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A novel electrochemical sensor for 17β-Estradiol from molecularly imprinted polymeric microspheres and multi-walled carbon nanotubes grafted with gold nanoparticles

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Abstract

Molecular imprinted polymer (MIP) is generally a more stable material for sensing application. The high selectivity and sensitivity of MIP for sensors can be achieved if the template molecule is imprinted in the polymer and this makes them an ideal alternative as a recognition element for sensors. A new electrochemical sensor based on molecularly imprinted polymeric microspheres (MIP) and multi-walled carbon nanotube/gold nanoparticles (MIP-MWCNT-AuNPs) modified carbon screen-printed electrode (SPE) for the rapid detection of 17β-estradiol (E) hormone in serum samples have been successfully developed. Hydropobic MIP was synthesized using photopolymerization in emulsion form. The multi-walled carbon nanotube grafted with gold nanoparticles was firstly deposited onto a carbon screen-printed electrode for the puspose of accelerating electron transfer to the surface of the electrode. The MIP microspheres specific to the 17β-estradiol hormone, prepared via facile photopolymerization technique, were coated onto the MWCNT-AuNPs -modified SPE. The presence of 17β-estradiol in biological samples could be detected with the sensor via absorption of 17β-estradiol into the deposited MIP and this was monitored by using differential pulse voltammetry (DPV) at 0.6 V for the reduction of 17β-estradiol. Under optimal conditions, the sensor could detect the concentrations of 17β-estradiol from 1.0×10^{-15}–1.0×10^{-6} M (R^2=0.9921), with a detection limit of 2.5×10^{-16} M. The sensor based on MIP microspheres and MWCNT-AuNPs modified electrode demonstrated stability of 55 days with good reproducibility (RSD <5 %, n=5) and regenerability (RSD <4 %, n=5). Using this sensor, the gender of the arowana fish determined via the level of 17β-estradiol using fish serum samples demonstrated good agreement with a conventional test kit based on immuno-assay method.

***Keywords**:* Molecularly imprinted polymers; 17β-estradiol; electrochemical sensor; multi-walled carbon nanotubes, gold nanoparticles

Introduction

The 17β-Estradiol (1,3,5(10)-estratriene-3,17β-diol) compound is a sex hormone classified as a phenolic steroid hormone. It is commonly used to fatten animals, due to its anabolic effect, and it is present in many aquatic environmental at low concentrations from ppt to ppb levels.[1] It was suspected to cause structural chromosomal aberration in human embryonic fibroblasts and inhibit vitellogenin production in male trout, which could result in adverse effects on the endocrine system of humans[2] and wildlife[3]. Therefore, the reliable, direct, and rapid determination of trace levels of 17β-estradiol hormone in fishery products and the aquatic system are essentially important for health protection and bio-safety.

Many conventional methods have been used for to detect 17β-estradiol hormone in aquatic systems. The reported method regularly used includes liquid chromatography-tandem mass spectrometry (LC–MS)[4,5], high performances liquid chromatography (HPLC)[6,7] and gas chromatography-tandem mass spectrometry (GC–MS)[8,9]. However, these methods commonly require sampling pretreatment process, expensive equipment, and are time-consuming. Moreover, analytical method based on the immunoassay technique is also commonly used to detect 17β-estradiol.[10] This method could provide high sensitivity for hormone detection, but it was low stability.

Currently, the detection of 17β-estradiol hormone based on molecularly imprinted polymer is of great interest due to its ability to recognize and bind specific molecules. MIP is a method that

involves reagents, including a functional monomer, crosslinker, initiator, and a template for it to be synthesized to became a rigid polymer matrix.[11] The embedded template in the MIPs could be removed by either chemical cleavage or solvent extraction, and the resulting MIPs should selectively recognizing the material to analytes possessing similar structures to that of the template. The main advantages of MIPs are its long-term stability, ease of preparation, low cost, large surface area, and reusability. These advantages has immensely contributed to the interest of the development of detection of 17β-estradiol hormone in an aquatic system.[1,12]

