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White light reflectance spectroscopy biosensing system for fast quantitative prostate specific antigen determination in forensic samples

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ABSTRACT

A label-free biosensor based on white light reflectance spectroscopy for the determination of PSA as semen indicator in forensic samples is presented. The sensor is based on a two-step immunoassay which employs the same polyclonal anti-PSA antibody as capture and detection antibody followed by reaction with streptavidin as a signal enhancement step. The whole assay time was set to 10 min; 5 min reaction of immobilized antibody with the PSA calibrators or the samples, 3 min reaction with the biotinylated anti-PSA antibody and 2 min reaction with streptavidin. Following this protocol, a detection limit of 0.5 ng/mL was achieved and the assay's linear response range extended up to 500 ng/mL. Thus, taking into account the quantification limit of 1.0 ng/mL and the average PSA concentration in semen (0.2–5.5 mg/mL), semen quantities of a few nanoliters could be detected. The accuracy of the sensor developed was demonstrated through recovery (% recovery ranged from 89.6 to 106) and semen dilution experiments. A linear correlation was found for semen dilutions ranging from 5000 to 360,000. The lack of interference by other bodily fluids was confirmed by analysing stains of blood, urine and saliva prior to and after the addition of semen. Finally, the sensor was evaluated by analysing 51 forensic casework samples which were also analysed with a semi-quantitative membrane strip test (Seratec® PSA), through microscopic detection of spermatozoa, and male DNA identification through detection of Y chromosome. The results obtained with the sensor were in excellent agreement with those provided by an immunoradiometric assay kit (PSA-RIACT) and in complete agreement with the findings using the membrane strip assay, spermatozoa and Y chromosome detection. The excellent analytical performance and small size of the instrument make the sensor developed an attractive tool for use in forensic evidence screening for semen detection.

1. Introduction

The detection of seminal fluid in forensic samples is essential data in cases of alleged sexual assault [1]. Although the detection of alleged assaulter's genetic material is considered as the strongest evidence of criminal activity [2–4], it relies on the number of spermatozoa that can be extracted by the sample. Thus, it can lead to false negative results in cases the ejaculation fluid originates from vasectomized, oligospermic, or aspermiac males where spermatozoa are either absent or sparse in the sample. Similarly, the microscopic examination of the sample for detection of spermatozoa, that is usually applied as a confirmatory method, could not resolve such cases, while is imposed to additional restrictions such as the presence of other types of cells in the sample (e.g., epithelial cells from the victim) or degradation of sperm cells that makes difficult their detection [5,6]. To address these cases and/or provide confirmatory results to genetic testing or sperm cells detection,

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two different but complimentary approaches have been exploited; spectrometric Raman analysis of suspected stains [1,7,8] or immunochemical detection of specific semen protein components in extracts from the forensic evidence [9]. The first approach has the advantage that is not destructive, nevertheless due to intrinsic heterogeneity of body fluids a mapping methodology along with appropriate statistical treatment of received spectra is required in order to differentiate between different body fluids (sperm, urine, blood, sweat, saliva, etc) [10]. On the other hand the detection of specific semen components could be destructive for the sample since it requires extraction but is more specific since the molecules detected are not found (at least in the same quantities) in other body fluids. The semen components usually detected are acid phosphatase, prostate specific antigen and semenogelin [1]. The determination of acid phosphatase enzymatic activity in forensic samples is one of the most widely employed methods in the investigation of sexual assault cases [11]. Nevertheless, acid phosphatase occurs naturally in the vagina at low concentrations leading to false positive results, and thus compromising the validity of a positive test as case-strong evidence in the court. Therefore, the detection of more semen-specific proteins like prostate specific antigen (PSA) and semenogelin has been proposed as more valid methods for the identification of semen in forensic evidence [12–14].

PSA is a 28.4 kD proteolytic enzyme produced by prostatic epithelial cells and mixed with the semen to achieve its liquefaction following ejaculation [15]. The classification of PSA as a semen-specific protein was made as early as 1978. Since then, the concentration ranges of PSA in different biological fluids have been established, with seminal fluid to contain the higher ones. According to the literature, the concentration of PSA in healthy men's seminal fluid ranges between 0.2 and 5.5 mg/mL. Nevertheless, PSA exists naturally to other biological fluids at concentrations < 0.4 ng/mL in saliva, < 20 ng/mL in blood, < 10 ng/mL in urine, < 2.1 μg/mL in breast milk, and < 10 ng/mL in vaginal fluid. Therefore, not only the detection but also the quantification of PSA in forensic samples is quite significant so as to assure that semen is present in the sample under examination.

The method most commonly employed by forensic laboratories to identify PSA is lateral flow immunochromatographic strips, which rely on a sandwich immunoassay format employing two monoclonal antibodies specific for PSA; one as capture and another labelled with gold nanoparticles as detection antibody [12,16]. The testing procedure involves extraction of PSA from the samples collected in the crime scene, application on the cartridge or immersion of strip onto the sample and reading of the result as a colored “test line” along with the “control” one after approximately 10 min. The main advantages of the method is the ease of use and the short response time and the main disadvantages the relatively low detection sensitivity and the increased occurrence of false negative results due to hook effect at very high PSA concentrations in the sample [17,18]. Thus it would be very useful to employ a method that could offer high analytical performance along with ease of use and short response time. Such a goal could be accomplished by the employment of biosensors [19]. A significant number of biosensor systems have been evaluated with respect to PSA determination in human serum or plasma samples, mainly driven from the wide use of PSA as a prostate cancer marker in clinical practice [19]. Nevertheless, to the best of our knowledge there is no report for PSA (or other protein markers) determination in forensic samples with biosensors. This could be ascribed to the fact that the samples could have diverse origin and/or be contaminated by other biological fluids or materials that might have a strong matrix effect on the sensor response. However, small-size instruments based on miniaturized biosensors that could provide for fast, sensitive and accurate measurement of PSA on-the-spot could be powerful tools in the hands of the forensic authorities [20,21]. The obvious benefits of such a system, to name just few, would be the decrease of the samples volume to be collected for analysis by the forensic laboratory, as well as the avoidance of protein markers deterioration in the sample that could lead to false negative results affecting the validity of the evidence.

