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Immunosensors for Estradiol and Ethinylestradiol Based on New Synthetic Estrogen Derivatives: Application to Wastewater Analysis

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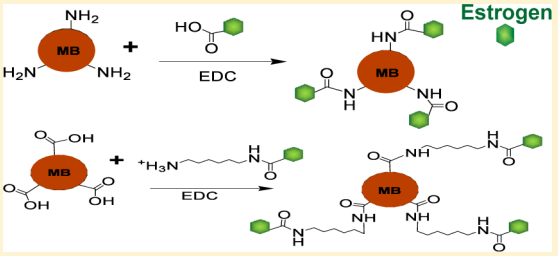
Supporting Information

ABSTRACT: Novel electrochemical immunosensors for sensitive detection of 17-β estradiol (E2) and ethinylestradiol (EE2) are described on the basis of the use of magnetic beads (MBs) as solid support and screen-printed electrodes as sensing platforms. Four synthetic estrogen derivatives containing either a carboxylic group or an amine group at the C-3 position were synthesized and covalently bound to MBs functionalized with amine or carboxyl groups, respectively. The assay was based on competition between the free and immobilized estrogen for the binding sites of the primary antibody, with subsequent revelation using alkaline phosphatase-labeled secondary antibody. Preliminary colorimetric tests were performed in order to validate the applicability of the synthetic estrogens to immunorecognition and to optimize different experimental parameters. In a second step, electrochemical detection was carried out by square wave voltammetry (SWV). Under the optimized working conditions, the electrochemical immunosensors showed a highly sensitive response to E2 and EE2, with respective detection limits of 1 and 10 ng/L. Cross-reactivity evaluated against other hormones demonstrated an excellent selectivity. The developed devices were successfully applied to analysis of spiked and natural water samples. These new immunosensors offer the advantages of being highly sensitive, easy, and rapid to prepare, with a short assay time.



Environmental water quality monitoring is an area of sustained scientific and technological interest. In the past decade, increased attention has been given to evaluate adverse effects of emerging contaminants like endocrine-disrupting chemicals (EDCs),[1,2] which interfere with the endocrine system of organisms by mimicking or antagonizing natural hormones.[3] Several substances are classified as EDCs, such as natural substances (phytoestrogens) and synthetic chemicals (alkylphenols, pesticides, phthalates, polychlorinated biphenyls, and bisphenol A). However, natural estrogens (17-β estradiol (E2), estrone, and estriol) as well as the synthetic estrogen ethinylestradiol (EE2) have been shown to display the highest affinity to nuclear estrogen receptors (ERs) and therefore present the greatest estrogenic potency.[4,5] Although these molecules are found in very low concentrations in water (ng/L range), they are suspected to induce adverse effects on aquatic, terrestrial organisms, and humans.[6,7] They are potentially involved in fish feminization, decreasing fertility of males,[8] and increased incidence of obesity and breast and testis cancers in humans.[9] The need to detect estrogenic compounds has been discussed in numerous publications, and environmental guidelines setting contamination limits have been developed by several regulatory authorities (REACH (2006), US EPA (2006)).

Conventional methods for the determination of estrogens in aqueous matrixes are based on gas chromatography coupled with mass spectrometry[10] or liquid chromatography with UV[11] or mass spectrometry detection.[12−14] Other detection techniques like fluorescence polarization and quenching resonance energy transfer technique have also been described, involving a preliminary derivatization of estrogens using fluorescein and lanthanide (III) chelates, respectively.[15] Although these methods are highly sensitive and specific, they are rather tedious and expensive and are not adapted to on-site measurement. Therefore, there is still a need of highly sensitive and consistent methods to perform rapid monitoring of real samples. For this purpose, a lot of research has been directed toward the development of immunoassays like enzyme-linked immunosorbent assays (ELISA),[16,17] chemiluminescence enzyme immunoassays,[18−20] and fluorescence immunoassays.[21,22] Several ELISA kits are already available on the market for estrogen monitoring (Abraxis kits, Biosense, Cayman) and have also been applied for research use.[23] However, these assays are time-consuming, involve multistep processes, and display quite elevated detection limits, close to 50 ng/L.

As an alternative, electrochemical sensors appear as promising tools for the monitoring of estrogenic substances due to their numerous advantages like reduced analysis time,

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low cost, portability, and possible remote use.[24] Although label-free methods allowing direct and specific monitoring of the interaction between antibody and antigen were recently reported,[25−27] most sensors use an indirect approach based on competition of free antigen and enzyme-labeled antigen for their binding to immobilized antibody. In this case, the detection principle is based on oxidation or reduction of an electroactive compound produced upon enzyme reaction.[28−34] Besides this classical configuration, another possible approach consists of a competition between free antigen and an immobilized antigen derivative for free primary antibody, an enzyme-labeled secondary antibody being used for the revelation. This strategy has been reported to be suitable for the detection of low molecular-weight targets, probably due to a better accessibility of the target to antibody binding sites.[35−38] Despite these advantages, the method has seldom been described for estrogen monitoring.

