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Ethinylestradiol quantification in drinking water sources using a fluorescent paper based inmunosensor

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Abstract

In this work we report a novel paper-based analytical device read-out via LED-induced fluorescence detection (FPAD) for the quantification of the emerging pollutant ethinylestradiol (EE2) in river water samples. The PAD was used as a reaction platform for a competitive enzyme immunoassay. For the PAD development, microzones of filter paper, printed by a wax printing method, were modified with amino-functionalized SBA-15 and subsequently, anti-EE2 specific antibodies were covalently immobilized. The determination of EE2 in water was carried out by adding a fixed concentration of EE2 conjugated with the enzyme horseradish peroxidase (HRP) to samples and standards. Then, the FPAD were added and incubated for 10 min. Finally, the detection was performed by the reaction of 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) whose oxidation is catalyzed by HRP in the presence of H_2O_2, obtaining the highly fluorescent resorufin (R). Resorufin was detected by LED excitation at 550 nm, observing emission at 585 nm. The EE2 concentration in the samples was inversely proportional to the relative fluorescence obtained from the enzymatic reaction products. The FPAD assay showed a detection limit (LOD) of 0.05 ng L^{-1} and coefficients of variation (CV) below 4.5 % within-assay and below 6.5 % between-assay, respectively. The results obtained show the potential suitability of our FPAD for the selective and sensitive quantification of EE2 in river water samples. In addition, it has the PADs advantages of being disposable, easy to apply and inexpensive.

Keywords: Ethinylestradiol, LED-induced fluorescence, paper based devices, amino-functionalized mesoporous silica particles

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1. Introduction

Emerging pollutants are residues of human activity such as personal care, healthcare, and industrial operations. There is no regulation on their presence and levels in the environment. Among them, there has been growing interest in endocrine disrupting compounds (EDCs). When EDCs are introduced into the aquatic environment they possess the ability to disrupt the endocrine system of animals by altering the natural balance of hormones [1]. The monitoring of these compounds is important since it has been shown that the exposure to EDCs, even at low concentrations, is related to some health problems: infertility, increase in tumor incidence, reproductive alterations and feminization in aquatic organisms [2, 3].

Ethinylestradiol (EE2) is a synthetic hormone with high estrogenic potency, and is one of the main components of oral contraceptives. EE2 has become an emerging pollutant in aquatic ecosystems because it is resistant to degradation by liver, therefore EE2 and its derivatives are introduced into the environment via wastewater. EE2 has a longer half-life than natural estrogens. In addition, EE2 has the tendency to accumulate in sediment and biota [4] where it is still endocrinally active, hence, EE2 is considered an EDC [5].

EE2 represents a risk as an EDC even at very low concentrations. Some studies proved its presence in water in the range of ng/L [6, 7]. EE2 has been quantified in water samples using standard instrumental methods such as gas chromatography–mass spectrometry or high performance liquid chromatography–mass spectrometry, often after pre-concentration by solid-phase extraction [8-10]. These methods achieve very low detection limits and high specificity. However, they are time-consuming, need large sample volumes and expensive equipment. In addition, they require an extensive sample clean-up and purification steps. For this reason, other strategies have been developed to determine estrogenic hormones in complex matrices. Among them, the competitive ELISA [11] is an interesting option.

As it is known, enzyme-linked immunosorbent assay (ELISA) is a technique that combines the specificity of antibodies with a high enzymatic activity to provide a specific and sensitive quantification method [12]. ELISA is extensively used in the development of analytical methods applied to clinical diagnosis [13], environmental monitoring [14, 15] and food testing [16]. There is a need to have rapid, selective and

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easy-to-use methods. In order to reach these requirements, paper-based ELISA (P-ELISA) methods have been developed as an interesting alternative to conventional ELISA. P-ELISA was firstly proposed by Cheng et al [17] and has provided a novel strategy for the detection of a wide variety of analytes with the combination of the advantages of ELISA and paper-based devices.

Paper-based analytical devices (PADs) have been widely exploited because paper surface is an inexpensive assay platform. Martinez et al. [18] proposed a method for hydrophobic patterning of paper and introduced paper as a microfluidic device platform. In the follow-up, this kind of supporting material caught attention in different fields for the development of methods to quantify several analytes by PADs [19]. Furthermore, the use of paper as support material generates a biocompatible and disposable analytical tool with small sample and reagent consumption, and simple operation [18, 20].

The immobilization surface area is higher in a PAD than in conventional ELISA wells due to the fact that in PADs the antibodies' immobilization is performed onto cellulose fibers. Moreover, this area can be enlarged with nanomaterials' incorporation. Among nanomaterials, mesoporous silica particles, especially Santa Barbara Amorphous type material, e.g. SBA-15, have become an attractive option due to their biocompatibility, large surface areas, and stable and organized mesoporous structures [21]. Besides, the hybrid functionalized mesoporous materials that can be easily obtained with specific organic functions have attracted attention for hosting a great variety of molecules [22]. The use of these materials has extended to fields such as drug delivery [23], biosensors [24], and immunosensors [25].