In an effort to address these goals, we implement a complete system based on White-Light Reflectance Spectroscopy (WLRS) for the fast and accurate determination of PSA concentration in forensic samples through a two-step immunoassay. The immunoassay employs a polyclonal antibody for capture of PSA on the chip surface, and the same antibody in biotinylated form in order to detect the immunoadsorbed onto the surface analyte. In a third step, streptavidin is used as a mean to enhance the immunoassay signal, improve detection sensitivity and decrease the assay time. The detection principle involved allowed the monitoring of the different assay steps in real time by continuously recording the specular reflectance spectrum and correlating the spectral shifts observed to the increase of the effective biomolecular adlayer thickness. The particular assay format has been applied in a previous report to determine the total- and free-PSA concentrations in human serum [22], and detection limits in the fM range have been achieved. Nevertheless, the assay required almost one hour to be completed. The implementation of the new microfluidic cell with improved fluid dynamics and smaller dimensions (previously used for the development of a dual-analyte assay for determination of C-reactive protein and D-dimer [23]) enabled a drastic reduction of the assay time without affecting its analytical performance. Moreover the new microfluidic cell allowed for significant reduction of the sample volume by a factor of 3 (from 300 μL to 100 μL). Furthermore, optimization of the cartridge allowed to perform the assay under ambient light conditions, a feature quite important for on-site determinations (e.g. crime scene). Thus, the small sensor size, thanks to the miniaturized optical, electronic and fluidic modules, make it very promising for on-site determinations. The proposed biosensor was employed for the determination of PSA in actual forensic casework samples, including swabs (vaginal or anal) or stains on fabrics suspected for semen, and the results were confirmed by an immunoradiometric assay kit for the determination of PSA in human serum. Furthermore, the validity of the results obtained with the sensor was confirmed by analysing the same samples with commercial semi-quantitative immunoassay membrane strips, through microscopic detection of spermatozoa, and male DNA identification through Y chromosome detection.

2. Materials and methods

2.1. Reagents

Four-inch Si wafers (< 100 >) were purchased from Si-Mat Germany (Kaufering, Germany) and cleaned with Piranha solution. Then a 1000-nm thick silicon dioxide layer was grown on the wafers by wet oxidation at 1100 °C in the Nanotechnology & MEMS Laboratory clean room facility of the Institute of Nanoscience and Nanotechnology of NCSR “Demokritos”. The fluidic compartments and the docking station were designed and manufactured by Jobst Technologies GmbH (Freiburg, Germany). Bovine serum albumin (BSA), prostate specific antigen (PSA) from human semen, (3-aminopropyl)triethoxysilane (APTES), and hematoxylin solution (Harris modified) were purchased from Sigma (St. Louis, MO). Eosin B was from Merck (Darmstadt, Germany). An affinity purified goat polyclonal antibody against human PSA (code GP079) was purchased from Scripps Laboratories (San Diego, CA). The calibration of PSA standard solutions was performed using a commercially available immunoradiometric assay kit for PSA determination in human serum (PSA-RIACT; CIS Bio International; Codolet, France). The same kit was used for the quantitative determination of PSA in forensic casework samples. Biotinylation of the goat anti-PSA antibody was performed following a previously published procedure [22]. Semi-quantitative immunoassay membrane strip tests for PSA (Seratec® PSA) were from Seratec GmbH (Goettingen, Germany). Differential DNA extraction was performed using the Differex™ System from Promega Corporation (Madison, WI). The

presence of Y chromosome was performed using the Quantifiler® Trio DNA Quantification Kit (Thermo Fisher Scientific Inc.; Waltham, MA). All other reagents were from Merck (Darmstadt, Germany).

2.2. Forensic casework samples

Forensic casework samples were provided by the DNA Subdivision of Forensic Science Division of Hellenic Police. Forty seven out of 51 samples were collected during the investigation of rape cases, 3 were from homicide cases, and 1 from kidnapping. The samples included 19 vaginal swabs, 5 anal swabs, 25 fabrics with stains suspected as semen, 1 tampon and 1 incontinence diaper. No further information about the cases was provided. All samples were kept in sealed bags in presence of desiccator. Fresh semen samples from anonymous donors, received after informed consent, were aliquoted in 20 μL samples and kept at −20 °C.