This paper presents the synthesis of four new estrogenic derivatives and their use for the development of immunoassays based on a competitive detection method. E2 and EE2 derivatives were chemically modified and functionalized for their selective immobilization on magnetic microbeads, that were chosen due to their numerous advantages such as easy fixation of the target, stability, large surface area, easy purification, and separation.[39−43] The systems were first optimized on microtitration plates and transferred to electrochemical sensing technology using square wave voltammetry (SWV) as the detection method. After testing their cross-reactivity, the electrochemical immunosensors were applied to the determination of E2 and EE2 in various water samples.

EXPERIMENTAL SECTION

Reagents and Materials. *Chemical Syntheses and Characterizations.* Estradiol (E2), ethinylestradiol (EE2), *p*-nitrophenyl phosphate (p-NPP), diethanolamine (DEA), 1-naphthyl phosphate (1-NP), *N*-hydroxysuccinimide (NHS), *N*-(3-dimethylaminopropyl)-*N*-ethyl-carbodiimide hydrochloride (EDC), *N*-Boc-1,6-hexanediamine hydrochloride, ethyl bromoacetate, sodium ethoxide, potassium bisulfate, acetyl chloride, and (benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate (BOP) were purchased from Sigma (France). All the reactions were monitored using TLC analysis, DC-Fertigfolien Alugram Xtra SIL G/UV_{254} (Macherey-Nagel Germany). The chemicals and organic solvents required for syntheses were purchased from Aldrich (France). All the ^{1}H and ^{13}C NMR spectra were recorded using a JEOL 400 MHz spectrometer. Mass spectral analysis was performed using electrospray ionization mass spectrometry (ESI-MS) (Thermo Scientific).

Immunoassays. E2 and EE2 was first dissolved in ethanol (1 g/L) and then diluted in phosphate buffer saline (PBS 1×). Buffer components, MES, Trizma base (tri[hydroxymethyl]-amino-methane), Tween 20, bovine serum albumin (BSA), casein blocking buffer, alkaline phosphatase (ALP)-labeled goat antimouse IgG antibody, and alkaline phosphatase (ALP)-labeled donkey antisheep IgG antibody were purchased from Sigma (France). Monoclonal antibody (anti-E2, developed in mouse) against E2 and polyclonal antibody (anti-EE2, developed in sheep) against EE2 was obtained from Thermo scientific. Magnetic beads (MBs) Dynabeads MyOne carboxylic acid (Invitrogen, USA) and Amino-Adembeads (Ademtech, France) were used as supports to immobilize the estrogens. U96 PolySorp Nunc-MicroWell Plates were obtained from Thermo Fisher Scientific. Adem-Mag 96 (adapted for 96-well microtiter plates) and Adem-MagSV (single magnet position adapted for 1.5 mL microtubes) were from Ademtech S.A (France). A horizontal shaker (IKA, Vibrax-VXR) was also used. Colorimetric measurements were performed with a lab systems Multiskan EX microtiter plate reader (Thermo Life Sciences, France).

Immunosensors Measurements. Square wave voltammetry (SWV) measurements were performed using an AUTOLAB PGSTAT100 potentiostat (Eco Chemie, Netherlands). Screen-printed electrode (SPE) systems, with graphite as working and counter electrodes and Ag/AgCl as a reference electrode, were fabricated using a DEK 248 screen-printing system.[44] A small 4 mm-diameter magnet was fixed on the backside of the working electrode to immobilize the MBs onto the electrode surface.

Synthesis of Modified Estrogens. *Synthesis of Ethyl Acetate Derivatives **1a** and **1b**.* 507 mg (7.4 mmol) of sodium ethoxide was added to a mixture of dry THF and ethanol (10:8 v/v) containing 1.68 mmol of E2 or EE2. After stirring for 30 min, 0.8 mL (4 equiv) of ethyl bromoacetate was added and the solution was heated at 80 °C for 24 h. The reaction mixture was then filtered, and the filtrate was concentrated under reduced pressure. The residue was diluted in 15 mL of ethyl acetate and evaporated under vacuum; this operation was repeated four times. The crude compound was purified by column chromatography with dichloromethane/ethyl acetate (8.5:1.5) as eluent to afford 330 mg (0.92 mmol, 55%) of ethyl-2-[(17β-hydroxy-estra-1,3,5(10)-trien-3-yl) oxy]-acetate (**1a**) or 350 mg (0.91 mmol, 54%) of ethyl-2-[(17α-ethinyl-17β-hydroxy-estra-1,3,5(10)-trien-3-yl) oxy]-acetate (**1b**).

