein), L-lactate sodium salt, 3,3′,5,5′-tetramethylbenzidine (TMB), Poly (allyl amine hydrochloride) (PAH), Poly(sodium 4-styrene sulfonate) (PSS) L-histidine monohydrochloride mono-hydrate, mucin (type II from porcine stomach), urea and the chromatographic paper for assay support (Whatman CHR 1, in sheets of 20×20 cm) were purchased from Sigma Aldrich (St. Louis, MO).

Sodium chloride, disodium hydrogen orthophosphate anhydrous, and sodium dihydrogen orthophosphate monohydrate were purchased from Carlo Erba Reagents S.r.l. (Milano, Italy).

Phosphate buffered saline (0.1 mol L^{-1} PBS, pH 7.5) was employed as a buffer. Artificial oral fluid at pH 7.2 was prepared by dissolving 0.6 mg mL^{-1} Na_2HPO_4, 0.6 mg mL^{-1} anhydrous $CaCl_2$, 0.4 mg mL^{-1} KCl, 0.4 mg mL^{-1} NaCl, 4.0 mg mL^{-1} mucin and 4.0 mg mL^{-1}

urea in deionized water according to Tlili et al. (2010). The colorimetric lactate enzymatic assay in the standard 96-well microtiter plate format (BioVision Incorporated L-Lactate colorimetric Assay Kit) was bought from BioVision Incorporated, Inc. U.S.A. and used according to the manufacturer's instructions. Salivettes cotton swabs for oral fluid sampling were purchased from Sarstedt, Germany.

2.2. Paper functionalization

A 1 mg mL^{-1} PSS solution in PBS was prepared by sonication. A 2 mmol L^{-1} TMB solution in PSS (PSS/TMB solution) was obtained by adding 50 µL of a 10 mg mL^{-1} stock solution of TMB in DMSO to 1 mL of PSS solution and shaking for 30 min. A 1 mg mL^{-1} solution of PAH in PBS was prepared by sonication. Enzymes solutions of HRP (2 mg mL^{-1}) and LOx (8 mg mL^{-1}) were prepared in PBS. The paper support (1×1-cm) was soaked in PSS/TMB solution for 30 min, then upon drying at room temperature, it was rinsed with PBS for 5 min to remove loosely adsorbed molecules. The PSS/TMBfunctionalized paper was then soaked in PAH solution for 30 min, then it was incubated in the HRP solution at 4 °C for 30 min for enzyme adsorption and washed with PBS for 5 min. The second enzyme adsorption was performed by immersion in PAH solution for 30 min, then incubation in LOx solution at 4 °C for 30 min. After washing with PBS for 5 min, the functionalized support was soaked in PAH solution for 30 min to obtain an external protective layer coating and then left to dry for 1 h at 4 °C. A schematic representation of the “wafer-like” structure of the functionalized paper is reported in Fig. 1.