The most commonly used detection system for ELISA is the colorimetric one. In order to improve the analytical figures of merit of immunoassay detection systems, various detection principles have been applied such as chemiluminescence [26], electrochemistry [27], surface plasmon resonance [28], Raman scattering spectroscopy [29], and fluorescence spectroscopy [30]. Among them, fluorescence is a good option due to its high detection sensitivity and fast response times [31, 32]. In addition to these advantages, fluorescence based on excitation by light emitting diodes (LED) has been widely used because of its low cost [33]. Although the LED beam is not as good as a laser, this drawback can be remediated with a previous pre-concentration step [34]. Furthermore, LEDs have long lifetime, and they are stable and easy to use [35].

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The aim of this study was the development of a paper-based competitive enzyme immunoassay (EIA) with read-out by LED-induced fluorescence detection for quantitative determination of EE2 in river water samples. In order to obtain the PADs, SBA-15 and anti-EE2 antibodies (anti-EE2-Ab) were covalently immobilized onto paper microzones obtained by wax printing. These PADs were suspended in standards and samples, in which previously a fixed concentration of HRP-conjugated EE2 was added. After incubation, fluorescence detection was carried out. HRP in the presence of H_2O_2 catalyzes the oxidation of 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) to highly fluorescent resorufin, which was measured using an excitation wavelength of 550 nm and recording emission at 585 nm. The relative fluorescence of the enzymatic reaction products was inversely proportional to the EE2 concentration. The proposed FPAD combines the selectivity of immunochemical recognition, the increase of the modification surface by the SBA-15 incorporation and the PADs advantages to obtain a novel reaction platform. According to the obtained results, the developed FPAD has a potential suitability for sensitive and selective quantification of EE2 in water samples.

2. Experimental

2.1. Reagents and solutions

All reagents were of analytical or biochemical grade. Ethinylestradiol and Whatman paper #1 (WCP#1) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Polyclonal rabbit anti-EE2-Ab (SA 2150) and enzyme conjugate (EE2 conjugated with horseradish peroxidase (HRP-EE2)) [36] were supplied by BAM Federal Institute for Materials Research and Testing, Berlin, Germany. Glutaraldehyde (25% aqueous solution) and hydrogen peroxide (H_2O_2) were purchased from Merck, Darmstadt, Germany. Pluronic P123, (3-Aminopropyl)trimethoxysilane (APTMS), Polyvinyl alcohol 47 kDa (PVA), graphite and ADHP were purchased from Sigma Aldrich, Argentina. Tetraethyl orthosilicate (TEOS, 98%) was from Fluka. All other reagents and solvents employed were of analytical grade, they were used without further purification and the presence of EE2 was not detected in the working range. All solutions were prepared with ultra-high quality water obtained from a Barnstead Easy pure RF compact ultrapure water system. The analyzed water samples were obtained from rivers of San Luis State (Trapiche and Potrero de los Funes rivers) which are sources for drinking water treatment plants. Also, EE2 was determined in tap water of

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the cities San Luis and Potrero de los Funes, as well as bottled water and ultrapure water.

2.2. Instrumentation

The paper microzones were printed using a ColorQube 8570 wax printer. The oxidation of the paper surface was carried out by oxygen plasma cleaner (Plasma Technology PLAB SE80 plasma cleaner). The morphology of SBA-15 modified paper microzones was studied by a LEO 1450VP scanning electron microscope (SEM). Energy dispersive x-ray spectroscopy (EDS) on the scanning electron microscope was used for the analysis of the elemental composition of the modified surfaces. Nitrogen adsorption–desorption isotherms at 77 K were measured by using a Micromeritics ASAP 2020 equipment. The infrared spectroscopy characterization studies were performed using a Nicolet Magna 560 instrument.

The fluorescence measurements were carried out by a LED-based excitation system which was constructed with a visible LED Series light source (LLS, Ocean Optics, USA). A 2 in 1 optical fiber assembly (Premium-grade Bifurcated Fibers 600 µm-UV/VIS, Ocean Optics, USA) was used. The fluorescence emission was filtered by a 570 nm long-pass filter and the fiber-optic measurements were detected by a QE65000-FL scientific-grade spectrometer (Ocean Optics, USA). This fluorescence detection system was assembled inside a box to eliminate ambient light.