*Synthesis of Acidic Derivatives **2a** and **2b**.* Sodium hydroxide (3 mL, 2 M) was added dropwise to a 10 mL stirred methanolic solution of **1a** or **1b** (0.78 mmol) at 0 °C. The solution was stirred for 1 h at RT and monitored by thin layer chromatography (TLC) (dichloromethane/ethyl acetate, 8.5:1.5), until full consumption of the starting material. The reaction mixture was diluted with water (5 mL) and acidified with $KHSO_4$ to pH 2. The mixture was extracted with ethyl acetate (2 × 20 mL), and the organic phase was washed with brine, dried over anhydrous sulfate, and evaporated to dryness, affording 220 mg (85%) of 2-[(17β-hydroxy-estra-1,3,5(10)-trien-3-yl) oxy]-acetic acid (**2a**) or 250 mg (90%) of 2-[(17α-ethinyl-17β-hydroxy-estra-1,3,5(10)-trien-3-yl) oxy]-acetic acid (**2b**). Throughout this text, these two derivatives will be called E2-COOH and EE2-COOH, respectively.

Addition of Hexanediamine Spacer. (Benzotriazol-1-yloxy)tris(dimethylamino) phosphonium hexafluorophosphate (BOP) (187 mg, 0.423 mmol) was added to a stirred solution of **2a** or **2b** (0.282 mmol) and triethylamine (78 μL, 0.564 mmol) in 5 mL of DCM and DMF mixture (4:1 v/v) at room temperature. After 10 min, *N*-Boc-1,6-hexanediamine hydrochloride (78.4 mg, 0.31 mmol) was added, followed by triethylamine (78 μL, 0.564 mmol). The resulting mixture was stirred for 4 h at room temperature. The reaction mixture was supplemented with ethyl acetate (10 mL) and then washed successively with HCl (1 M, 3 × 5 mL), H_2O (1 × 5 mL), $NaHCO_3$ (1 M, 3 × 5 mL), and H_2O (2 × 5 mL). The organic phase was dried over anhydrous sulfate and concentrated to provide the protected compounds *N*-Boc-(6-aminohexyl)-2-[(17β-hydroxy-estra-1,3,5(10)-trien-3-yl)oxy]-acetamide (E2-NH-Boc, **3a**) (122 mg, 77%) and *N*-Boc-(6-aminohexyl)-2-[(17α-ethinyl-17β-hydroxy-estra-1,3,5(10)-trien-3-yl)oxy]-acetamide (EE2-NH-Boc, **3b**) (110 mg, 70%) as colorless powders.

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Deprotection of Boc Group. Deprotection of **3a** derivative: 100 mg (0.189 mmol) of E2-NH-Boc was dissolved in 2 mL of DCM and TFA (1:1 v/v). The reaction mixture was stirred at room temperature for 1 h and monitored by TLC (ethyl acetate/DCM, 7:3). The reaction mixture was then evaporated under vacuum, and residual TFA was eliminated by coevaporation with cyclohexane. The residue was triturated with anhydrous ether (2 × 20 mL) to provide 89 mg (86%) of *N*-(6-aminohexyl)-2-[(17β-hydroxy-estra-1,3,5(10)-trien-3-yl) oxy] acetamide (E2-NH_2, **4a**). Deprotection of **3b** derivative: To a solution of EE2-NH-Boc (100 mg, 0.18 mmol) in ethyl acetate (5 mL), 4 mL of 4 M solution of hydrochloride in ethyl acetate was added dropwise. The reaction mixture was stirred at room temperature for 60 min. The reaction mixture was evaporated under vacuum, and the residue was dissolved in 10 mL of ethyl acetate. The solution was again evaporated, and the residue was triturated with anhydrous ether, leading to 83 mg (94%) of *N*-(6-aminohexyl)-2-[(17α-ethinyl-17β-hydroxy-estra-1,3,5(10)-trien-3-yl) oxy] acetamide (EE2-NH_2, **4b**).

Immobilization of Estrogens Amine Derivatives on Magnetic Beads. *Fixation of E2-NH2 or EE2-NH2 to Carboxyl-Functionalized Magnetic Beads.* MBs coated with carboxylic acid (carboxyl-MBs) were used as support for immobilization of E2-NH2 or EE2-NH2. 200 μL of carboxyl-MBs was collected and washed twice with 200 μL of binding buffer (MES, 25 mM, pH 6). Estrogen immobilization was carried out by mixing the MBs with 45 μL of MES and 5 μL of estrogen solution (10 g/L) in ethanol, followed by 30 min of gentle stirring. 30 μL of EDC (10 mg/mL) in cold MES buffer (25 mM) was then added, and the solution was incubated on a roller overnight at 4 °C. To eliminate unbound estrogens, the activated MBs were washed twice with 200 μL of Tris 50 mM containing BSA 0.5% and Tween 0.1%. The estrogen-coated MBs (E2-hexa-MBs or EE2-hexa-MBs) were then resuspended in the storage buffer (Tris 50 mM) and stored at 4 °C until use.