2.3. Instrumentation and sensor preparation

The WLRS optical setup consisted of: a) a visible-near infrared light source (AvaLight; Avantes, The Netherlands), b) a spectrometer (Maya 2000Pro, Ocean Optics; Duiven, The Netherlands), and c) a reflection probe of custom design coupled to the light source and the spectrometer for the vertical illumination of the chip and collection of the reflected light for further analysis. The biosensor consisted of the chip (Si dies of 5.0×15 mm^2 size with 1-micron thick thermal SiO_2 layer) sealed with the microfluidic cell. The thickness of SiO_2 layer has been optimized in a previous report where it was found that thicknesses from 0.7 to 3.0 μm provided the highest signals during bioreaction [22] due to the spectral range of operation and the number of fringes required for accurate measurement of minute spectral shifts in the interference spectrum. The chips were cleaned and hydrophilized by O_2 plasma treatment (10 mTorr) for 30 s and then immersed for 20 min in a 2% (v/v) aqueous APTES solution. To complete the silanization procedure, the chips were gently washed with distilled water, dried under a nitrogen stream, and cured by heating at 120 °C for 20 min. The APTES-modified chips were then biofunctionalized by spotting the polyclonal antibody against PSA. For this purpose, 15 lines of 35 spots each with a spot-to-spot distance of 100 μm from a 200 μg/mL antibody solution in carbonate buffer, pH 9.2, were deposited using the Bio-Odyssey Calligrapher™ microarray spotter (Bio-Rad Laboratories, Hercules, CA) equipped with solid pins with a tip diameter of 375 μm (SSP015, Arrayit Corporation, Sunnyvale, USA). Thus, an antibody zone of 1.7×3.7 mm^2 was created. The spotted chips were incubated overnight at 4 °C in a controlled humidity chamber (75% humidity), washed with 10 mM Tris-HCl, pH 8.25, 9 g/L NaCl (washing solution), and then immersed in blocking solution (20 mg/mL BSA in 0.1 M $NaHCO_3$, pH 8.5) for 2 h at RT. After that, the chips, called hence forward biochips, were washed with washing solution and distilled water, dried under nitrogen flow, and used either immediately or kept at 4 °C in a desiccator until use. Prior to the assay, the biochip was assembled with the fluidic compartment and then positioned on a milled docking station incorporated to a non-transparent jacket that provided also for the correct alignment of the antibody zone on the biochip with the reflection probe. The delivery of the solutions to the fluidic compartment was accomplished through a custom made computer controlled peristaltic micropump operating in suction mode at a constant rate of 20 μL/min. Images of the whole sensing system (Fig. 1a) and details of the docking station (Fig. 1b) are provided in Fig. 1. All measurements were performed at room temperature using the FR-Monitor software (ThetaMetrisis, Athens, GR) for recording (every 1 s) and processing of the reflectance spectrum. In order to calculate the effective thickness of the biomolecular layer from the reflectance spectrum shift, the spectrum was fitted in the 450–800 nm spectral range with the Levenberg-Marquardt algorithm considering a layer stack consisted of washing buffer/biomolecular layer/SiO_2/Si. The thickness of the SiO2 layer was considered constant throughout the measurement, whereas for the biomolecular layer a refractive index of 1.45 was used based on literature data [24]. This way the evolution of the biomolecular layer thickness versus was monitored in real-time, whereas the individual spectra were stored for further off-line processing if needed.

2.4. PSA extraction from forensic samples

The samples used in the present study were cotton swabs with either vaginal or anal samples or fabrics of different composition collected from crime scenes bearing stains of biological fluids. Fiber fragments of the swabs or fabric pieces in the area of semen-suspected stains (approximately 0.25 cm^2) were cut and placed in 1.5 mL tubes. Then, 1 mL of 50 mM Tris-HCl buffer, pH 7.8, containing 5 mg/mL BSA, and 9 g/L NaCl (assay buffer) were added per tube, and after short vortexing the samples were extracted passively for 20 min at room temperature. The eluate was then diluted 10-times with assay buffer and analysed in the WLRS sensing system.

2.5. Determination of PSA in buffer and forensic samples with the WLRS sensing system

At first, assay buffer was run over the biochip for 3 min to acquire stable baseline. Then, the PSA calibrators or extracted and diluted forensic samples were introduced and run for 5 min, followed by a 10 μg/mL biotinylated anti-PSA antibody solution in assay buffer for 3 min, and a 5 μg/mL streptavidin solution in the same buffer for 2 min. Finally, the biochip was regenerated by running 0.1 M glycine/HCl buffer, pH 2.5, for 3 min, followed by washing and equilibration with assay buffer prior to the next run.

2.6. Sample analysis by immunochromatographic strip test and immunoradiometric kit for detection of PSA

The immunochromatographic Seratec® PSA test was performed according to the manufacturer's instructions for both the extraction and the measurement process. In brief, swabs and fabric samples were transferred to 1.5-mL tubes and extracted using 1 mL of buffer provided with the kit. The extraction time was in all cases 2 h. Approximately 120 μL (3 drops) from each sample were transferred to the sample well of the Seratec® PSA cassette and the results were read after 10 min. In case of positive sample the three test lines (test result line, internal standard and control line) appeared as red-colored zones. In case of negative samples (PSA < 1 ng/mL), only the internal standard and control line were visible.

Positive samples were also analysed with the PSA-RIACT immunoradiometric kit to quantitatively determine the PSA concentration in these samples. For this purpose, samples extracted and diluted as described in Section 2.4 were assayed following the manufacturer's instructions. Radioactivity was measured using a 12-well gamma-counter (LB 2111; Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany).

2.7. Spermatozoa and Y chromosome detection in forensic samples

Sperm cells were extracted from casework samples (swabs or piece of cloths) through incubation with a 10% (v/v) aqueous acetic acid solution for 10 min. Then, 20 μL of the extract were transferred on microscope slides and fixed by heating with an alcohol-burner. The slides were then stained by applying the hematoxylin solution for 3 min, followed by washing with doubly distilled water and 30% (v/v) ammonia solution. After that, a 1% (w/v) aqueous eosin solution was applied for 1 min and the slides were washed with water and dried. The sperm cells were observed under an optical microscope (Axio Imager 2; Carl Zeiss, Germany) equipped with a Sony Cyber-Shot digital camera.