Zhang et al.,[13] utilized MIPs functionalized 6-mercaptonicotinic acid-platinum nanopartilces (MNA-PtNPs) to develop a sensor that detects 17β-estradiol in environmental waters. The hormone sensor based on MIPs-MNA-PtNPs was grafted onto the glassy carbon electrode, resulting in a low detection limit >5.0 ppb, with good reproducibility and rapid response time. Wen et al.,[14] have developed sandwich-type electrochemical 17β-estradiol sensor based MIPs modified graphene oxide-platinum nanoparticles, whereby MNA was used to crosslink the hydrogen terminal of 17β-estradiol to the PtNPs. The sensor demonstrated a wide linear response range and low detection limits. Yuan et al.,[15] proposed other MIPs functionalized platinum nanoparticles-glassy carbon electrode to detect 17β-estradiol in an aquatic environment, and obtained detection limits as low as sub-nanomolar (nM) level with excellent reproducibility. Azevedo et al.,[16] reported an amperomtric 17β-estradiol sensor based on MIPs grafted onto conducting polyaniline membrane modified gold electrode. A 17β-estradiol sensor based on conducting membrane was synthesized via a simple electropolymerization, and then the MIP layer was photochemically embedded onto the polyaniline membrane via N,N-diethyldithiocarbamic acid benzyl ester activation of methacrylamide groups. The analytical performance of a polyaniline membrane based sensor had a detection limit of < 0.7 μM level.

The use of MIPs in developing a 17β-estradiol sensor have many advantages, including low detection limits, wide linear response range, good reproducibility, and high stability and reusability.

There are several reports on the use of multi-walled carbon nano-tube with MIP as sensing materials. Yola et al.,[17] developed a sensor based on MIPs for the sensitive determination of cefexime in human plasma by employing Fe@Au nanoparticles and 2-aminoethanethiol functionalized multi-walled carbon nanotubes. The MIPs-modified Fe@Au-MWCNT demonstrated very low detection limits, down to 25 pM. Gupta et al.,[18] reported a molecular imprinted polypyrrole modified glassy carbon electrode (GCE) for the determination of tobramycin and found a good linear response. Yola and Atar,[19] proposed a voltametric sensor based aminothiophenol functionalized multi-walled carbon nanotubes coated onto gold nanoparticles used for the evaluation of quercetin and rutin. The voltametric sensor was capable of low-level detections of quercetin and rutin at < 340 pM.

Yola and Co-worker proposed a sensitive molecular imprinted surface plasmon resonance (SPR) sensor based on gold surface-modified with allyl mercaptane. The surface Plasmon resonance sensor fabricated via template-imprinted poly(2-hydroxyethyl methacrylate–methacryloylamidoglutamic acid) (p(HEMA–MAGA) film generated on the gold surface was capable for low detection limits of citrinin at >1 ppt level,[20] amoxicillin in human plasma at > 1 ppb level,[21] amikacin in human plasma at >1 ppb level,[22] and triclosan in wastewater at > 1 ppb level,[23] while MIPs based quartz crystal microbalance (QCM) have been prepared via self-assembling monolayer formation of allylmercaptane on QCM chip surface for the detection of lovastatin in red yeast rice, kaempferol in fruit juices, atrazine in wastewater, and tobramycin in pharmaceuticals. The QCM nanosensor was prepared via template-imprinted poly(2-

hydroxyethyl methacrylate–methacryloylamidoglutamic acid), and low detection limits of 30 pM for lovastatin,[24] 60 pM for kaempferol,[25] and 28 pM for atrazine,[26] 5.7 pM for tobramycin,[27] and long term stability (40 days). Yola et al.,[28] designed a tyrosine sensor based on MIPs grafted onto gold nanoparticle-graphine oxide for the detection of tyrosine in milk. The tyrosine sensor employed 2-aminoethanethiol functionalized graphene oxide modified the glassy carbon electrode to enhance electron acceleration, resulting in low detection limits at > 150 pM, long term stability, and good reproducibility. Generally, the fabricated sensor based on MIPs demonstrated improvements in terms of LOD, wide linear response range, good reproducibility, and long-term stability. This is probably contributed by the large surface area and the homogeneity of the nano/micro sized MIPs, as well as excellent diffusion of MIPs on the SPE surface.