Fixation of E2-COOH or EE2-COOH to Amine-Functionalized Magnetic Beads. 200 μL of amine-MBs (10 mg/mL) was prepared and washed 3 times with 200 μL of MES. 160 μL of solution of EDC (4 mg/mL) in MES and 5 μL of solution of E2-COOH or EE2-COOH (10 g/L) in ethanol were added to the beads suspension and incubated on a roller for 4 h at room temperature. The supernatant was then removed, and 200 μL of solution of BSA 0.05% in PBS (1×) was added and incubated on a roller for 30 min at room temperature. Unbound estrogens were eliminated by washing the activated MBs twice with 200 μL of PBS 1× Tween 0.1%. Activated MBs were resuspended in the PBS 1× and stored at 4 °C until use.

Colorimetric Immunoassay Protocol. *Colorimetric Assays Using EE2-MBs or EE2-Hexa-MBs.* To avoid nonspecific interactions, a preliminary blocking step was performed by introducing in each microwell 200 μL of PBS (1×) containing 0.5% BSA. After 1 h incubation, the blocking buffer was discarded and either 30 μL of EE2-MBs (at dilution 1/10) or 20 μL of EE2-Hexa-MBs (at dilution 1/10) were deposited in the wells. The solution was then removed, the magnetic beads being maintained using the Adem-Mag 96 magnetic support. The competition step was then performed for 50 min using 50 μL of EE2 standard solutions at different concentrations and 50 μL of anti-EE2 solution in PBS (1×). It was shown that the optimum dilutions of anti-EE2 were 1/1340 and 1/2000, using, respectively, EE2-MBs and EE2-Hexa-MBs. 100 μL of ALP-labeled donkey antisheep secondary antibody solution at 1/30 000 in PBS (1×) was then added in each well. After 40 min of incubation, the bound enzyme activity was revealed by addition of 100 μL of a 4 mg/mL p-NPP solution in 10% DEA buffer (pH 9.5). After 30 min of reaction, the absorbance of the yellow product was measured at 405 nm. All the experiments were carried out at room temperature with constant shaking. Washing steps were performed between each step using 200 μL of PBS (1×) containing 0.05% of Tween 20.

Colorimetric Assays Using E2-MBs or E2-Hexa-MBs. In this case, the blocking step was carried out by adding 200 μL of PBS (1×) containing 1% casein for 1 h. 10 μL of E2-MBs at dilution 1/10 or E2-Hexa-MBs at dilution 1/50 were deposited in the microwells. The buffer was then removed, and the competition step was performed for 50 min using 50 μL of E2 standard solutions at different concentrations and 50 μL of anti-E2 solution at dilution of 1/3750 in PBS (1×). Then, 100 μL of ALP-labeled goat antimouse secondary antibody solution was added at dilution of 1/2500 in PBS (1×) for 40 min. Washing and revelation steps were performed as described for EE2.

Electrochemical Immunosensor Protocol. *Electrochemical Immunosensor for EE2.* 10 μL of the EE2-Hexa-MBs at dilution 1/10 was added on the surface of working electrode, and a blocking step was performed by adding 150 μL of PBS (1×) containing 0.5% BSA for 1 h. The competition step was performed using 50 μL of EE2 standard solutions at different concentrations and 50 μL of anti-EE2 solution at dilution 1/1340 in PBS (1×) for 30 min. Then, 100 μL of ALP-labeled donkey antisheep secondary antibody solution was incubated at dilution 1/30 000 in PBS (1×) for 30 min. ALP activity was revealed by adding 100 μL of a 0.5 mg/mL 1-NP solution in 10% DEA buffer (pH 9.5). After 2 min of reaction, the electrochemical detection was performed by scanning the potential between 0 and 0.5 V (frequency 8 Hz, step potential 0.01 V, modulation amplitude 0.05 V, standby potential 0 V). The height of the resulting peak was recorded and plotted against target concentration to give a calibration curve. Washing steps were performed by adding 100 μL of PBS (1×) between each step, a small magnet being used to retain the MBs on the surface of working electrode. Assays were performed in duplicate.

Electrochemical Immunosensor for E2. 10 μL of the E2-Hexa-MBs at dilution of 1/50 was added on the surface of working electrode, and a blocking step was performed by adding 150 μL of PBS (1×) containing 1% casein for 1 h. The competition step was performed using 50 μL of E2 standard solutions at different concentrations and 50 μL of anti-E2 solution at dilution of 1/3750 in PBS (1×) for 30 min. Then, 100 μL of ALP-labeled goat antimouse IgG secondary antibody solution was added at dilution of 1/2500 in PBS (1×) for 30 min. Washing and measurement steps were performed as described for EE2.

Calibration of Immunoassays and Immunosensors. Standard calibration curves were obtained using E2 or EE2 standard solutions prepared in PBS (1×). The matrix effects were assayed using blank samples without the target. Recovery was calculated by spiking the blank samples with a known amount of target standard solution.