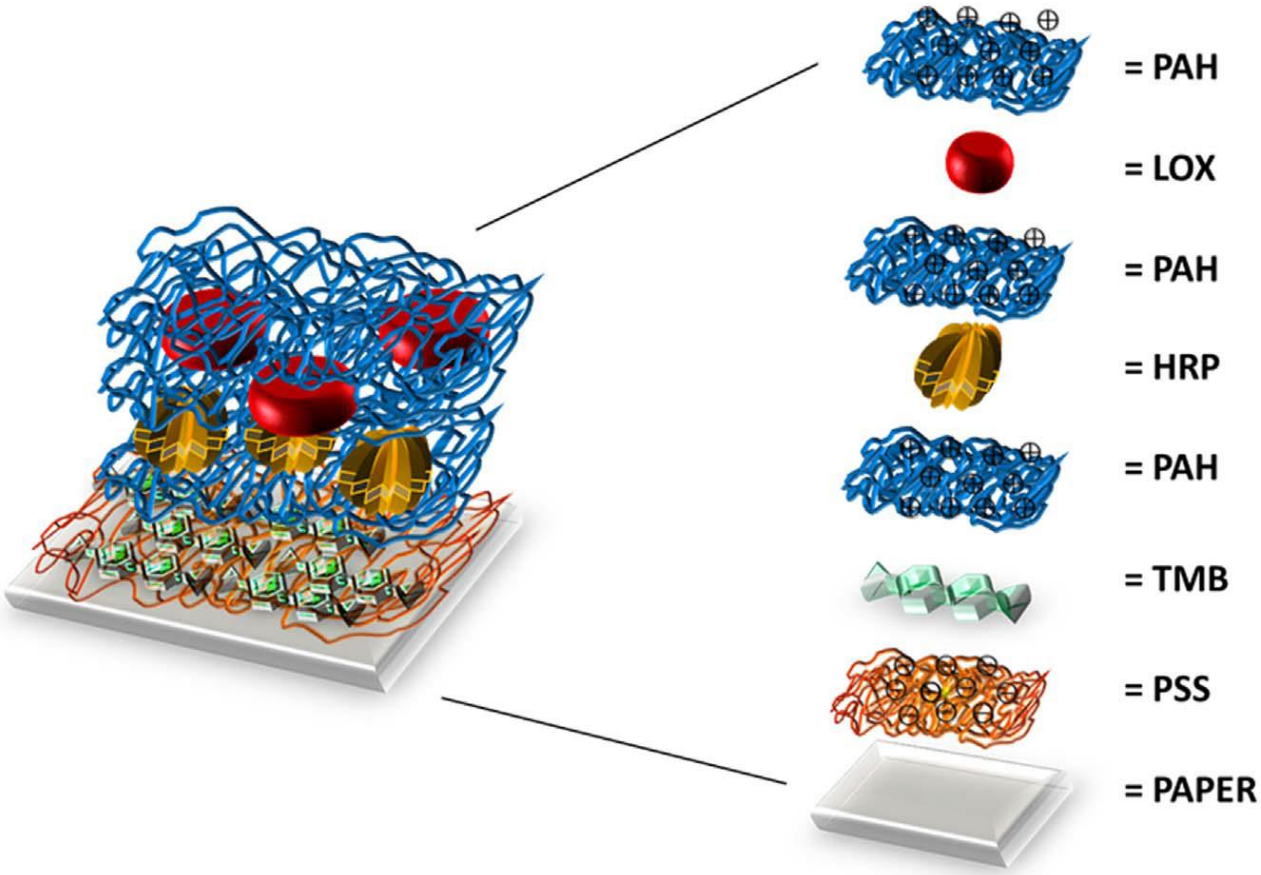

Fig. 1. "Wafer-like" structure of the functionalized paper. PAH (poly(allylamine hydrochloride)); PSS (poly(styrene sulfonate)); LOx (L-lactate oxidase); HRP (horseradish peroxidase); TMB (3,3′,5,5′-tetramethylbenzidine).

2.3. 3D printed smartphone accessory fabrication

The analytical device (Fig. 2), made of black acrylonitrile-butadiene- styrene (ABS) polymer, was fabricated using a low-cost commercial 3D printer (Replicator 2X Desktop 3D Printer, MakerBot Industries, New York, NY). The device was designed using the opensource SketchUp software (Trimble Navigation). Files, exported in stl file format, were then fed to MakerWare v.2.4 software, which employs an algorithm to digitally slice the object into thin layers for 3D printing and defines printing options and settings. The smartphone accessory was designed in two versions, one compatible with ASUS Zenfone 2 smartphone and one

with iPhone 6. The device consists of three separate components: (a) a disposable analytical cartridge, (b) a mini dark box, and (c) a smartphone cover-like adapter. The analytical cartridge (20 mm×10 mm) contains one reaction chamber (diameter 4 mm, depth 3 mm) in which the 1×1-cm paper support functionalized with PAH/PSS polyelectrolyte bilayer film entrapping LOx/HRP/TMB was positioned. The adapter includes a flash diffuser (4 mm diameter, consisting of a 5-mm thick polydimethylsiloxane (PDMS) membrane) and a plano-convex plastic lens (6 mm diameter, 12 mm focus) placed in front of the smartphone camera; it also allows, together with the mini dark box, positioning the reaction chamber in front of the camera and at the correct focal distance for images acquisition.

2.4. Oral fluid collection

Oral fluid was collected with Salivette® kits (Sarstedt, Germany) by chewing the cellulose swab for 1 min and then centrifuging. Samples were obtained from healthy volunteers during a 20-min running exercise. In particular, oral fluid was sampled before running and then after 10-min and 20-min running performance. When not analyzed on the sampling day, oral fluid samples were stored at −20 °C.

2.5. Assay procedure

L-lactate standard solutions were prepared in artificial oral fluid at concentrations ranging from 0.6 to 10 mmol L−1 and analyzed in triplicates. To perform the assay, an image of the assay chamber was acquired for background subtraction, then 50 μL of oral fluid or Llactate standard solution was applied in the reaction chamber of the cartridge (Fig. 2B). The cartridge was then inserted back into the mini dark box camera snapped to the smartphone through the adapter (Fig. 2) and a reflectance image was acquired 60 s after sample loading, employing the smartphone camera and setting the “flash automatic mode” to exploit the smartphone built-in flash as a light source. Suitable smartphone photography apps (Camera

FV-5 Lite app for Android-based smartphones and NightCap Pro for iPhone) were used to control white balance (AWB), sensitivity of the sensor to light (ISO) (automatic setting), exposure compensation (0.0) and the exposure time (1 s) long enough to achieve the required detectability and for image handling.

2.6. Reflectance image analysis

Images quantitative analysis was performed using the open source software ImageJ v.1.46 (National Institutes of Health, Bethesda, MD). For each image, a region of interest (ROI) corresponding to the sample chamber (area =458201 pixels2; diameter =764 pixels) was selected, then the RGB values were computed and converted to HSV. The Hue (H) value of HSV color space obtained from the image acquired before sample application (background signal) was then subtracted from the H value obtained 60 s after sample application, to obtain a corrected H value. Each L-lactate concentration was analyzed in triplicate and the average H value was calculated as the mean of these 3 images. Calibration curves were obtained employing GraphPad Prism v. 5.04 (GraphPad Software, Inc., La Jolla, CA) by plotting the inverse of average corrected H values (1/H) against L-lactate concentration and fitting the experimental data with a two-parameter linear function. To obtain the analyte concentration in real samples, the average 1/H value was calculated as described above and interpolated on the calibration curve.