This study involved the use of MIP microspheres and multi-walled carbon nanotubes grafted with gold nanoparticles (MWCNT-AuNPs) for the development of a new electrochemical 17β-estradiol sensor. The advantage of using MIP microspheres is to provide large surface area for binding reactions while the MWCNT-AuNPs will enhance electron transfer during the electroanalysis of 17β-estradiol. The MIP microspheres for the specific determination of 17β-estradiol was prepared using 17β-estradiol as a template molecule, methacrylic acid (MAA) as a functional monomer, and ethylene glycol dimethacrylate (EGDMA) as a cross-linker. The microspheres were deposited onto the MWCNT-AuNPs modified SPE electrode. The presence of 17β-estradiol hormone in fish plasma or aquatic environments should be recognized by MIP microspheres via hydrogen binding between the hydroxyl (OH) functional group of 17β-estradiol with the oxygen at carbonyl (C=O) functional group of MIP. The proposed MIP based sensor provided an alternative but practical method in 17β-estradiol analysis.

Experimental

Chemical and apparatus

Multi-walled carbon nanotube (MWCNT), sulfuric acid (H_2SO_4), nitric acid (HNO_3), gold(III) chloride ($AuCl_3$), tri-sodium citrate, photoinitiator 2-2-dimethoxy-2-phenylacetophenone (DMPP), methacrylic acid (MAA), and cross linker ethylene glycoldimethacrylate (EGDMA) were purchased from Aldrich, while 17β-Estradiol (E) and testosterone were procured from Across. Sodium dodecyl sulfate (SDS), acetone and acetonitrile were purchased from Systerm, J.J Baker, and Merck, respectively.

Differential pulse voltammetry (DPV) experiment was performed using a potentiostat Autolab PGSTAT 12 (Autolab, Metrohm). Molecularly imprinted polymers (MIPs) nanospheres functionalized multi-walled carbon nanotube-gold nanoparticles and grafted onto screen-printed electrode (SPE) (Scrint Technology (M) Sdn. Bhd) was used as working electrode. Carbon pencil and Ag/AgCl electrodes (containing 3.0 M KCl) were used as auxiliary and reference electrodes, respectively. All potentials measured in this study refer to the Ag/AgCl electrode, and a homogeneous mixture of material solutions were prepared using sonicator bath Elma S30H.

Preparation of multi-walled carbon nanotubes grafted with gold nanoparticles (MWCNT -- AuNPs)

Multi-walled carbon nanotube (MWCNT) was prepared using strong acids, heating, sonication, and UV irradiation techniques according to the method reported by Saeedfar et al.,[29]. About 5.0 mg of unmodified MWCNT was added to 1 mL of quantified $H_2SO_4/HNO_3/H_2O$ (3:1:3) solution and sonicated carefully at 75 °C for 90 min to allow for oxidization to take place. The oxidized MWCNT was then sonicated for another 3 min under UV light at similar temperatures. The carboxylic-functionalized MWCNT was collected via centrifugation at 4000 rpm for 20 min, and neutralized via the addition of deionized water to a pH around 6.0. Then, the modified MWCNT was frozen and dried for 2 h. Approximately 1 mg of MWCNT was immediately mixed with 1 % $AuCl_3$ solution, and sonicated for 10 min at ambient temperatures. The mixture of MWCNT/$AuCl_3$ was diluted with 10 mL deionized water and heated using microwave irradiation for 3 min. Around 400 µL of tri sodium citrate (1%) was then added into the heated MWCNT/$AuCl_3$ solution and microwave-irradiated for another 5 min under boiling conditions. The MWCNT-coated gold nanoparticles were collected via centrifugation at 4000 rpm for 20 min and dried with the freeze-drying method.

Synthesis of MIP microspheres and 17β-estradiol absorption study

The molecularly imprinted polymer was synthesized via a photo-polymerization technique. A mixture of 68.1 mg 17β-Estradiol (E), 339.16 µL functional monomer methacrylic acid (MAA), 631.82 µL ethylene glycoldimethacrylate (EGDMA), 6.89 mg 2-2-dimethoxy-2-

phenylacetophenone (DMPP), 1 mg sodium dodecyl sulfate (SDS), and 20 μL deionized water was directly sonicated at room temperature for 10 min. The obtained emulsion was then photocured for 600 s with ultraviolet light under continuous nitrogen gas flow. Then, the templated 17β-estradiol into MIPs microspheres were finally collected using centrifugation at 13,000 rpm for 30 min, and carefully washed with K-phosphate buffer thrice and left to dry at ambient temperature. The produced MIPs were then divided into MIPs-A and MIPs-B. The size of MIP microspheres was recorded using a scanning electron microscope (SEM) (LEO 1450VP).