The absorbance values were converted into their corresponding test inhibition values (A/A_0) as follows: % (A/A_0) = (A/A_0) × 100 where A is the absorbance or the intensity values of competitive assay and A_0 is the absorbance or the intensity value of the noncompetitive assay. The results were calculated

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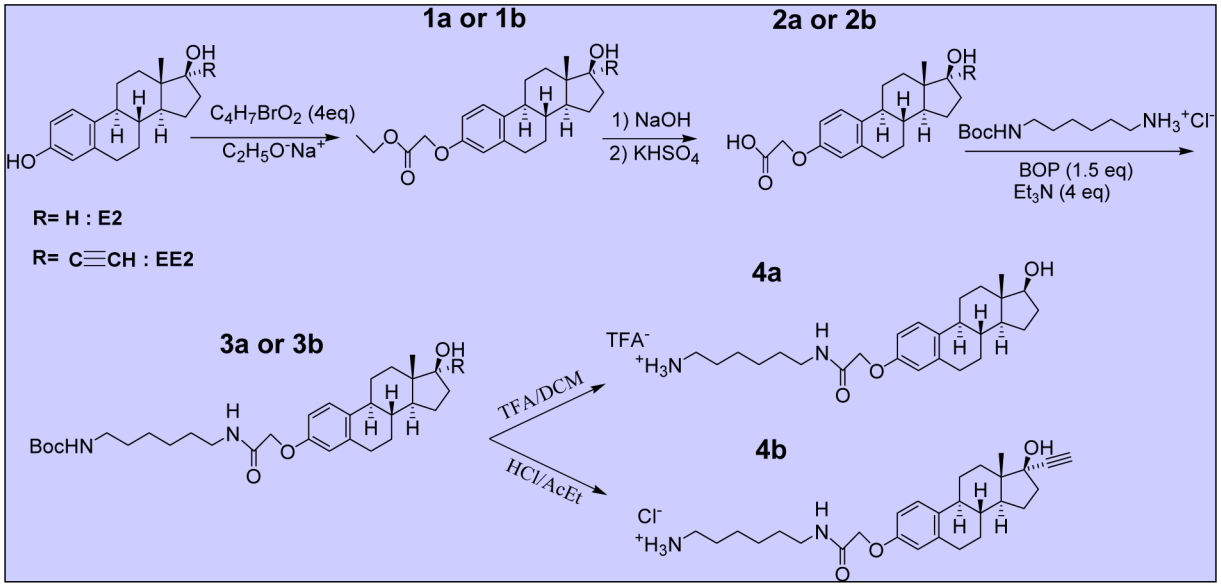

Figure 1. Synthesis of carboxyl (E2-COOH and EE2-COOH) and amine (E2-NH2 and EE2-NH2) estrogen derivatives.

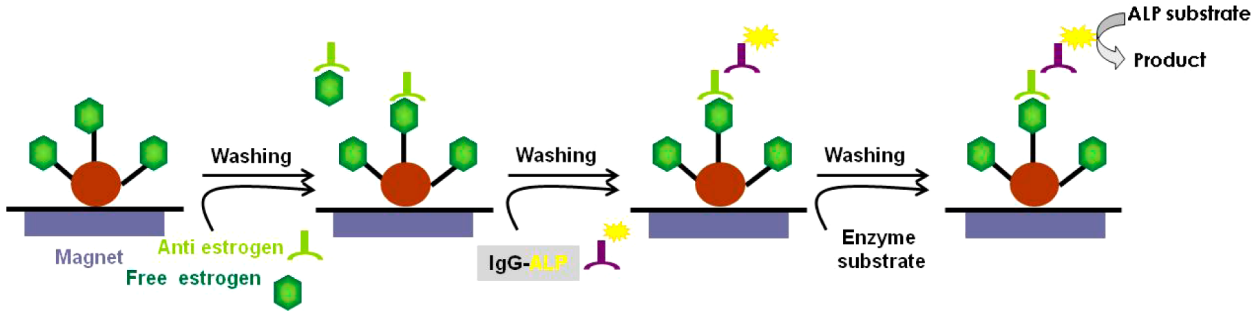

Figure 2. Strategy for indirect competitive assay for both colorimetric and electrochemical detection based on magnetic nanoparticles.

as the relative response compared to the response in the absence of free target, expressed in percentage.

RESULTS AND DISCUSSION

Synthesis of Estrogens Amine Derivatives E2-NH_2 (4a) and EE2-NH_2 (4b). As C_{17} seems to be the more likely binding epitope of the antibody, the modification of the estrogen was performed on a remote position by selective alkylation of E2 or EE2 at position-3, leading to the title compounds **4a** and **4b** (Figure 1). The 3-hydroxyl group of E2 or EE2 was first alkylated by ethyl bromoacetate to provide ethylestradiol-3-oxylacetate (**1a**) or ethylethinylestradiol-3-oxylacetate (**1b**) in 55% and 54% yield, respectively.[45] Subsequent saponification of **1a** and **1b** allowed their conversion into the corresponding acids **2a** (85% yield) or **2b** (90% yield). The carboxylic acid group was then coupled to the *N*-Boc-1,6-hexanediamine hydrochloride using the BOP reagent, yielding 77% and 70% of **3a** and **3b**, respectively. Deprotection of **3a** *N*-Boc amine was rapidly accomplished using TFA in methylene chloride, yielding 86% of product **4a**. The deprotection of **3b** was performed using hydrochloride in ethyl acetate, yielding 94% of product **4b**.