2.3. SBA-15 synthesis and functionalization

The SBA-15 was synthesized following previous work [37]. Briefly, 12.6 g of Pluronic P123 were dissolved in 89.6 g of water at 38 °C under continuous stirring. Then, 359.6 g of a 2 M HCl solution and 25.6 g of TEOS were added at 35 °C, and the mixture was allowed to react while stirring for 20 h. Later, it was incubated at 90°C without stirring for another 24 h. After this time of aging, the gel obtained was filtered and washed with distilled water and finally with ethanol. The precipitate was dried at 60 °C for 3 h and was calcined by a heating ramp of 1 °C/min and then left for 6 h at 500 °C.

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The SBA-15 sample obtained was functionalized with APTMS in order to obtain an amino-functionalized hybrid material. The process was carried out using toluene as the reaction medium. 150 mg of the obtained SBA-15 were added to 20 mL of dry toluene. When the SBA-15 was dispersed, 180 µL of APTMS were added and the mixture was allowed to react for 24 h with constant stirring at room temperature. Then, the solid was filtered, and washed with toluene and ethanol to later be dried at 60 °C for 24 h. Finally, the product was heated at 130 °C for 48 h in order to stabilize the functionalization of the SBA-15 (SBA-15-N).

The obtained SBA-15 and SBA-15-N were characterized by SEM, nitrogen adsorption–desorption and FTIR.

2.4. Fabrication and modification of paper microzones

Firstly, to obtain the paper microzones, a 5 mm diameter circular shape was drawn using CorelDraw software version 11.0. Then, the microzones were printed onto filter paper surfaces by a ColorQube 8570 wax printer. Finally, a hydrophobic barrier was generated by exposing the printed design to heat, which caused the wax to penetrate the paper.

PVA has a good compatibility with inorganic materials. The hydroxyl groups present in the polymeric matrix form strong interactions with the residual silanol groups in the SBA-15 [38]. Furthermore, PVA is expected to interact with the hydrophilic surface of the paper, forming hydrogen bonds. Due to these features, PVA was chosen as a material for fixating SBA-15-N on the paper surface. A solution of 0.1% PVA and 0.5% SBA-15-N was prepared and 15 µL of this solution were added to the paper microzones and left to dry under a heating lamp (Fig. 1a). When they had dried, the polymer was crosslinked for 2 h using a 5% glutaraldehyde solution in acetone and acid medium. After this time, the paper microzones were washed with bi-distilled water until neutral and dried with a blotting paper. Next, 15 µL of glutaraldehyde, pH 10, were added on the paper microzones to activate the amino groups of the SBA-15-N, and allowed to react for 90 min. Later, 5 µL of a 1:100 solution of anti-EE2-Ab were placed and the paper microzones and incubated for 30 min at room temperature (Fig. 1b), followed by a wash with phosphate-buffered saline solution (PBS) (0.01 M, pH 7.2) to remove excess antibody. Non-specific binding was blocked by 1% of bovine serum

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album (BSA) in PBS (Fig. 1c). Finally, the microzones were washed three times with PBS.

The modified paper surface was characterized by SEM and EDS. The immobilization surface increases, due to the SBA-15-N incorporation, were studied modifying three different paper microzones with HRP. The first one was an unmodified paper, the second one was previously oxidized with plasma (which generates aldehyde groups in the cellulose of the paper to which the amino groups of the enzyme bind covalently [39]), and the third one was modified with SBA-15-N. Later, the fluorescence of the reaction products of the ADPH oxidation in presence of H_2O_2 was measured by LED-induced fluorescence.

2.5. Sample preparation

For EE2 quantification in river water samples by the developed FPAD, these were passed through filter papers (Whatman) and by vacuum through 934-AHTM RTU glass microfiber filters (Whatman), following the work of Schneider et al. [9]. Finally, the samples were adjusted to pH 7 using 0.1 M phosphate buffer.

The EE2 determination was carried out by a competitive EIA. For this reason, enzyme conjugate was added to standards and samples in a fixed concentration in order to obtain a final dilution of 1:10000.

2.6. EE2 determination

Steps d) and e) in Fig. 1 are schematic representations of the process carried out by the FPAD for the determination of EE2 in water samples. Briefly, a modified paper microzone was placed into 10 mL of conditioned sample and it was left while stirring for 10 min. In this step, the EE2 present in the sample competes with the HRP-EE2 for the binding sites of the immobilized antibodies. Then, the paper microzone was washed three times with PBS and dried. For the relative fluorescence (RF) measurement, 5 µL of substrate solution were added to the modified microzone. The substrate solution was prepared from a stock solution of ADHP (10 mM in dimethyl sulfoxide (DMSO)), which was diluted to 1 mM with 0.1 M phosphate–citrate buffer (pH 5.05) and H_2O_2 with a concentration of 0.1% was added. The HRP of the conjugated EE2 in the

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presence of H_2O_2 catalyzes the oxidation of ADHP to R which was measured using excitation at 550 nm and emission at 585 nm. Therefore, the relative fluorescence obtained was inversely proportional to the EE2 concentration in the sample.