The obtained MIPs-A (30 mg) and MIPs-B (30 mg) were separately saturated with 1.0 mL of 17β-estradiol (2 mM) in acetonitrile solution for 48 h. The saturated MIPs-A and MIPs-B were then obtained from centrifugation at 10.000 rpm for 5 min, and thoroughly washed with deionized water thrice. The MIPs-A was air-dried overnight at room temperature and used as a control sample, while MIPs-B was directly incubated into fresh acetonitrile (1 mL) for 10 min and sonicated for 5 min to release 17β-estradiol from MIPs-B microsphere suspension. The suspended MIPs-B was collected with centrifugation at 13.000 rpm for 5 min and left to dry at room temperature for 16 h. Approximately 1 mg of dried MIPs microspheres were then suspended in 100 μL deionized water for further use in sensor fabrication.

Fabrication of 17β-estradiol sensor

The fabrication of 17β-estradiol sensor was begun with the deposition of 20 μL MWCNT-AuNPs (1 mg/450 μL) suspension in deionized water onto a carbon screen-printed electrode (SPE) and left to dry at ambient temperatures. Then, ~5 μL of MIP microspheres (MIPs-B) was depostied onto the MWCNT-AuNPs modified carbon paste screen-printed electrode (MIPs-MWCNT-

AuNPs-SPE). The newly prepared sensor was incubated in 300 µL of 17β-estradiol (2.0 mM) solution in deionized water and carefully rinsed with 0.05 M K-phosphate buffer solution (pH 7.0) to remove any remaining unbound 17β-estradiol. All the measurements of DPV peak current were conducted in 4.5 mL K-phosphate buffer (0.05 M, pH 7.0) solution at a temperature of 25 ^{o}C. Another sex hormone, 11-testosterone was also used to test for selectivity of the sensor.

Optimization of 17β-Estadiol sensor responses

The hormone sensor response was optimized to get the best working conditions for the detection of 17β-estradiol. MWCNT-AuNPs loading was optimized, from 0.11×10^{-4} to 5.56×10^{-4} mg, whilst MIPs were deposited between 0.1×10^{-3} and 2.5×10^{-3} mg onto MWCNT-AuNPs modified SPE surface. The optimization of the response time, MIPs was dipped into the 17β-estradiol solution from 5–90 min. The effect of temperature on the hormone sensor response was conducted by altering the temperature operation from 4–60 ^{o}C.

The evaluation of 17β-estradiol sensor

The 17β-estradiol sensor response was evaluated in a series of hormone concentrations from 1.0×10^{-6} to 1.0×10^{-16} M to obtain a linear response range and the lowest detection limit of the 17β-estradiol sensor. The electrochemical response was collected after 15 min of hormone reaction, starting at 25 ^{o}C. Additionally, the reproducibility of the sensor response was conducted using two different 17β-estradiol concentrations within the hormone sensor linear response range i.e. 1.0×10^{-7} and 1.0×10^{-9} M. For reusability, the hormone sensor was used by immersing the

hormone electrode into a 17β-estradiol (1.0×10^{-5} M) solution for 15 min at room temperature, followed by collecting the PDV peak current signal. The hormone sensor was dipped into a 400-μL acetonitrile regeneration solution for 15 min and rinsed vigorously with K-phosphate buffer (pH 7.0, 0.05M) for 60 s, which is quickly followed by current response measurement. Similar experimental procedures were repeated five times to regenerate the 17β-estradiol sensor. The stability of 17β-estradiol sensor was tested using 52 electrodes, which kept in a refrigerator at 4 ^{o}C. The DPV peaks current of the three units of the electrodes were periodically measured by using concentration of 1.0×10^{-5} M 17β-estradiol within 90 days of experimental period.

Determination of Asian arowana fish gender in real serum samples

To evaluate the feasibility of the hormone sensor in determining the gender of arowana fish based on the content of 17β-estradiol hormone concentration in the fish serum samples, 14 samples of juvenile arowana fish (*Scleropages formosus)* serum were utilized for the evaluation of the sensor. The samples were collected from the Freshwater Fisheries Research Institute, Glami Lemi, Negeri Sembilan, Malaysia. The use of the fish specimens is in accordance with regulations by IUCN Red List and the CITES, where only cultured specimens are allowed to be used for the studies. The use of the cultured specimens were also approved by the Department of Fisheries, Malaysia when the project involving Arowana fish was initiated. The fish serum was directly taken using the caudal puncture procedure in heparinised plastic Ependorf-type centrifuge tubes. The isolated fish plasma was aliquoted and kept at -20 ^{o}C. The serum was used to evaluate 17β-estradiol using the sensor. Approximately 9 μL of fish serum was diluted using 900 μL of acetonnitrile. The diluted serum was carefully sonicated for 15 min to release 17β-