H NMR of compounds **2a** and **2b** are characterized by the presence of peaks at δ= 4.5 ppm that correspond to the methylene groups of the introduced acetyl moieties. On the other hand, signals of amide protons at δ = 7.6−8.0 ppm confirm the conjugation of the estrogen moiety to the hexanediamine present in compounds **4a** and **4b**. Furthermore, all synthesized compounds were analyzed by HPLC, showing a purity of at least 95%. Structural confirmation was assessed by ESI+ mass spectroscopy (Supporting Information).

Grafting of E2 or EE2 to MBs was performed either using carboxyl or amine estrogenic derivatives. The carboxylic group of E2-COOH or EE2-COOH was activated using EDC and linked to an amine group of MBs, leading to, respectively, E2-MBs or EE2-MBs. Using a comparable method, the amine group of E2-NH_2 or EE2-NH_2 was conjugated to the carboxyl group of MBs via a hexamethyl spacer arm, providing, respectively, E2-hexa-MBs or EE2-hexa-MBs.

Colorimetric Detection of E2 or EE2 in Competitive Assays. Colorimetric immunoassays were carried out as a rapid method to confirm the covalent attachment of MBs to synthetic estrogens (E2 or EE2), to assess the affinity of bound estrogens (E2-MB or EE2-MB) to their respective antibodies, and to optimize experimental factors. As described before, the assay was based on the competition of free and immobilized estrogens for their binding to antibodies used in solution. As these primary antibodies were not labeled, a secondary anti-IgG antibody labeled with ALP was used for performing the colorimetric detection via hydrolysis of p-NPP into the yellow-colored p-nitrophenol (p-NP) (Figure 2). In order to optimize the different experimental parameters, first, assays were performed in the absence of free estrogen. Estrogen-coated MBs were magnetically immobilized in microtiter plate wells

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Table 1. Optimal Experimental Parameters Applied in Colorimetric and Electrochemical Detection

methods	MBs	MBs volume (μL), dilution	primary antibody dilution	incubation time, min	secondary antibody dilution	incubation time, min	blocking buffer
colorimetric immunoassay	E2-MBs	10, 1/10	1/7500	50	1/2500	40	casein 1% 1 h
	E2-hexa-MBs	10, 1/50	1/7500				
	EE2-MBs	30, 1/10	1/2680		1/30000		BSA 0.5% 1 h
	EE2-hexa-MBs	20, 1/10	1/4000				
electrochemical immunosensor	E2-hexa-MBs	10, 1/50	1/7500	30	1/2500	30	casein 1% 1 h
	EE2-hexa-MBs	10, 1/10	1/2680		1/30000		BSA 0.5% 1 h

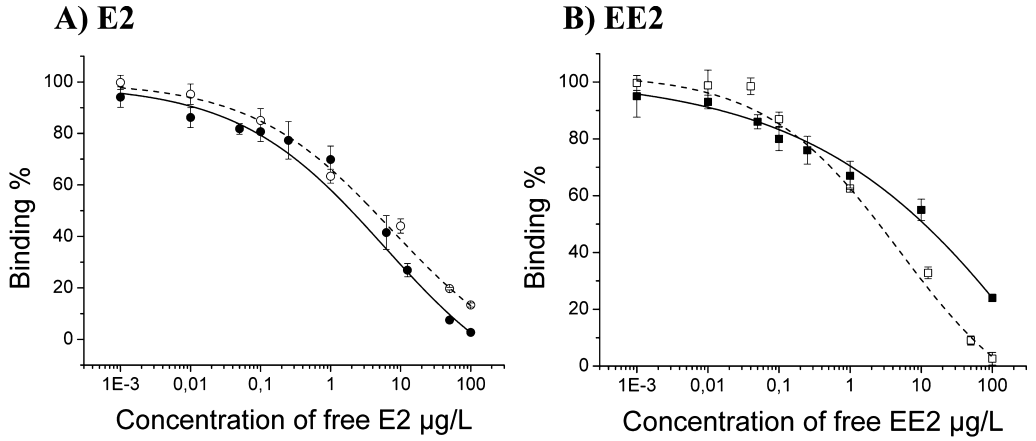

Figure 3. Calibration curves of E2 (A) and EE2 (B) obtained by colorimetric assays using estrogen-hexa-MBs (● or ■) and estrogen-MBs (○ or □).

using Adem-Mag 96 magnetic support; the optimized contact time was 5 min. The blocking step was carefully studied, since this is a key point in immunoassays to avoid nonspecific adsorption of antibodies. Casein blocking buffer (1%) and BSA (0.5%) were selected as blocking solutions for E2 and EE2, respectively. Experiments with different dilutions of primary antibody and labeled secondary antibodies were also performed. According to the theory, high antibody concentrations increased the signal intensity but decreased the sensitivity of immunoassays and generated elevated background signal.[27] The best working dilutions obtained for each estrogen derivative are presented in Table 1.