3. Results and discussion

The aim of this work was to develop a simple, fast, cheap and portable paper-based biosensor for assessing L-lactate concentration in oral fluid employing reflectance measurements performed through a smartphone BI-CMOS. Through image analysis, the color change deriving from the enzymatic oxidation of L-lactate catalyzed by LOx coupled with the TMB/H_2O_2/HRP system was quantitatively related to L-lactate salivary

concentration. The main advantages of this colorbased biosensor are its utmost assay simplicity and the fast and reproducible response, as all the components and reagents are entrapped on a paper support in different layers with a “wafer-like” structure and the assay is completed in few minutes upon simple sample addition.

3.1. Functionalization of the paper support

The main limitation of previously reported paper-based colorimetric enzyme assays is the heterogeneity of the color distribution in the detection area, mainly due to the mobility of enzymes and reagents towards the edge of the detection area observed upon sample application.

This strongly affects color detection reproducibility, thus ultimately compromising assay accuracy and precision. In this work, we provide a simple and efficient way to selectively entrap and immobilize on paper all the reagents and enzymes needed to complete the analysis, through a “wafer-like” bilayer of polyelectrolytes (PAH/PSS) coating (Fig. 3A and B), thus offering a ready-to-use bioanalytical system.

As shown in Fig. 3B, the color distribution of the PAH/PSS modified paper is more homogeneous than that obtained by applying reagents and enzymes on unmodified paper. Thanks to this functionalization, the enzymes and the chromogenic reagent TMB were entrapped on the surface of the paper support by a “host-guest”-like interaction established between the reagents and the charged polyelectrolyte polymers. In this way, the enzyme mobility and the release of chromogenic reagent are reduced based on complementary electrostatic forces.

In particular, the first PSS layer is negatively charged due to the presence of the sulfate groups, while the second layer of PAH polyelectrolyte polymer bears a positive charge due to the presence of ammonium groups. Thus, the adsorption of the positively charged PAH

film onto the paper, as well as entrapment of the oxidized 3,3′,5,5′- tetramethylbenzidine (TMBox, produced by the oxidation of TMB with H_2O_2 catalyzed by HRP), is favored by the electrostatic attraction with PSS layer. On the other hand, the positively charged PAH layer is used to trap LOx and HRP enzymes, which are negatively charged in PBS (pH 7.5) being their isoelectric point (pI) 4.6 and 3.5, respectively.

Thanks to electrostatic interactions all the enzymes and the colored product of the reaction are evenly distributed and stably immobilized in the layer shells, avoiding their heterogeneous diffusion on the paper.

Moreover, another advantage of this method is the improved preservation of enzymatic activity due to the prevention of the biomolecule conformational structural changes and the related denaturation that may occur as a result of a bioconjugation reaction involving covalent bonds formation (Moraes et al., 2009; Ram et al., 2001). In our system, enzymes retained 70% of original activity after 6 weeks, when stored at 4 °C in the dark (to avoid oxidation of the TMB reagent). This is in line with previously reported results (Moraes et al., 2009; Ram et al., 2001; Snyder et al., 2012).