estradiol hormone, and centrifugated immediately at 4000 rpm for 10 min at 4 ^{o}C. The extracted 17β-estradiol was then measured using the sensor, and each experiment was performed in triplicates at ambient temperatures. The gender determination of arowana fish based on content of 17β-estradiol quantity was performed using EIA immunoassay kit (Ann Arbor, MI, USA) for comparison purposes.

Results and Discussions

MIPs and its electrochemical characteristics on MIPs-MWCNT/AuNPs-SPE

The entire process of preparing MIP microspheres (MIPs) for the 17β-estradiol molecules is shown in Fig. 1. MIPs were traditionally prepared via the photo-polymerization method. The synthesis of MIPs involved mixing the template (E), monomer (MAA), cross-linker (EDGMA), initiator (DMPP) and surfactant (SDS), and directly emulsifying it via a sonication technique. The use of MAA as a functional monomer resulted in a donation of its carboxylic group acting as a proton donor and hydrogen bond acceptor, suitably providing for templates, such as 17β-estradiol containing Bronsted-basic or hydrogen bonding. The vinyl group of the cross-linker (EDGMA) could act as a stabilizer for the template-functional monomer complex in the polymerization process, and is mostly related to the provision of enough binding site accessibility and rapid mass transfers.[30] The size and morphology of the MIPs was determined using the SEM (Fig. 2 The size and morphology of the MIPs was determined using the SEM (Fig. 2). The photo-polymerization technique for the synthesis of MIPs produced particles

smaller than 2.0 μm. In view of the homogeneous size distribution of MIP microspheres, they were further used for the subsequent development of a 17β-estradiol sensor.

The DPV peak current response of the released 17β-estradiol hormone from MIPs was determined via the extraction method using acentronitrile solution, as illustrated in Figure 3. The highest DPV peak current signal was found for the MIP-A electrode. This indicated that the 17β-estradiol, as a template in the synthesis of MIPs, have been successfully produced via a simple polymerization technique. No current response was obtained for the MIP-B electrode, due to the 17β-estradiol hormone being removed by the extraction with fresh acetonitrile solution, demonstrating that almost all of the 17β-estradiol could be released from MIPs matrices. No sensor response was expected for the modified electrode alone without MIPs.

The cyclic voltammograms and differential pulse voltammograms of a hormone sensor post-immersion into a 17β-estradiol solution is described in Figure 4. In the presence of the 17β-esrtadiol, the electrode clearly demonstrated high current response compared to the modified electrode or bare SPE. This demonstrated that the 17β-estradiol hormone has absorbed chemically onto MIPs microspheres and formed hydrogen bonds between the hydroxyl (OH) group of 17β-estradiol with oxygen at the carbonyl (C=O) group of MIP. The binding interaction of 17β-estradiol to the MIP was selectively bonded with the imprinted selective sites from the polymeric backbone of MIPs matrices.[11] No sensor response was found for the electrodes without MIP microspheres due to the 17β-estradiol hormone not being chemically adsorbed onto the electrodes.

The optimization of analytical conditions

In order to determine the best condition of the hormone sensor response, parametric optimization were carried out including the quantities of MWCNT-AuNPs, MIPs, incubation time and temperature. As shown in Fig. 5A, DPV peak current response increased with the MWCNT-AuNPs loading increased from 0.11×10^{-4} mg to 2.22×10^{-4} mg, due to the increasing electron transfer rate from the 17β-estradiol sensors. When the MWCNT-AuNPs amount was deposited above 2.22×10^{-4} mg, the hormone sensor response declined due to the excess coverage of MWCNT-AuNPs on SPE surface, which limits electron transfer, resulting in lowered analytical performance of 17β-estradiol sensor response. In order to evaluate the effect of the amount of MIP microspheres on the modified electrodes, electrodes were loaded with various concentrations of MIPs, and the obtained results are illustrated in Fig. 5B. The current response increased as the MIPs loading increased from 0.1×10^{-3} mg to 1.0×10^{-3} mg, due to the larger amounts of MIPs on the modified electrode could increase the recognition sites of the electrode's surface. Further increase of the quantities of MIPs on the electrode surface from 1.0×10^{-3} mg to 2.5×10^{-3} mg resulted in declining DPV peak current response, due to the creation of an insulating layer that could impede electron transfer rates to the electrode's surface. Hence, the optimal MWCNT-AuNPs at 2.22×10^{-4} mg and MIPs at 1.0x10-3 mg were used to prepare the 17β-estradiol sensor.