On the basis of the optimized parameters, calibration curves were established of E2 and EE2 using each estrogen derivative (Figure 3). As expected, the intensity of the response was inversely proportional to the concentration of free estrogens (E2 or EE2) in each assay. Due to the low standard deviation of the calibration curve (between 1% and 4%), the LOD was defined as the free estrogen concentration inducing a 15% decrease of the maximum signal. The calibration curves were fitted by the sigmoidal logistic four parameter-equations (Origin Pro 8.6). Table 2 summarizes the main characteristics of the colorimetric assays with the two different types of MBs charged with each estrogen (E2 and EE2). The correlation coefficient R, the B_{50}, and LOD values derived from the regression equations are summarized in Table 2. The B_{50} was defined as the estrogen concentration inducing a 50% decrease of the signal. Results showed that LOD values were 2-fold lower using estrogen-hexa-MBs (E2-hexa-MBs or EE2-hexa-MBs) when compared to estrogen-MBs (E2-MBs or EE2-MBs). This difference can be explained by the presence of the hexamethyldiamine spacer arm, which allows minimizing steric hindrance and favors accessibility of immobilized antigen to antibody binding sites.

Table 2. Curve Parameters Derived from the Nonlinear Four-Parameter Logistic Regression Fitting of the Plots Obtained for Both Colorimetric and Electrochemical Detections, Using the Two Types of Magnetic Beads (MBs)

methods	MBs	LOD (ng/L)	B_{50} (ng/L)	R
colorimetric immunoassay	E2-MBs	80	4000	0.999
	EE2-MBs	100	2500	0.99
	E2-hexa-MBs	36	1970	0.996
	EE2-hexa-MBs	50	9640	0.997
electrochemical immunosensor	E2-hexa-MBs	1	237	0.977
	EE2-hexa-MBs	10	220	0.99

Electrochemical Immunosensors for E2 or EE2 Detection. On the basis of the higher sensitivity of estrogen-hexa-MBs in colorimetric assays, this method was transferred to immunosensor format, using screen-printed electrodes (SPEs) as electrochemical transducers. SWV was used as detection method, based on oxidation (+210 mV vs Ag/AgCl/Cl^-) of 1-naphthol produced upon hydrolysis of 1-naphthyl phosphate by ALP. When compared to other electrochemical techniques like cyclic voltammetry and differential pulse voltammetry, this method was shown to be more rapid, efficient, and suited to the development of electrochemical immunosensors.[27] The main parameters of E2-hexa-MBs based immunoassays were thus transferred to immunosensors (Table 1); the incubation time of

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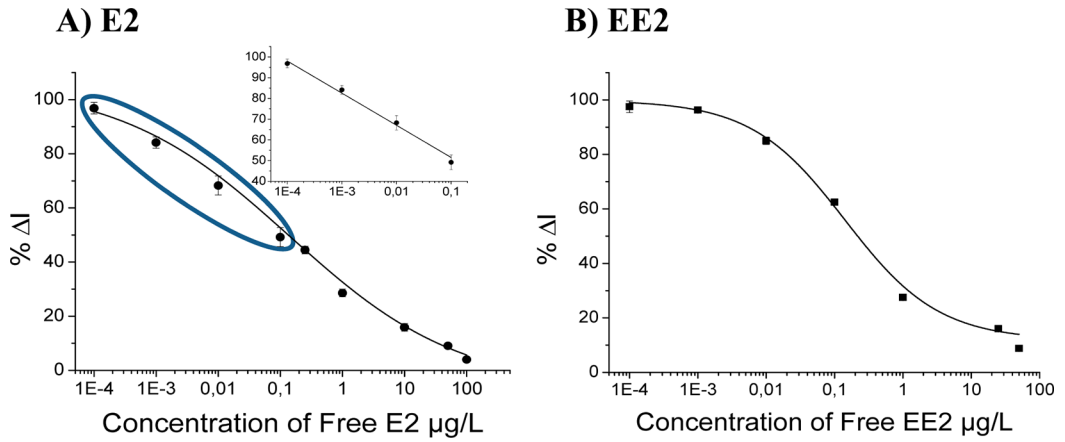

Figure 4. Calibration curves of E2 (A) and EE2 (B) obtained using electrochemical biosensor.