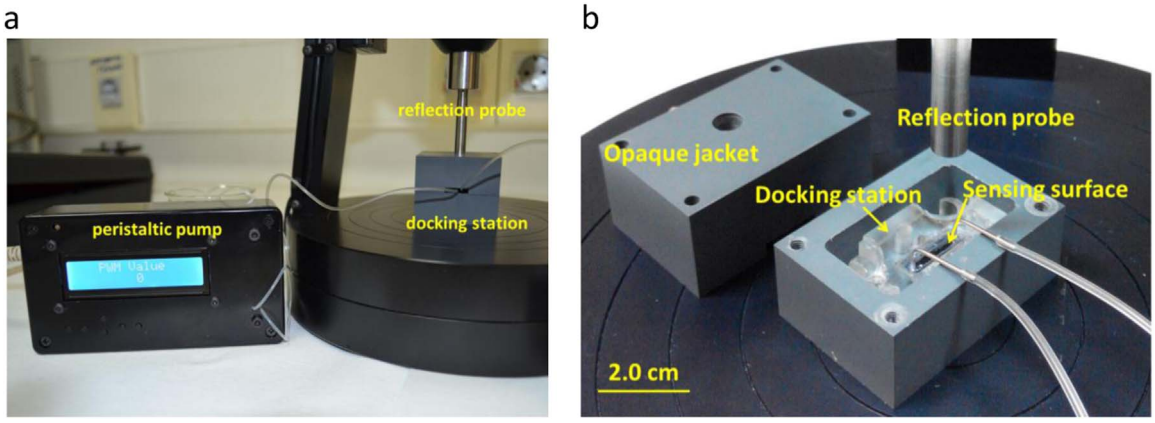

Fig. 1. Image of (a) the whole sensing system and (b) detailed view of the docking station loaded with a biochip assembled with its fluidic compartment and the reflection probe above them.

Images were processed using the ImagePro v6.0 software (Media Cybernetics, Inc.; Rockville, MD).

Sperm DNA extraction was performed using the Differex™ System following the manufacturer's instructions. The extracted sperm DNA was then quantified using the Quantifiler® Trio DNA Quantification Kit in an ABI Prism 7000 Sequence Detection System (Thermo Fisher Scientific Inc.; Waltham, MA). The kit involved the use of three primers, two for whole human genomic DNA quantification and degradation assessment, and one for Y chromosome detection and quantification.

3. Results and discussion

In a previous work, our group reported a method for the detection of free- and total-PSA in human serum samples using an immunosensor based on the WLRS detection principle [22]. The device used in that work involved a complicated cartridge and chips with surface of 4 cm^2, mainly due to cartridge and fluidic compartment design. Although in a more recent work, targeting the detection of C-reactive protein and D-dimer, the size of these components has been decreased [23], the measurements had to be performed under conditions of stable low illumination and the alignment of the biochip with the reflection probe was done manually, thus making difficult the use of the system for field testing.

In the present study further improvements of the set-up have been made to make the system more robust, and therefore appropriate for applications outside the analytical lab. Thus, a two-component milled jacket for the docking station was fabricated in grey opaque Plexiglas (Fig. 1a); the lower part of the jacket had dimensions that fitted exactly those of the docking station, whereas the upper part had an appropriately drilled hole that allowed the exact positioning of the reflection probe with respect to the biochip and eliminated all possible interferences from changes of the environment lighting. Moreover, a small size and low power peristaltic pump was employed in order to shrink down the footprint of the set-up towards a portable system suitable for at-field determinations of PSA or other markers.

3.1. Development of PSA assay

For the detection of PSA in forensic samples, a goat-polyclonal anti-PSA antibody was used both as capture and as detection antibody following a two-step immunoassay format. In order to further increase the assay sensitivity, the detection antibody was biotinylated and an additional reaction step with streptavidin was introduced, as it is schematically depicted in Fig. 2a.

The assay parameters, such as coating and blocking conditions, assay and washing buffer compositions, as well as the antibodies concentration have been optimized, and the optimum ones are mentioned in the Materials and Methods section (see Figs. S1 & S2, Supplementary material). A critical parameter studied was the kinetics of the different assay steps. In Fig. 2b, the evolution of the effective biomolecular adlayer thickness (as calculated by fitting of the reflectance spectrum) versus time obtained upon assaying a 100 ng/mL PSA calibrator is illustrated. From this graph is obvious that for the first immunoreaction at least 10 min were required in order to reach maximum signal values. The net signal (equivalent biomolecular adlayer thickness) achieved at this time was approximately 0.1 nm. Regarding the second step, namely the reaction of the detection antibody with the immunoadsorbed PSA molecules, plateau signal values were obtained after 3 min of reaction providing an additional signal increase of about 0.17 nm. The subsequent reaction with the streptavidin was completed in about 2 min and resulted in a signal increase of about 0.55 nm (a two-fold increase compared to the signal achieved by both the first and the second immunoreaction). This signal enhancement could be ascribed to the fact that streptavidin could bind to multiple sites of the biotinylated antibody, leading to a denser biomolecular layer and to higher signal. Thus, with a 15-min overall assay duration maximum signal values could be achieved. However, in an attempt to reduce the whole assay duration, shorter first step immunoreaction times were tested. It was found, that by running the PSA calibrators for 5 min, followed by 3-min reaction with biotinylated detection antibody and 2 min with streptavidin, adequate analytical sensitivity and assay dynamic range for the determination of PSA in forensic samples was achieved, despite the fact that the net specific signal was reduced by approximately 50%.