For the response time effect, the electrochemical sensor response increased progressively with increasing incubation times, from 5.0–15.0 min (Fig. 5C), indicating increased quantities of 17β-estradiol attached to the MIP microspheres. The current response stabilized after 15.0 min of incubation time due to the imprinted selective sites of MIPs assumed to have fully occupied with the 17β-estradiol hormone via hydrogen bonding. On the other hand, the DPV peak current response gradually increased with the temperature, from 4–25 ^{o}C (Fig. 5D), indicating increased

reaction of 17β-estradiol with the MIPs matrices. The DPV peak current response declined sharply from temperatures beyond 40 ^{o}C due to the denaturation of the hormone structure of 17β-estradiol. Therefore, a response time of 15 min and a temperature at 25 ^{o}C were selected as the optimum incubation times and temperature for the 17β-estradiol sensor, respectively.

Analytical performance of the 17β-estradiol sensor

To investigate the analytical performance of the sensor for the determination of 17β-estradiol, the prepared electrochemical sensor was dipped in the 17β-estradiol solution at different concentrations, from 1.0×10^{-15} M to 1.0×10^{-6} M for 15 min. As shown in Fig. 6, the DPV peak current response proportionally increased with increasing 17β-estradiol hormone quantities due to the increased recognition sites of 17β-estradiol to the MIPs matrices on the surface electrode. The current response to 17β-esradiol concentrations were linear, varying from 1.0×10^{-15} M to 1.0×10^{-6} M and LOD was 2.5×10^{-16} M based on the 3σ values of blank signals.

A comparison of the proposed electrochemical sensor based on MIPs with other previously reported ones for the evaluation of 17β-estradiol is summarized in Table 1. The 17β-estradiol sensor based on MIPs-MWCNT-AuNPs screen-printed electrode showed large improvements in terms of sensitivity, linear response range, and detection limit compared to previously published electrochemical-based electrodes on MIPs modified gold nanoparticles[13], MIPs-functionalized graphene oxide[14] and MIPs modified mercaptonicotinic acid-platinum nanoparticles composites[15]. This probably contributed to the homogeneity and biocompatibility of the micro-

sized MIP microspheres that improved surface area of binding and diffusion of MIPs-MWCNT-AuNPs on SPE surface.

Table 1 A comparison between the sensor developed and other previously reported electrochemical sensors for the determination of 17β-estradiol.

Material and electrode design	Linear range (M)	LOD (M)	Response times (min)	Reference
MIPs-MWCNT/AuNPs-SPE	1.0×10^{-15}–1.0×10^{-6}	2.5×10^{-16}	15	This work
MIPs-AuNPs-GCE	3.7×10^{-15}–3.7×10^{-10}	3.7×10^{-15}	15	13
MIPs-rGO-PtNPs-GCE	4.0×10^{-9}–6.0×10^{-8}	2.0×10^{-9}	5	14
MIPs-MNA-PtNPs-GCE	3.0×10^{-8}–5.0×10^{-5}	1.6×10^{-8}	-	15

Note: Molecularly imprinted polymers (MIPs), multi wallet carbon nanotube (MWCNT), screen printed electrode (SPE), glassy carbon electrode (GCE), reduced graphene oxide (rGO), platinum nanoparticles (PtNPs), 6-mercaptonicotinic acid (MNA).