3. Results and discussion

3.1. SBA-15 and SBA-15-N characterization

The SEM image (Fig. 2a) confirms obtaining elongated particles (500 ± 70 nm length and 400 ± 50 nm width) with a high mesoscale order due to channel-like structures. Also, in order to confirm the SBA-15 obtaining the nitrogen adsorption isotherms at 77 K were performed for SBA-15 and SBA-15-N samples. Isotherms type IV with H1 hysteresis loops were obtained, which is in accordance with the behavior studied for SBA-15 [37]. Table 1 shows the BET surface area and pore diameter obtained by the BJH method (isotherms and pore diameter distribution are provided as Supplementary Material). SBA-15-N exhibited a reduction in these properties compared to SBA-15 due to the presence of the organic agent.

FTIR measurements were performed to characterize the SBA-15 and SBA-15-N. Fig. 2b shows the characteristic bands of the inorganic framework Si–O–Si at 800 cm^{-1} and in the 950–1300 cm^{-1} zone, for both materials [40]. The absorbance of the Si–O–H bands at 960 cm^{-1} SBA-15 turns into a shoulder when the material is functionalized, indicating the presence of aminosilane groups in SAB-15-N. The modified material showed an increase in the signal at the ranges of 2800-3000 cm^{-1} and 3000-3500 cm^{-1} attributed to the vibrations -C-H and stretch N-H superimposed on the water bands, respectively. This confirms the presence of organic groups in SBA-15. However, the most relevant signal in the spectrum of SBA-15-N is the band corresponding to the asymmetric flexion of N-H + at 1560 cm^{-1}. These large N-H vibrations are typical of N-containing species with a strong H-binding interaction with the surface of the pores [41].

3.2. Characterization of the modified paper microzones

The SBA-15 was incorporated to the PAD in order to increase the immobilization surface. Fig. 3a shows a typical SEM micrograph in which SBA-15-N

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particle aggregates deposited on paper fibers can be observed. Fig. 3b shows the EDS spectrum obtained for the samples modified with the mesoporous nanoparticles. The elemental composition was analyzed along the samples and with different magnifications, demonstrating a regular presence of Si with its corresponding peak at 1.74 keV. Hence, these data confirm that the immobilization was successful, and that the SBA-15 N loading was even along the samples.

3.3. SBA-15 incorporation effect on the obtained signal

The amplification effect of the obtained signal due to the SBA-15 incorporation was evaluated. For this purpose, a 1 mM ADHP + 1μL mL^{-1} H_2O_2 solution was added to three paper microzones with HRP immobilized: one was an unmodified paper microzone; other was an oxidized paper microzone; the last one with SBA-15-N incorporated. The relative fluorescence of the enzymatic response were measured and Fig. 4a represents the obtained signals. As can be observed, the obtained signal of the enzymatic response was higher when the PAD was modified with SBA-15-N.

3.4. Parameter optimization

For a good performance of the FPAD several parameters were optimized. One of the important parameters is the anti-EE2-Ab concentration used for the immobilization. It was studied in a range of 1:2500 to 1:50. The immobilization was carried out as described in section 2.4. Fig. 4b shows that the relative fluorescence obtained increased with the anti-EE2-Ab concentration to a value of 1:100 from which it remained constant. Hence, a 1:100 concentration of anti-EE2-Ab was considered as the optimal value.

The incubation time was also optimized. Several assays were carried out with different incubation times, in a range between 5 and 30 min. It was observed that for times longer than 10 min, there was no significant increase in the amount of EE2 bound. For this reason, the incubation time used was 10 min.

Finally, the time of the fluorescence measurement was studied. This refers to the waiting time between adding the substrate solution and reading the relative fluorescence obtained. As can be observed in Fig. 4c, when the substrate solution was added, the RF

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increased but after 5 min a constant RF value was obtained. Thus, all the fluorescence measurements were obtained at 5 min.

3.5. Determination of EE2

The FPAD was developed for the quantitative determination of EE2 in water samples. Firstly, a calibration curve was obtained within the concentration range 0-100 ng L^{-1} of EE2 standard solutions. The linear regression equation was RF = 10274.14 – 84.43 x C_{EE2} with a correlation coefficient r = 0.998. The limit of detection (LOD) obtained was 0.05 ng L^{-1}, considering LOD as the concentration that gives a signal 3 times the standard deviation of the blank above its signal.