In Fig. 3a, the real time responses obtained following the above mentioned assay duration for PSA calibrators with concentrations ranging from 1 to 1000 ng/mL are presented. As shown, the first immunoreaction provides signal distinguishable from the zero calibrator signal for PSA concentrations equal to or higher than 100 ng/mL. Following the second immunoreaction the lowest detectable PSA concentration goes down to 20 ng/mL, while after reaction with streptavidin the response provided by a 1.0 ng/mL calibrator is clearly distinguished from that of zero calibrator. A typical calibration curve obtained following the described assay conditions is provided in Fig. 3b. The assay detection limit, calculated as the analyte concentration corresponding to +3 SD of 20 replicate measurements of zero calibrator (non-specific binding; NSB), was 0.5 ng/mL, while the linear dynamic range extended up to 500 ng/mL. The detection limit achieved is of the same magnitude with that reported in our previous work regarding PSA determination in human serum samples (0.2 ng/mL). Nevertheless, the assay duration was suppressed from 70 to only 10 min. This was due to the implementation of a better designed fluidic cover that allowed for faster diffusion of the molecules run over the

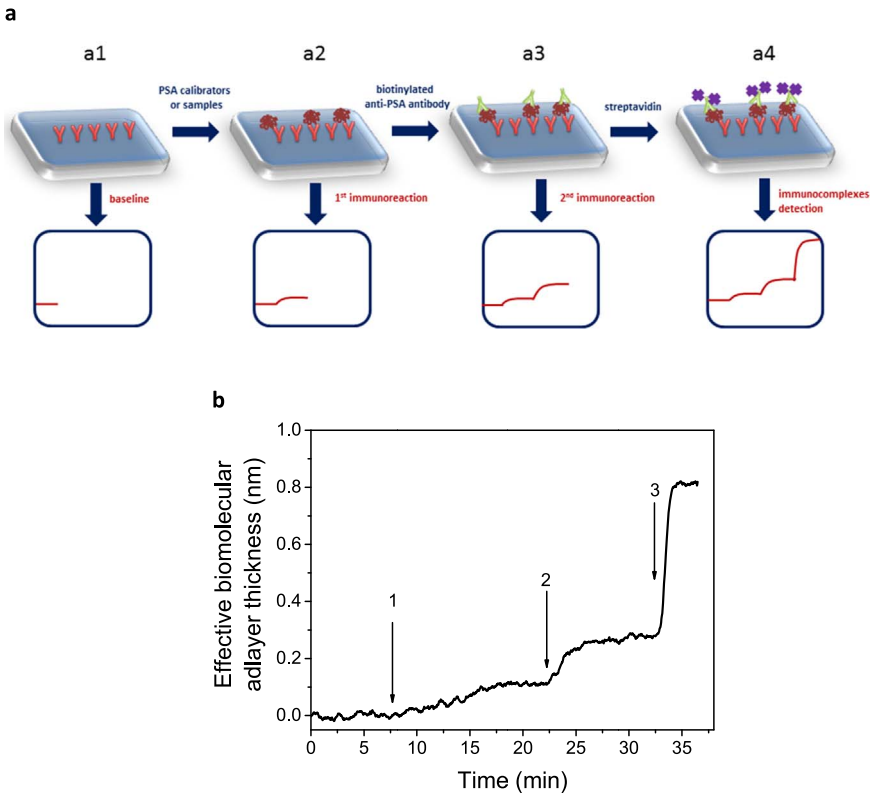

Fig. 2. (a) Schematic of the two-step non-competitive PSA immunoassay (upper panel) and expected sensor response (lower panel). (b) Signal evolution with time due to: the capture of the PSA from a 100 ng/mL calibrator by the immobilized onto biochip antibody (arrow 1–2; step a2), the biotinylated goat anti-PSA antibody binding to immunoadsorbed onto the biochip PSA (arrow 2–3; step a3), and finally streptavidin binding to immunocomplexes formed onto biochip surface through reaction with the biotinylated anti-PSA antibody (arrow 3 to end of run; step a4).

biochip surface (PSA, biotinylated anti-PSA antibody, and streptavidin) to the already bound ones, as well as to the new docking station that protected the system from the effect of extremal light and helped to standardize the measurement procedure.

3.2. Accuracy, reproducibility, stability and reusability of the sensor developed

Critical features for a system aimed for application in a demanding field such as that of forensic analysis are the high accuracy and reproducibility of the results obtained. The accuracy of PSA determination in forensic samples using the developed sensing system was determined through recovery experiments. For this purpose, one PSA-negative and two PSA-positive fabric samples were spiked with known amounts of PSA and left to completely dry for at least 24 h prior to the extraction. The PSA concentration in these samples prior to and after addition of exogenous PSA was determined with the developed sensing system. The results are presented in Table 1. The % recovery values, calculated as the ratio of amount determined to the amount expected, ranged from 89.6% to 106%, demonstrating the accuracy of the sensing system developed.

The accuracy of the developed sensor was also assessed though dilution linearity experiments. For this purpose, a semen sample was serially diluted 5000–360,000 times with assay buffer. The dilutions