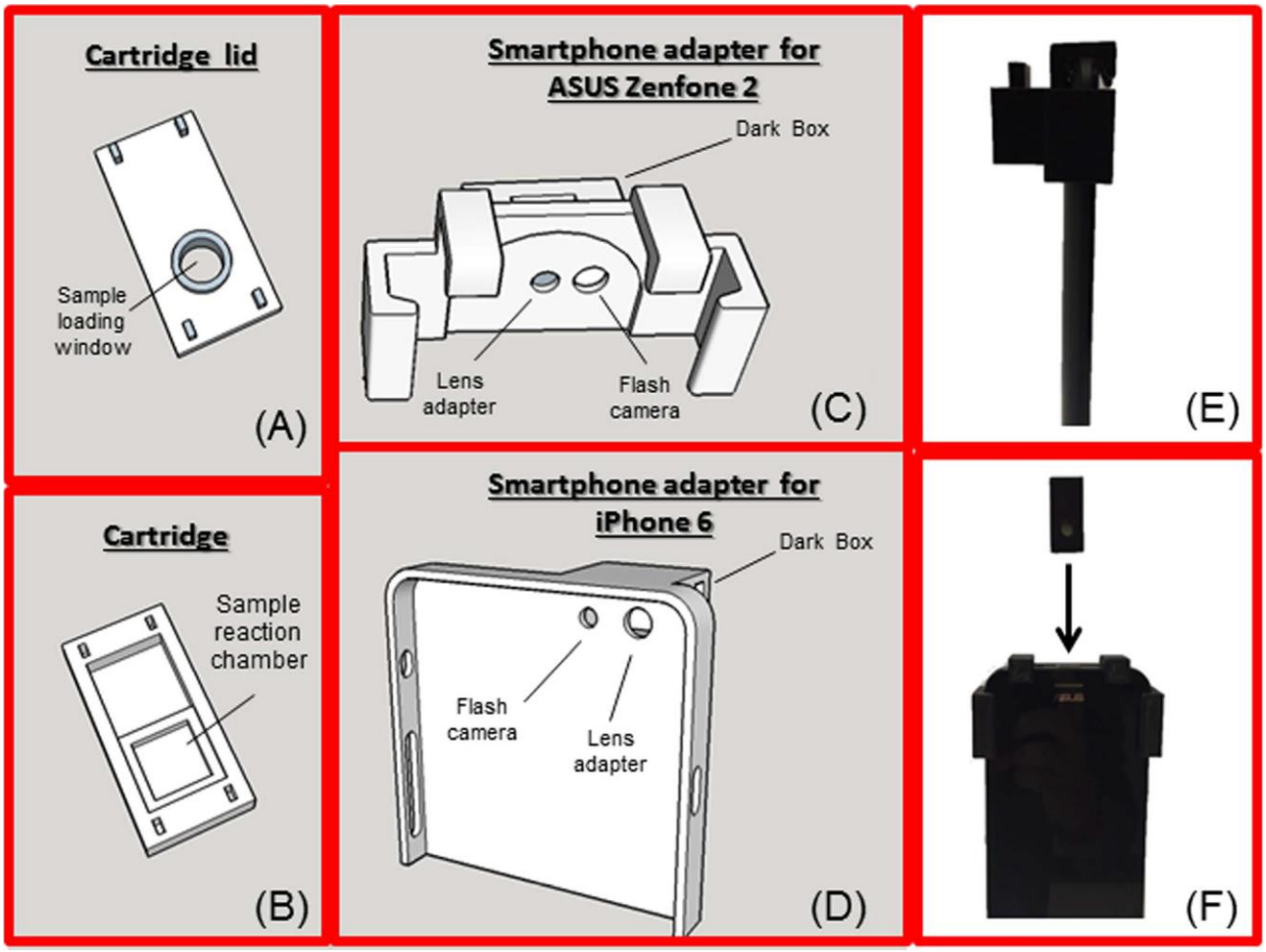

Fig. 2. The 3D printed analytical device made of black acrylonitrile-butadiene-styrene (ABS) polymer consisting of three separate components: a disposable analytical cartridge, a mini dark box and a smartphone adapter. (A) Cross-sections of the cartridge lid and (B) the analytical cartridge showing the internal sample reaction chamber. (C) Photo of the smartphone adapter for ASUS Zenfone 2 and (D) for iPhone 6. Photos of smartphone integrated within smartphone-based device accessory for ASUS Zenfone 2 (E) cross-wise and (F) front-wise.

3.2. The smartphone accessory fabrication and implementation

The smartphone accessory is fabricated in black ABS exploiting the 3D low cost printing technology, which offers the possibility to realize devices of different geometries, adaptable and upgradable to various smartphones and analytical methods in a simple and economic way.

The accuracy of the reflectometric color quantification depends on the smartphone devices and on the type of illumination source. As all next-generation smartphones are equipped with a built-in Auto White Balance function and being the analytical signal of this biosensor ratiometric, the method is independent of the used camera. As concerns illumination, ambient light should be avoided because reproducible and accurate reflectance measurements require uniform and calibrated illumination; furthermore inaccurate imaging can be obtained if the smartphone BI-CMOS is not placed at the proper distance from the test strip. The geometry of the accessory was thus designed to ensure better uniformity of lighting on the circular detection area of the paper, while the mini dark box accepts the analytical cartridge ensuring the correct positioning of functionalized paper and avoiding interference from ambient light (Fig. 2). The smartphone cover-like adapter, that can be snapped onto the smartphone to correctly position the dark box, integrates an optical lens that focuses the image of the paper support onto the smartphone BI-CMOS camera. In order to improve the sensitivity of the system and to reduce the effect of support misalignment into the device, we have incorporated a PDMS light diffuser over the flash. PDMS often used for the fabrication of optical components, thanks to its elastomeric properties, optical transparency and low attenuation (Cai et al., 2013). The large difference in refractive index between air and PDMS (mixture with a weight ratio of 10:1 of base and curing agent, n=1.420) makes the incident light to be refracted at large angle (Park and Khang, 2016; Shih

et al., 2006). Moreover, structural imperfections inside the diffuser act as secondary light sources following the Huygens scattering principle (Azmayesh-Fard et al., 2013).

The flash diffuser allows to homogenize the light toward the detection area and to uniformly illuminate the paper, reducing the variations in the image quality (Fig. 3C and D). Indeed, in the absence of the diffuser, the support appears as white with either 100% or 0% saturation levels (Oncescu et al., 2014). A saturated color image would not allow to differentiate between slight color differences, thus to distinguish between two neighbouring concentrations, and this would thus decrease assay sensitivity. The geometry and the distance from the support (12 mm of distance) allow to decrease the intensity of the flash light, to fully illuminate the support and to discriminate the different intensity of the color differences.