The precision of the method was evaluated by applying the FPAD to EE2 standard solutions of 10, 60 and 120 ng L^{-1} concentration. The measurements were repeated 5 times a day and the series of analyses was repeated on 3 consecutive days. The FPAD assay showed a relative standard deviation (RSD) with intra-day values that were below 4.39 %, and the inter-day values below 5.74 %, respectively (Table 2). The selectivity of the anti-EE2 antibodies used in this work was evaluated by Schneider et al. [42]. They tested the cross-reactivity (CR) of estradiol, several metabolites of estradiol and ethinylestradiol as well as the immunogen and a synthetic precursor of the immunogen with the EE2 antiserum. The antiserum proved to be very specific for EE2 and only low cross-reactivities were observed (CR<0.5%) for most of the compounds studied.

The EE2 quantitative determination in river water samples by this new method, also has a short total assay time. The time achieved was shorter than 20 min.

3.6. Application to real water samples

The developed FPAD was applied for the EE2 quantification in natural and spiked river water samples. The reliability of the proposed method was studied, data is summarized in Table 3. It can be observed that the recovery values obtained from spiked samples ranged from 98.5% to 103.4%. Therefore, the accuracy of the FPAD is appropriate for the quantitative detection of EE2 in river water samples.

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Furthermore, the FPAD was compared with a spectrophotometric ELISA for the EE2 quantification in water samples [11]. Fig. 5 shows the correlation between both methods and a slope close to 1 was obtained which indicates a good correlation between the developed FPAD method and the ELISA.

According to our search, paper based analytical devices has been used for water analysis. Almeida et al. [43] had summarized the application of microfluidic PADs for the determination of nutrients, heavy metals, organic contaminants, such as pesticides and polycyclic aromatic hydrocarbons, and microorganisms in water samples. Despite these studies, the quantification of endocrine disrupting compounds in water samples by PADs has not been described. Recently, our group reported an electrochemical paper-based immunocapture assay (EPIA) applied to the EE2 quantification in water samples [44]. In the present work, we used the known benefits of the paper-based immunocapture assay to improve the traditional competitive EIA. Table 4 shows a comparison of this work and reported immunoassays for EE2 determination. Coille et al. [45] proposed two different fluorescence immunoassay methods for EE2 determination. One was based on total internal reflection fluorescence (TIRF) and the other was a competitive homogeneous energy transfer immunoassay (ETIA). Kanso et al. [46] developed a chemiluminescence immunoassay based on the derivatization of the estrogen to biotinylated ethinylestradiol. Compared to these methods, the present FPAD has several advantages, such as a lower detection limit and a simpler methodology.

4. Conclusion

This article describes the development of a novel paper-based platform for a competitive EIA, which was applied for EE2 quantitative determination in river water samples. The developed FPAD combines the specificity of immunochemical binding, the increase of the immobilization surface due to the incorporation of amino-functionalized SBA-15 and the high sensitivity of fluorescence detection methods with the inherent advantages of PADs, such as the possibility to obtain disposable, easy-to-apply and inexpensive devices. According to the results obtained, our FPAD achieved a selective, quantification of EE2 with good precision, accuracy and LOD. Furthermore, this method has a great potential to be applied for water samples with only a

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conditioning step of the sample and immersing the PAD in the solution, so a more laborious and expensive separation technique is not necessary.

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Figure captions

Figure 1. Schematic representation of the covalent immobilization process and EE2 determination by FPAD. The paper microzones were modified with PVA+SBA-15-N (a). The amino groups of the SBA-15 were later activated by glutaraldehye. The paper microzones were incubated with an anti-EE2-Ab solution (b). Non-specific binding was blocked with 1% BSA solution (c). Modified paper microzones were introduced into the conditioned water sample for the capture and pre-concentration of EE2 (d). Then, a 1 mM ADHP + 1μL mL^{-1} H_2O_2 solution was added and the RF of the resorufin (R) resulting from the enzymatic reaction was measured (e).

Figure 2. (a) SEM image obtained for SBA-15 and (b) FT-IR spectral of SBA-15 and SBA-15-N.

Figure 3. Characterization of the paper microzones modified with SBA-15-N. (a) SEM image, (b) EDS spectrum.

Figure 4. (a) Relative fluorescence of the enzymatic ADHP substrate turnover of HRP immobilized on an unmodified paper microzone, an oxidized paper microzone and one with SBA-15-N incorporated. (b) Relative fluorescence obtained as a function of the anti-EE2-Ab concentration studied for two EE2 standard solution (100 and 50 ng L^{-1}). (c) Study of the fluorescence measurement time using 100, 50 and 5 ng L^{-1} EE2 standard solution.

Figure 5. Correlation between proposed immunosensor and spectrophotometric ELISA for the EE2 quantification.

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Table 1. Surface area and pore diameter obtained for SBA-15 and SBA-15-N.

Sample	Surface area (m^2/g)	Pore diameter (nm)
SBA-15	473.1	5.2
SBA-15-N	220.8	4.6

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Table 2. Intra-day and inter-day precision for the developed FPAD.