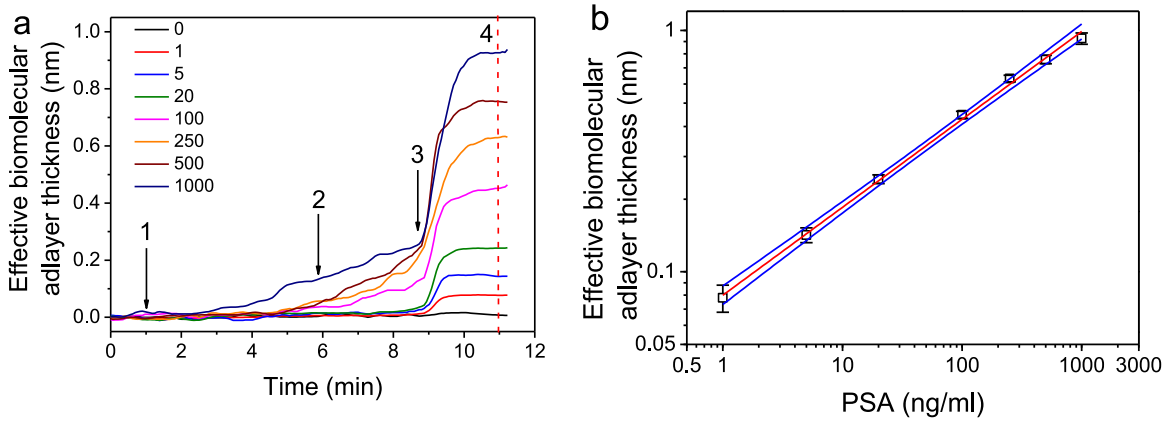

Fig. 3. (a) Effective biomolecular adlayer thickness monitored in real-time from a chip coated with goat-polyclonal anti-PSA antibody upon running: start to arrow 1: assay buffer; arrow 1–2: PSA calibrators with concentrations ranging from 0 to 1000 ng/mL in assay buffer; arrow 2–3: biotinylated anti-PSA antibody solution (10 μg/mL in assay buffer); arrow 3–4: streptavidin solution (5 μg/mL in assay buffer). The vertical red dashed line indicates the end time point of each measurement. (b). (b) Typical PSA calibration curve. Each point is the mean value of four measurements ± SD. The linear regression equation is logY=0.364(± 0.007)logX – 1.097(± 0.015), R^2=0.997. Red line corresponds to linear regression curve; Green lines correspond to ± 95% confidence limits. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Recovery.

Sample #	Amount added (ng/mL)	Amount determined (ng/mL)	Amount added determined (ng/mL)	Recovery %
1	0	0	–	
	10	10.2	10.2	102
	50	49.6	49.6	99.2
2	0	73.0	–	–
	50	126	53.0	106
	100	167	94.0	94.0
3	0	51.0	–	–
	50	95.8	44.6	89.6
	100	149	98.0	98.0

were selected so as, taken into account the mean PSA semen content, the expected values in the diluted samples to fall within the assay linear response range. As shown in Fig. 4a, where the sensor responses obtained for different semen dilutions against the dilution factor are presented, there is a linear correlation of the sensor responses for semen dilution ranging from 320,000 to 5000, which provided values in the linear working range of the calibration curve. The fact that PSA could be detected at semen dilutions as high as 320,000 signifies that the proposed system is able to detect semen quantities of less than one nanoliter. More concentrated samples (1000 to 10-times diluted) provided slightly higher values. The concentration of PSA in the undiluted semen was approximately 3 mg/mL, thus even at PSA concentrations of 300 μg/mL no high-dose hook effect was observed. This finding is attributed to the two-step assay configuration selected.

The assay reproducibility was evaluated by repetitive measurements of three semen samples appropriately diluted so as to correspond to three different levels of PSA (low, medium and high) in the range of calibration curve, both at the same day (intra-assay reproducibility), as well as at different days (inter-assay reproducibility). The intra-assay reproducibility, expressed as the % relative standard deviation (RSD) of the values determined by running each of the samples 3 times in the same day ranged from 3.1% to 7.3%. The inter-assay reproducibility, expressed as the % RSD of the measurements performed in duplicate for each one of the samples at five different days over a period of one month, ranged from 4.5% to 9.2%.

The reproducibility of chips activation and biofunctionalization procedure was also determined by running a total number of 40 biosensors prepared over a period of 2 months in batches of five. The biosensors were tested using the same semen sample and the % relative standard deviation (RSD) of the values determined was less than 7.6%. Thus, it can be claimed that the procedure involved for chip activation and biofunctionalization provided for repeatable results and conformity of the assay developed with the specifications of analytical methods aimed to the determination of biological markers.

The potential for reuse of the same biochip was also exploited as a further evaluation of sensing system robustness and good performance. In particular, 24 analysis/regeneration cycles were performed in a single biochip using a 250 ng/mL PSA calibrator and the responses obtained were shown in Fig. 4b. The mean value of the 24 measurements is indicated by the horizontal red line and the ± 1 standard deviation (SD) limits by the dashed green lines. All 24 measurements fall within ± 1SD limits, demonstrating the excellent robustness and stability of the biochip. Thus, the same biochip could be used for multiple measurements; a feature that could facilitate screening a high volume of evidence at the crime scene, decreasing considerably the analysis cost and the volume of evidence for analysis by the forensic laboratory.

3.3. Optimization of the PSA extraction procedure

The procedure for PSA extraction from semen stains on fabrics or swabs is another important issue to be considered during the development of a method for use by the forensic laboratories. The simpler method is the immersion of the sample into a buffer, preferably the one used for the assay, and direct use of the extract for the analysis without further processing. To test for the most efficient PSA extraction method clean swabs and pieces of cotton fabric were implemented. A 10-μL drop of semen was applied, and all specimens were left to dry overnight at room temperature. Then, they are immersed in 1 mL of assay buffer for 2–60 min. The eluate received was diluted 500-times with buffer and analysed by the sensing system developed. The PSA values determined for these samples were compared to those determined in the initial semen sample after 50,000-times dilution with assay buffer, so as to have the same dilution factor with the swab/fabric samples. As shown in Fig. 5a, almost 100% recovery was achieved for both swabs and fabrics after 10 min incubation in the assay buffer. This finding could be helpful for fast analysis of samples at the crime scene. However, in our study a 20-min extraction time was adopted for the analysis of the forensic samples in order to ensure high recovery of PSA for all types of samples. This extraction time is 6-times shorter than that recommended for extraction of samples prior to analysis by PSA immunochromatographic strip, which is commonly employed in the forensic laboratories, in order to have the higher possible detection