3.3. Smartphone camera detectability and quantification of L-lactate in oral fluid samples

Smartphone BI-CMOS cameras were designed to reproduce images in a manner similar to human eyes and they therefore have Bayer color filters (a grid of red, green and blue filters) over the photo sensor array. Images are thus captured and stored in a RGB scheme. In order to determine the quantitative correlation between the variation of color and the concentration of the analyte, we used the hue saturation value (HSV) color space system, which allows discriminating even small variations of color in a more selective manner, as the scoring does not only depend on the color (as in the case of the RGB color space), but also on the intensity and brilliance of the color. In particular, the H component (hue) has been used as an analytical parameter for bitonal optical sensors (Chang, 2012; Oncescu et al., 2013, 2014) because it provides a numeric precise value independent of the thickness of the material, amount of chromogenic agent, detector spectral responsivity and illumination (Cantrell et al., 2009).

Our biosensor is based on an end-point enzymatic colorimetric assay, thus incubation time after sample application must be long enough to ensure that the enzyme reactions are complete. Therefore, the color change was monitored upon application of 50 μL of an artificial oral fluid sample with a 2 mmol L^{-1} L-lactate concentration, that is considered as the salivary concentration of anaerobic threshold. As it is shown in Fig. 4B, the color was observed to stabilize in about 60 s, therefore this time was employed for all the measurements. In order to quantify the color-based reaction that occurs on paper, a calibration curve was constructed by plotting the inverse of the corrected hue coordinate of HSV color space (1/H) with respect to Llactate concentration in oral fluid (range 0.6–10 mmol L−1) (Fig. 4A), thus correcting any possible interference of oral fluid matrix on color change result and TMB/H2O2/HRP colorimetric system. A limit of detection (LOD, calculated as the ratio between three times the standard deviation of blank and the curve slope, 3σ/m) of 0.1 mmol L−1 (corresponding to 0.9 mg dL−1) and limit of quantification (LOQ, calculated as the ratio between ten times the standard deviation of blank and the curve slope, 10σ/m) of 0.3 mmol L−1 (corresponding to 2.7 mg dL−1) of L-lactate were obtained in artificial oral fluid. These values, as compared with salivary concentration of anaerobic threshold (2 mmol L−1), show that the biosensor can detect L-lactate in oral fluid in the relevant range of concentrations.