Control sample	Intra-day test		Inter-day test	
	Concentration measured ± SD	**RSD (%)**	**Concentration measured ± SD**	**RSD (%)**
5 ng L^{-1}	5.07 ± 0.17	3.34	5.12 ± 0.24	4.81
50 ng L^{-1}	50.65 ± 2.22	4.39	49.83 ± 1.77	3.56
100 ng L^{-1}	98.84 ± 2.80	2.83	102.21 ± 5.86	5.74

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Table 3. Analytical results for EE2 in natural and spiked water samples.

Sample	EE2 ($ng L^{-1}$)		RSD (%)	Recovery (%)
	Added	Found [a]		
River water (Potrero de los Funes, San Luis, Argentina)	–	8.84 ± 0.21	2.37	–
	5.0	14.01 ± 0.47	3.35	103.4
	10.0	19.16 ± 0.64	3.34	103.2
River water (Trapiche, San Luis, Argentina)	–	7.57 ± 0.33	4.36	–
	5.0	12.69 ± 0.37	2.91	102.4
	10.0	17.42 ± 0.63	3.62	98.5
Tap water (Potrero de los Funes, San Luis, Argentina)	–	2.51 ± 0.09	3.58	–
	5.0	7.64 ± 0.33	4.31	102.6
	10.0	12.38 ± 0.45	3.63	98.7
Tap water (San Luis, San Luis, Argentina)	–	1.75 ± 0.05	2.86	–
	5.0	6.91 ± 0.21	3.04	103.2
	10.0	11.97 ± 0.44	3.80	102.2
Bottled water (San Luis, Argentina)	–	1.13 ± 0.04	3.54	–
	5.0	6.28 ± 0.17	2.71	103.0
	10.0	11.05 ± 0.44	3.80	99.2

[a] Mean of six determinations ± SD

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Table 4. Comparison of immunoassays for determination of EE2

Method	LOD (ng L^{-1})	Linear range (ng L^{-1})	Reference
Chemiluminescence EIA	0.2	0.8-100	[11]
Fluorescence immunoassay based on total internal reflection fluorescence (TIRF)	70	60-1.839 x10^4	[45]
Competitive homogeneous energy transfer immunoassay (ETIA)	10	40-2x10^5	[45]
Chemiluminescence immunoassays	1.2	1.2-50	[46]
Bioseparation procedure coupled with electrochemical detection	0.01	0.02-70	[47]
Paper-based immunocapture assay (EPIA)	0.1	0.5-120	[44]
Fluorescence paper-based immunosensor	0.05	0.1-100	This paper