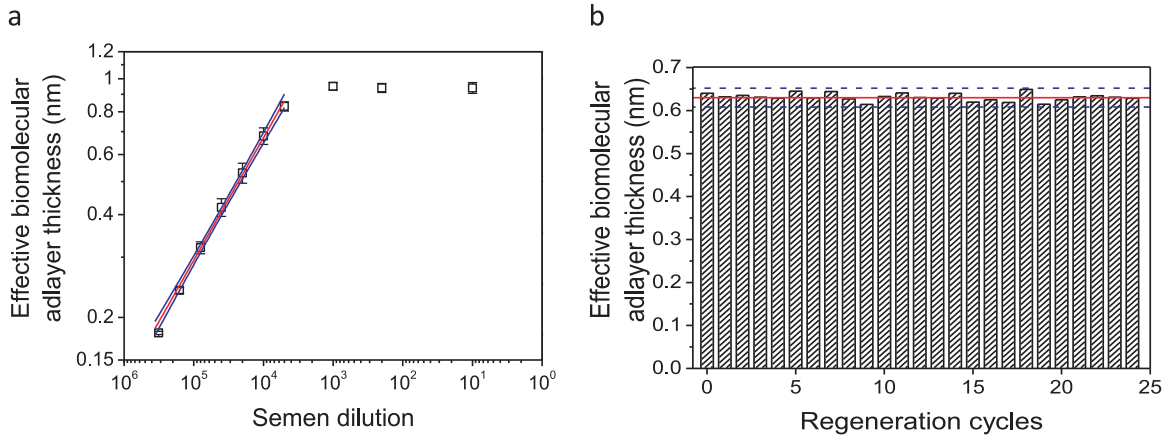

Fig. 4. (a) Effective biomolecular adlayer thickness values determined for the PSA assay when running semen diluted 5000–360,000 times with assay buffer versus the dilution factor applied. Each point is the mean value of four measurements ± SD. The linear regression curve equation is logy = 0.359(± 0.007)logx+1.26(± 0.03), R^2=0.997, p < 0.001. Red line corresponds to linear regression curve; Green lines represent the 95% confidence limits. (b) Responses obtained from a single chip functionalized with anti- PSA antibody through 24 consecutive assay/regeneration cycles performed in a single day. A 250 ng/mL PSA calibrator was used in all runs. Red line corresponds to the mean of the 24 measurements and dashed green lines to mean ± 1SD limits. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

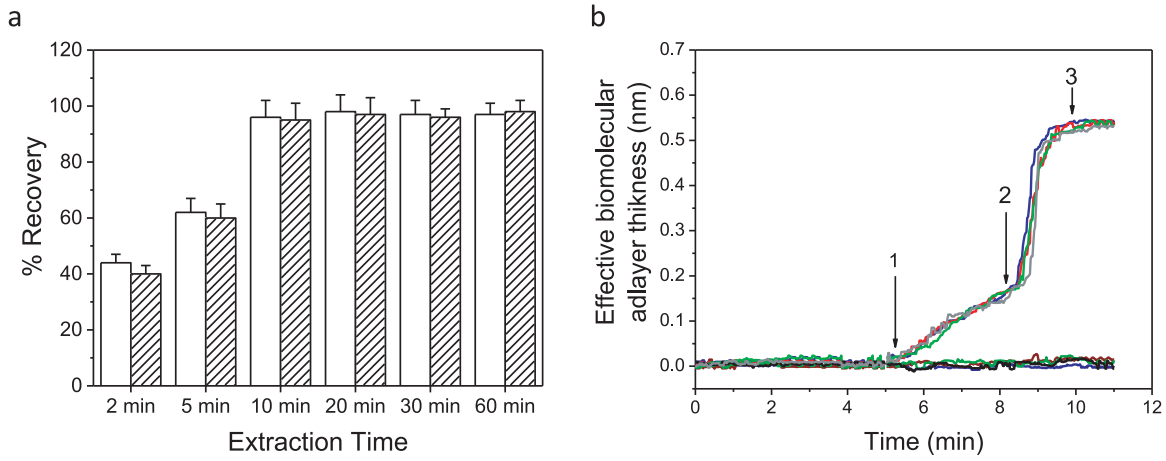

Fig. 5. (a) Percent PSA recovery values determined from swab (white columns) or fabric samples (striped columns) spiked with diluted semen versus the incubation time with the assay solution. Each value is the mean of four determinations ± SD. (b) Real-time signal responses obtained when running: assay buffer without semen (dark blue line), assay buffer spiked with semen (light blue line), blood without semen (dark red line), blood spiked with semen (light red line), urine without semen (dark green line), urine spiked with semen (light green line), saliva without semen (black line), and saliva spiked with semen (grey line). The sequence of solutions run are as follows: start to arrow 1: semen spiked or non-spiked sample 100-diluted with assay buffer; arrow 1–2: biotinylated anti-PSA antibody solution (10 μg/mL in assay buffer); arrow 2–3: streptavidin solution (5 μg/mL in assay buffer); and arrow 3 to end: assay buffer. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sensitivity.