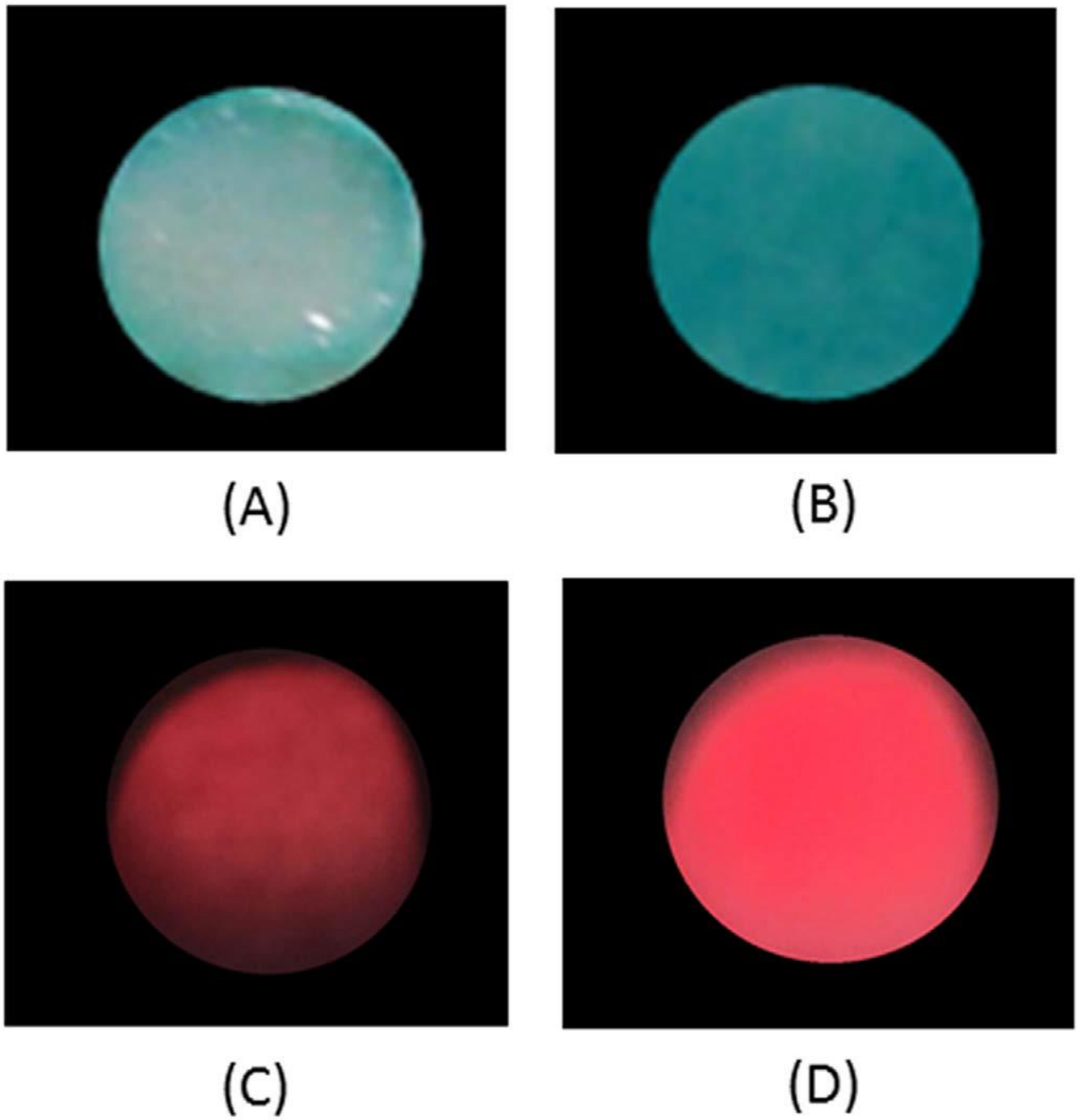

Fig. 3. Pictures of paper supports treated with LOx, HRP and TMB and exposed to Llactate (1 mmol L−1): (A) unmodified paper support; (B) paper support functionalized with polyelectrolites “wafer-like” bilayer films. Paper support functionalized with polyelectrolites “wafer-like” bilayer films entrapping LOx, HRP and TMB and exposed to L-lactate (10 mmol L−1): pictures acquired with (C) or without (D) flash diffuser.

3.4. Validation of the method and application on real oral fluid samples

To evaluate the assay accuracy, the lactate concentration measured with smartphone-based biosensor was compared with a validated commercial colorimetric lactate enzymatic assay in a standard 96-well microtiter plate format. Fig. 4C and Table 1 report the correlation between results obtained when analyzing ten samples containing Llactate (range 1–10 mmol L^{-1}) in artificial oral fluid with the standard colorimetric L-lactate assay and the smartphone-based L-lactate biosensor. The good correlation observed between the results obtained with the two methods r2=0.9902 in a L-lactate concentrations ranging from 1 to 10 mmol L^{-1} demonstrates the ability of the assay to provide accurate results, without any matrix effects. To further verify the reliability and the specificity of enzymatic biosensor for L-lactate, spike recovery experiments were performed. In particular, five real oral fluid samples were analyzed prior and after spiking with L-lactate (between 1 and 3 mmol L^{-1} levels). As shown in Supplementary material, recovery in the range 80–110% was obtained, confirming the biosensor specificity and accuracy (Table S1).

The compound mainly expected to exhibit and interfering effect is ascorbic acid, which could interfere with H2O2 reduction. Nevertheless, the salivary concentration of ascorbic acid is in average 4 μM (Mäkilä and Kirveskari, 1969), which is negligible with respect to salivary Llactate levels and also to ascorbic acid concentrations able to elicit an inhibitory effect on the substrate/H2O2/HRP system (Pogačnik and Poklar Ulrih, 2012). Furthermore, previous studies have demonstrated that the immobilization of LOx and HRP in a layered structure network decreases the interfering effect of ascorbic acid towards enzymes (Hasebe et al., 2005).

To demonstrate the applicability of the smartphone-based biosensor for monitoring L-lactate levels during physical exercise, we measured L-lactate in oral fluid collected during the running track performed by a volunteer. Fig. 5 shows the oral fluid L-lactate profile measured in the volunteer; the values plotted in the graph represent the mean of three independent

measurements performed on each collected oral fluid sample ± the standard deviations (SD). A coefficient of variation below 20% was obtained in these measurements, proving the reliability and practical applicability of the proposed smartphone reflectance paper-based device for quantitative determination of Llactate in oral fluid samples.