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Figure 1

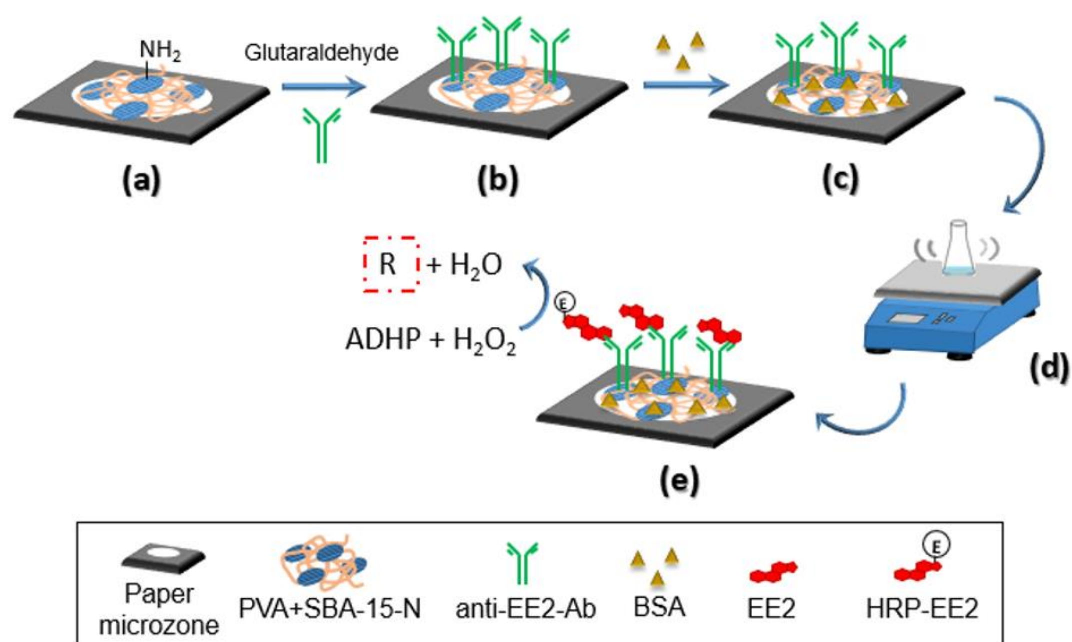

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Figure 2

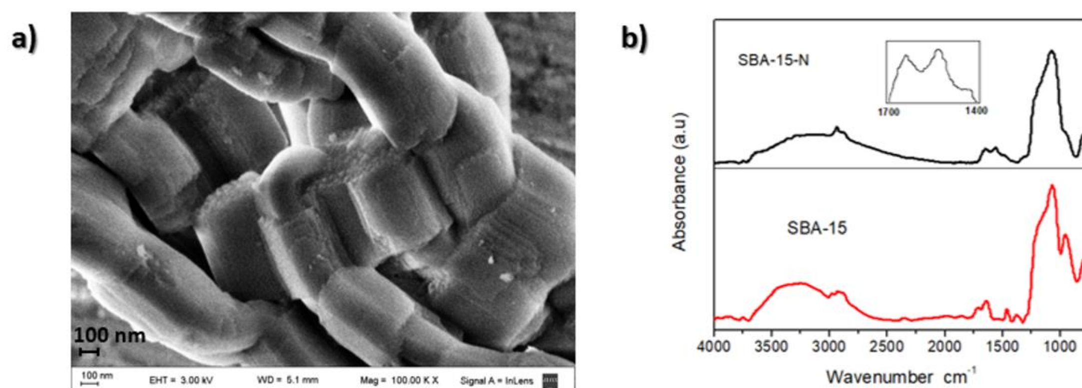

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Figure 3

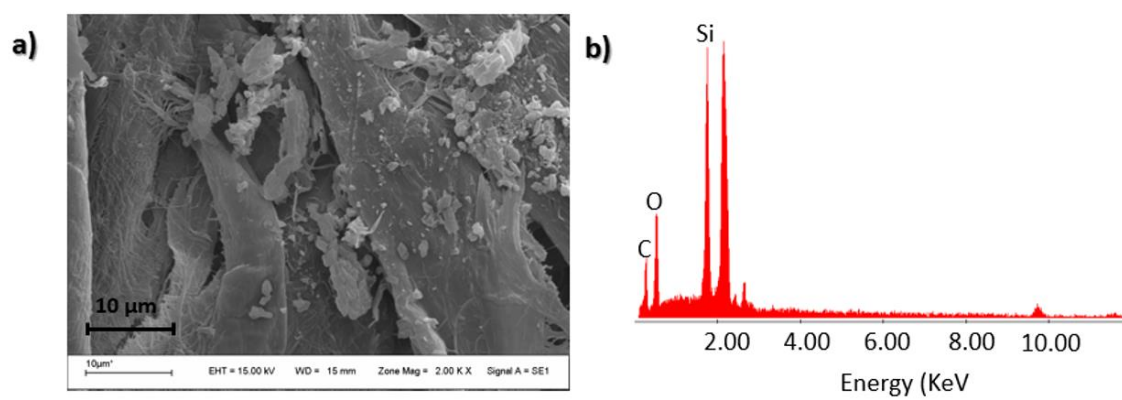

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Figure 4

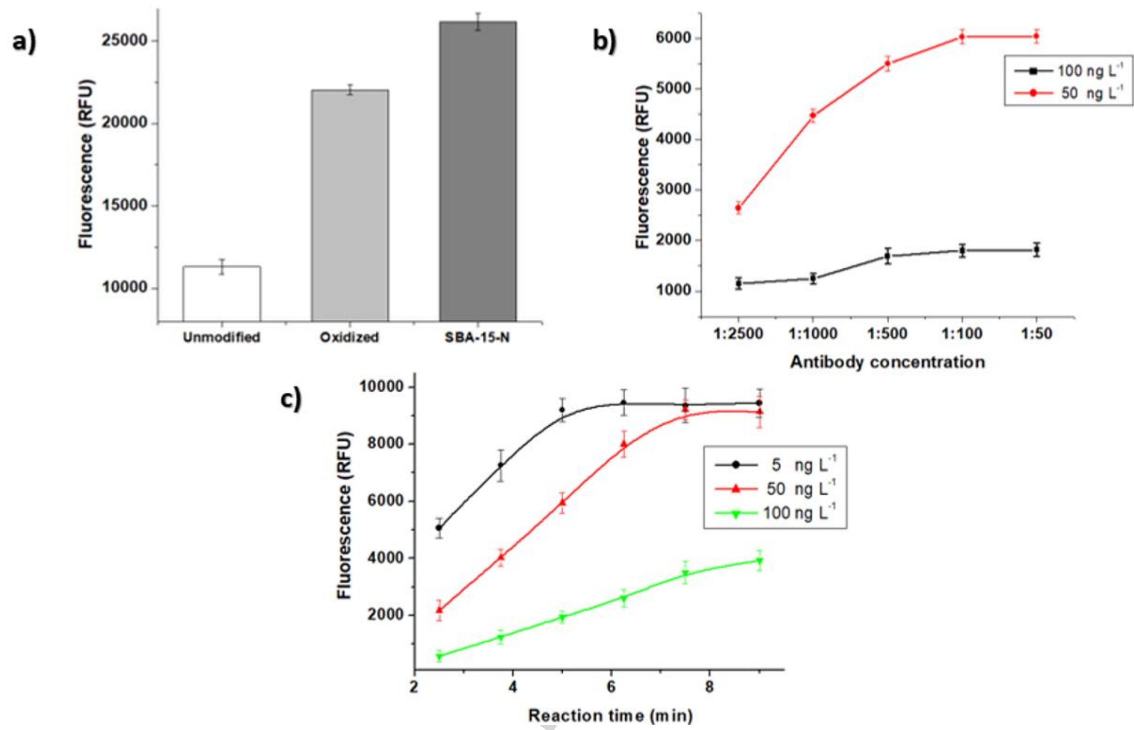

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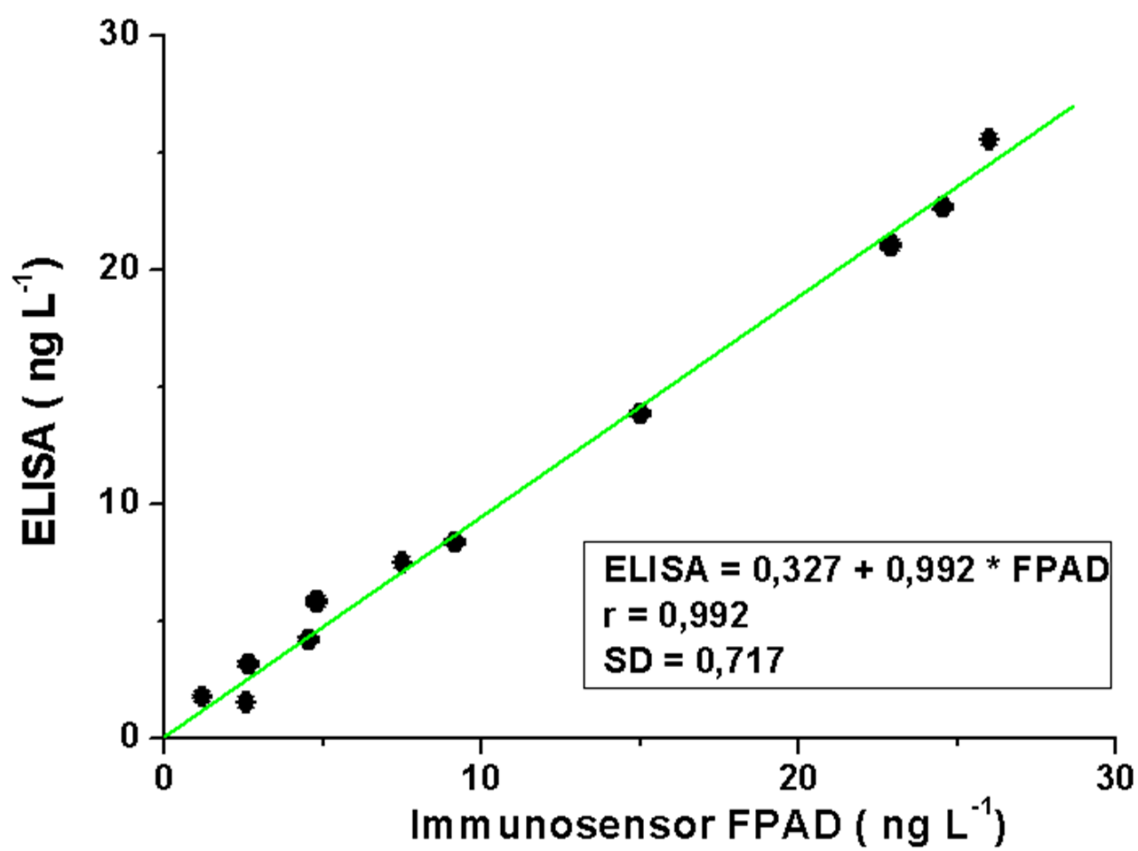
Figure 5



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Highlights

- Fluorescent paper based analytical device for ethinylestradiol detection.
- Increasing the immobilization surface for the immunoassay platform by using mesoporous silica particles.
- Paper immunoaffinity support and SBA-15-N generate high sensitivity and selectivity
- The developed method represents a novel strategy to ethinylestradiol determination in water samples determination.

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