3.4. Interference from other body fluids

Forensic samples collected from crime scenes and especially alleged rape cases might contain mixtures of biological fluids (semen, blood, urine, saliva) that could affect the detection of PSA. Therefore, the effect of the presence of other biological fluids, like blood, urine and saliva, to the determination of semen PSA with the reported sensing system was evaluated by spiking a blood, urine, or saliva sample with semen so as to prepare samples containing 10% (v/v) semen. Ten microliters of the spiked samples were spotted on cotton fabric and left to dry. An area containing the whole stain (approximately 0.5 cm^2) was cut out and extracted using 200 μL of assay buffer. The extract was further diluted 100-times with assay buffer and assayed using the sensor developed. The responses obtained were compared with those received by net blood, urine or saliva samples prepared following the same procedure, as well as with those received by a 10% (v/v) semen sample prepared in blood, urine, or saliva, respectively. As shown in Fig. 5b, the responses obtained when running extracted blood, urine or saliva to which no semen was added were very low and comparable with those obtained when running assay buffer. On the other hand, the responses obtained by the respective samples containing 10% (v/v) semen were similar to that received for the 10% (v/v) semen sample in assay buffer. These results clearly demonstrate the absence of any interference from extracted blood, urine or saliva and the ability of the system developed to determine PSA in presence of other biological fluids.

3.5. Analysis of forensic casework samples

Fifty one forensic casework samples collected by 29 alleged sexual assault scenes or related evidence were examined for the presence of semen through determination of PSA with the system developed. Forty seven out of the 51 samples refer to rape cases, 3 to homicides, and 1 to kidnapping. Examined samples included vaginal and/or anal swabs, stains in fabrics including mainly underwear and regular clothes as well as bed sheets. Moreover, in several of these samples additional biological materials, like blood, saliva, urine, menstruation blood, anus wastes, etc., were present.

All samples were run on the sensor after extraction following the procedure described in Section 3.3. The presence or absence of semen in the samples was also confirmed by analysis with the Seratec® PSA membrane strip test that is used from the majority of forensic laboratories for PSA detection, as well as by microscopic detection of spermatozoa and male DNA identification through detection of Y chromosome. The positive samples were furthermore assayed using an immunoradiometric kit for the determination of PSA in human serum so as to compare the results of the proposed method on a quantitative basis. The sensor response during analysis of a positive fabric sample along with a microscope image showing the detected spermatozoa are provided in Fig. S3. Forty three out of the 51 samples were found negative for PSA by the sensor developed, the strip PSA test, spermatozoa and Y chromosome detection (Table S1, Supplementary material). Only 8 samples were found positive by all methods. For these samples, the PSA concentrations determined by the sensor developed along with the values determined by the immunoradiometric assay kit are provided in Table 2. As shown, the PSA values determined with the sensor developed are in good agreement with those determined with the immunoradiometric assay kit. These findings further support the accuracy of the system developed with respect to determination of PSA for semen detection in forensic evidence samples.

As it has been stressed out in a recent review [25], concerning the development of PoC devices, the analytical accuracy is a major and indispensable feature for any system that foresees to enter this very competitive market. Nevertheless, other aspects should be also taken into account when an actual product and not a laboratory prototype is envisioned. The system size and robustness is of outmost importance. Both these aspects have been taken into account in the design of the next generation system that will be built so as to accommodate all the

Table 2
PSA concentrations determined by the sensor developed and an immunoradiometric assay kit in 8 extracted and 100-times diluted forensic samples found positive by Seratec® PSA test, spermatozoa and Y chromosome detection.

Sample#	Case	Sample type	WLRS (ng/mL PSA)	RIA kit (ng/mL)
2	Rape	stain on bedsheet	80	83
3	Rape	stain on bedsheet	112	110
4	Rape	stain on bedsheet	73	71
10	Rape	stain on skirt	36	35
15	Rape	stain on shirt	51	54
40	Rape	stain on trousers	115	119
43	Rape	vaginal swab	6.6	6.1
44	Rape	stain on underwear	140	136

optical components and the pumps needed for the fluids circulation in an instrument with a footprint smaller than an A4 page. In addition, the reagents required for the performance of the assay (e.g., assay buffer for equilibration, streptavidin solution, etc.) will be incorporated onto an appropriately designed single-use cartridge that will ensure minimum interaction of the permanent fluidic circuit with the sample in order to avoid cross-contamination. The form of the stored reagents (liquid or dried) and their self-life should be also investigated since it will define to a great degree the design and size of the cartridge. The biochip will be part of this cartridge and the user will have to load the sample and select the protocol from the device menu. The device accompanying software is also to be improved, through the inclusion of pre-determined calibration curves and algorithms for the calculation of PSA concentration in each particular sample. Different data transfer capabilities should be also considered, taking into account the particularities of the forensics, e.g., who will have access to the results in order not to jeopardize the evidence credibility."

4. Conclusions

The detection of PSA as semen indicator in forensic casework samples using a biosensor based on white light reflectance spectroscopy has been demonstrated. The assay is completed in 10 min and the detection limit achieved was 0.5 ng/mL of PSA. Thus, compared to immunochromatographic membrane strip tests, the biosensor developed requires the same amount of time, is more sensitive, provides quantitative determinations of PSA and it is high-dose hook effect free. In addition, the measurements performed with the developed biosensor were accurate and reproducible, while no interference by other body fluids, including blood, urine and saliva, was detected. The results of the analysis of 51 forensic casework samples with the developed sensor were in agreement with those provided by a semi-quantitative membrane strip test (Seratec® PSA). No false-negative or false-positive results were observed, while the concentration of PSA in the positive samples was well correlated with those determined in the same samples by a commercial immunoradiometric assay kit. Taking it overall, the excellent analytical performance and small size of the instrument make the sensor developed an attractive tool for use in forensic evidence screening for semen detection.

Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.talanta.2017.07.074.

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