Table 3. Determination of E2 and EE2 in Spiked and Unspiked Water Samples[a]

	estradiol (ng/L)			ethinylestradiol (ng/L)		
sample	added	found	recovery (%)	added	found	recovery (%)
mineral water (Evian)		0			0	
	1	1.3 ± 0.6	130	10	10.3 ± 2.7	103
	10	9.8 ± 2	98	20	21 ± 3	105
	20	20.5 ± 2.5	102.5	30	31 ± 4	103.3
lake water (La Raho)		0.7 ± 0.15			0	
	1	1.5 ± 0.3	80	10	11 ± 3	110
	10	10.5 ± 0.5	98	15	14.5 ± 4	96.7
	20	22 ± 3	106.5	30	33.5 ± 6	111.7
WWTP raw water		74 ± 5			18.8 ± 1.2	
	20	102 ± 7	140	20	42 ± 1	116
WWTP treated water		42 ± 4.5			15.5 ± 2	
	20	65 ± 6	115	20	41	127.5

[a]WWTP = wastewater treatment plant.

antibodies was reduced to 30 min, leading to a total preparation time of 120 min, compared to 280 min using classical immunoassays.

The stability of the system was studied using electrodes coated with estrogen-MBs and treated using the blocking buffer. The prepared electrodes stored at 4 °C were tested after 1 week of storage with no detectable loss of activity and 80% of activity remained after 3 weeks of storage. This good stability was related to the covalent binding of estrogen amino derivative on carboxylated MBs.

Competition assays were performed using free targets (E2 or EE2). The calibration curves obtained for estrogen-hexa-MBs are presented in Figure 4. The wider linear range was obtained for the detection of E2, for concentrations ranging from 0.1 to 100 ng/L (Figure 4A). Due to the low experimental error (5%) associated to electrochemical detection, LOD was calculated considering the estrogen concentration inducing a signal decrease of 15%. LOD values of 1 and 10 ng/L and B_{50} values of 237 and 220 ng/L were obtained using E2-hexa-MBs and EE2-hexa-MBs, respectively. The achieved LODs were in the same order of magnitude of those obtained in other works[28] and much lower than those of commercially available detection kits, whose detection limits for estradiol were between 19 and 50 ng/L. When compared to colorimetric assays, it was shown that electrochemical detection allowed one to increase the sensitivity of detection for both types of modified MBs (Table 2).

Tests of Cross-Reactivity and Real Sample Analysis. Various natural and synthetic hormones (E2, EE2, estrone, stigmasterol, and ergosterol) were tested as potential interfering substances for the immunosensor response toward E2 and EE2. The cross-reactivity was tested using a maximum of 250 ng/L of each compound, and all the tested species gave very low percent of cross-reactivity, with values lower than 0.01%. These results demonstrated the high selectivity of the developed immunosensors. The developed immunosensors were then tested for analyzing various waters, spiked with known concentrations of estrogens. Three real samples were tested: a mineral water (Evian), a lake water (lac de la Raho, France), and water from a local wastewater treatment plant (Pia, France). Each sample was filtered through a 0.45 μm membrane and tested under the same procedure as described before. The concentration of estrogen (E2 or EE2) was calculated by correlation with the corresponding calibration curve. The comparison of the results obtained using spiked and nonspiked mineral and lake waters clearly demonstrated the absence of matrix effect in estrogen determination (Table 3). The values of recovery tests were acceptable and adequate. Results were slightly different with the samples obtained from the wastewater treatment plant; a weak matrix effect was observed inducing a small overestimation of estrogen content (increase of the recovery rate). As expected, raw waters contained elevated concentrations of the natural hormone E2 and lower but significant amounts of the synthetic EE2. After

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the wastewater treatment process, the concentration of E2 decreased from 74 to 42 ng/L. This diminution may be likely explained by the oxidation of E2 to estrone,[7] even if adsorption on clay and precipitation may also occur. The first hypothesis was consolidated by the results observed for EE2, which is not easily oxidized and eliminated during the wastewater treatment process (Table 3).

CONCLUSIONS

In this paper, we have synthesized new estrogens derivatives: carboxyl estrogens (E2-COOH or EE2-COOH) and amine estrogens with hexamethylamine spacer arm (E2-NH_2 or EE2-NH_2). These new derivatives were covalently immobilized to MBs. Results demonstrate that chemical modification on the C3 does not change the interaction with their respective antibodies. A lower LOD was obtained using amine estrogens with hexamethylamine spacer arm, probably due to the increase of the accessibility of antibody to their target.

The combination of MBs as immobilization platform and SPE as transducer allowed numerous advantages over other analytical techniques in terms of analysis time, sensitivity, and cost effectiveness. Moreover, the use of SPE allows one to miniaturize the system and make the device portable. These new immunosensors were successfully applied for surface water and wastewater analysis, demonstrating their potential application for estrogen surveillance monitoring. In the future, the synthesized estrogen derivatives could be easily immobilized on other surfaces for the development of electrochemical label-free immunosensors based on optical (surface plasmon resonance), gravimetric (quartz crystal microbalance), or electrochemical (electrochemical impedance spectroscopy) methods.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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