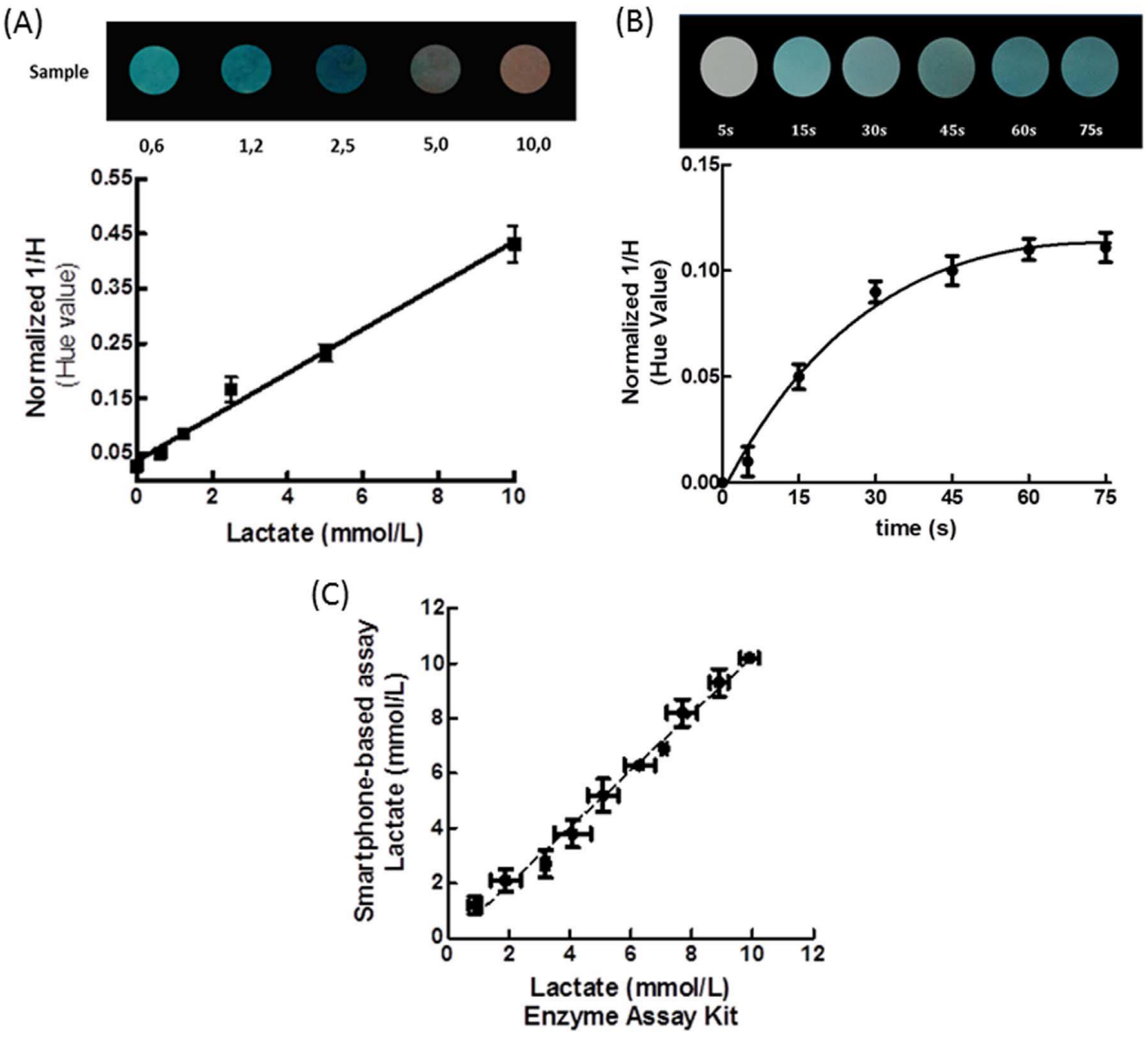

Fig. 4. (A) L-lactate calibration curve obtained in artificial oral fluid (range 0,6–10 mmol L−1). Hue values were subtracted of the H value obtained for the blank (before sample application) of the same cartridge. Data points represent the mean ± SD of three replicates. (B) Color images acquired at different times upon application of L-lactate (2 mmol L−1) on a functionalized paper support. (C) Comparison of the L-lactate concentrations measured in samples of artificial oral fluid with 10 standard concentrations of L-lactate (range 1– 10 mmol L−1) using the smartphone-based L-lactate biosensor and a BioVision Incorporated L-Lactate colorimetric Assay

Kit performed in the standard 96-well microtiter plate format. Each data represents the mean ± SD of three replicates.

Table 1

Comparison of the L-lactate concentrations measured in 10 samples of artificial oral fluid using the smartphone-based lactate biosensor and a reference method.

Method	L-Lactate standard samples									
	1 mmol L^{-1}	2 mmol L^{-1}	3 mmol L^{-1}	4 mmol L^{-1}	5 mmol L^{-1}	6 mmol L^{-1}	7 mmol L^{-1}	8 mmol L^{-1}	9 mmol L^{-1}	10 mmol L^{-1}
Reflectance smartphone-based assay	1.2 ± 0.3	2.1 ± 0.4	2.7 ± 0.5	3.8 ± 0.5	5.2 ± 0.6	6.3 ± 0.2	6.9 ± 0.1	8.2 ± 0.5	9.3 ± 0.5	10.2 ± 0.2
Reference colorimetric enzyme assay kit	0.9 ± 0.2	1.9 ± 0.5	3.2 ± 0.1	4.1 ± 0.6	5.1 ± 0.5	6.3 ± 0.5	7.1 ± 0.1	7.7 ± 0.5	8.9 ± 0.3	9.9 ± 0.4

Comparison of the L-lactate concentrations measured in 10 samples of artificial oral fluid with 10 standard concentrations of L-lactate (range 1–10 mmol L−1) using the smartphonebased lactate biosensor and a BioVision Incorporated L-lactate colorimetric Assay Kit performed in the standard 96-well microtiter plate format. Each data represents the mean ± SD of three replicates.

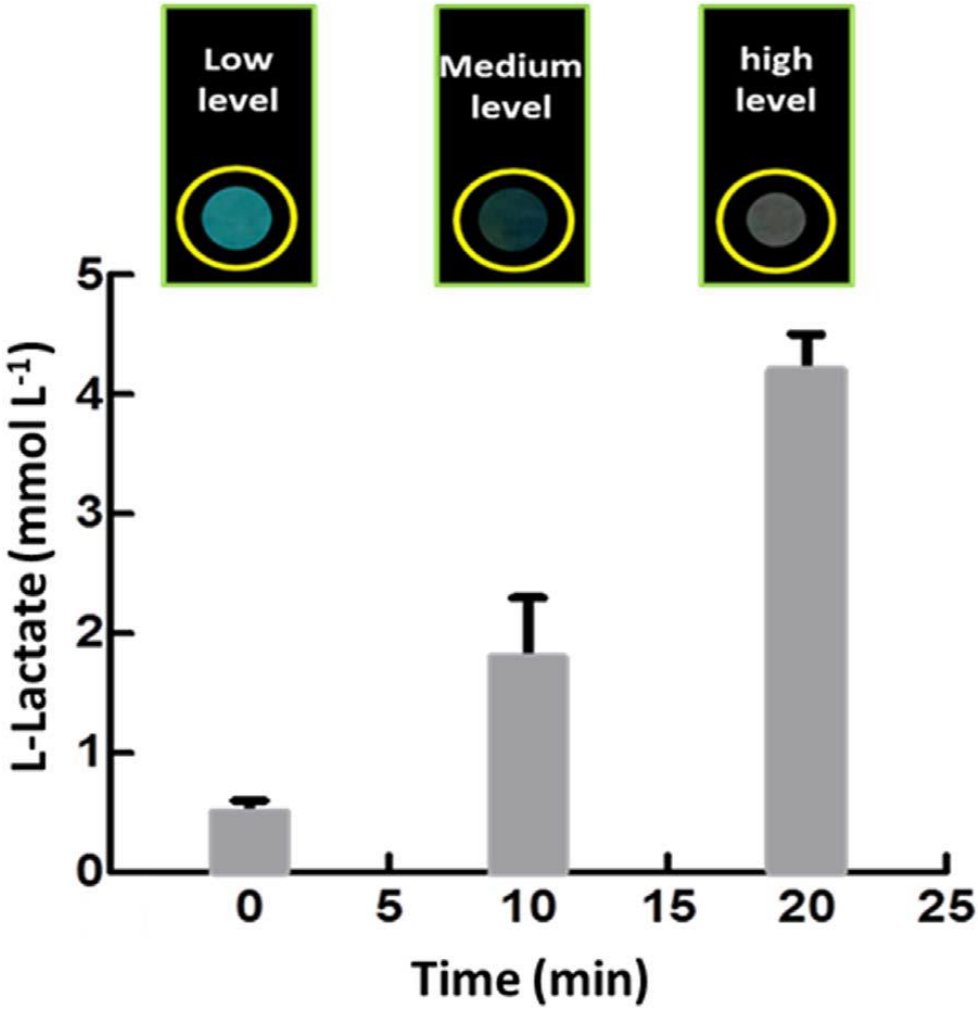

Fig. 5. Images and results of L- lactate levels in oral fluid monitored by smartphonebased biosensor during the running track performed by a volunteer.

4. Conclusions

We have successfully developed and optimized a reflectometry enzyme biosensor for rapid and accurate L-lactate quantitative detection in oral fluid employing a layer-by-layer functionalized paper support enclosed in a simple cartridge, placed in front of the smartphone camera for color reflectance measurements. Chemical modification of cellulose by deposition of PAH/PSS polymers in a “wafer-like” structure allowed realizing a portable ready-to-use analytical system and to enhance reagents stability and the homogeneity of color distribution, thus improving assay portability, robustness, accuracy and precision. The biosensor exploits BI-CMOS sensors integrated into modern smartphones and 3D printing technology to obtain a very rapid, simple, and sensitive assay that can be conveniently applied to POC environment, with reduction in cost and response time. The method allows quantitatively detecting L-lactate in oral fluid samples in the relevant physiological range

(LOD 0,1 mmol L−1), thus finding applications in endurance sports performance evaluation and critical care patients and diabetics monitoring.

The extreme simplicity of the device which contains all the reagents widens its applicability and makes it suitable for the detection of many other analytes of clinical interest thank to enhanced sensitivity in respect to previous methods associated with a fast and simple assay. This system could be expanded for multiplex format, for the analysis of other analytes such as glucose, cholesterol, and ethanol, exploiting their specific H2O2-producing oxidases. The system can be integrated in several camera-based technologies, such as smartphones, tablets, mini-portable scanners, and smart glasses to further improve the device prototype and to design different analytical formats.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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