The selectivity properties of the fabricated sensors were performed to obtain the specificity of the hormone sensor in determining the content of 17β-estradiol hormone in environmental samples. The sensor was incubated at various concentrations of 17β-estradiol, 11-testosterone, and 17β-estradiol-11-testosterone mixture for 15 min. As illustrated in Fig. 7, the current response increased linearly with increasing 17β-estradiol and 17β-estradiol-11-testosterone concentrations, from 1.0×10^{-15} to 1.0×10^{-8} M, and the slope signal for 17β-estradiol and 17β-estradiol-11-testosterone were 100% (4.285 nA/decade, R^2 = 0.9923) and 99.58% (4.267 nA/decade, R^2 = 0.9728), respectively. There are no significant differences between 17β-

estradiol and 17β-estradiol-testosterone mixture current responses. This implies that the 11-testosterone mixed into 17β-estradiol did not demonstrated interference to the electrochemical sensor responses. The proposed sensor was also exposed to various concentrations of 11-testosterone solution (Fig. 6), resulting in a slope value of 28.33% (1.214 nA/decade, R^2 = 0.8129), relative to the slope 17β-estradiol response. The developed sensor exhibited a low response to the 11-testosterone hormone, due to the structural similarity of 11-testosterone and 17β-estradiol prompting MIP microspheres to absorb low amount of 11-testosterone, however, a functional group differences in the ability of different DPV current is also evident. With the observed low response to 11-testosterone, it can be said that the proposed hormone sensor is highly selective to 17β-estradiol hormone.

Reproducibility, reversibility and stability studies

The reproducibility of the sensor was evaluated by measuring the current response of 10 different electrodes towards the same 17β-estradiol concentration. The electrochemical sensor response was observed at two analyte concentrations, i.e. 1.0×10^{-9} M and 1.0×10^{-11} M 17β-estradiol. The obtained reproducibility RSD values of sensor were in the range of 1.8-4.7 (n=5). These RSD values were low enough for us to regard the MIPs as reproducible utilizing the developed method in the present work.

In order to evaluate the sensor response after incubation into a regeneration solution and return to baseline response, the treated sensor was made to undergo generation study. The regeneration profile of the sensor is illustrated in Fig. 8. The sensor response decreased by 38.19–40.83% of its initial response after being immersed in absolute acetonitrile for 15 min.

This indicated that the 17β-estradiol hormone were desorped from MIP in the presence of acetonitrile. When the sensor was re-incubated with 1.0×10^{-6} M 17β-estradiol for 15 min, the sensor was capable of retaining its initial response with a reversibility RSD (relative standard deviation) values in the range of 1.48–3.64% (RSD, *n=5*). This implied that the sensor response is reversible, and could be re-used to determine the concentration of 17β-estradiol in aquatic samples.

The lifespan of 17β-estradioal sensor is > 90 days as described in Fig. 9. The 17β-estradiol sensor response maintained at 82.9 % of its initial response after 50 days of storage period at 4^{o}C, and the sensor response decreased to 54.9–25.8 % relative to its original response between 60 days and 90 days of storage time. This indicates that the sensor possesses good stability.

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Evaluation of 17β-estradiol in arowana fish serum samples

The Arowana fish, a highly priced ornamental fish found in South-East Asia, is known to be difficult in sex identification, especially at juvenile stage. The sex determination has important application for the breeding of the fish for commercial purposes. The MIPs-based hormone sensor was used to determine the 17β-estradiol content of the arowana fish serum in order the establish the gender of the fish. A total of 14 samples of arowana fish serum were evaluated by the sensors. The performance of the sensor and the immunoassay kit method for the determination of 17β-estradiol and hence the gender of arowana fish was summarized in Table 2. The results after statistical testing showed good agreement between those obtained from 17β-estradiol sensor and those from conventional immunoassay method for the determining 17β-estradiol in the serum samples of arowana fish. Hence this leads to confirmation of the gender of

the fish of all samples when compared with standard test kit using immunoassay method. Therefore, the proposed amperomentric sensor has potential for the evaluation of the gender of arowana fish based on determination of 17β-estradiol hormone.

Table 2.

The performance of the MIP based sensor for the evaluation of 17β-estradiol in arowana fish to determine the gender using serum samples and comparison with EIA kit method (n=3).

No	Samples	Sensor method					EIA Kit method
		Current (nA)	RSD	Baseline current±SD	*t test*	Gender	
1	Sample 6	14.70±0.99	6.78	19.80±1.63	14.812**[a]	M	M
2	Sample 12a	12.37±1.19	8.23	19.80±1.63	16.409**[a]	M	M
3	Sample 12b	15.39±1.20	7.83	19.80±1.63	3.834**[a]	M	M
4	Sample 13	12.70±1.12	8.82	19.80±1.63	13.954**[a]	M	M
5	Sample 15	37.45±1.62	4.33	19.80±1.63	22.044**[b]	F	F
6	Sample 16	18.13±1.38	7.59	19.35±1.38	0.865	M	M
7	Sample 17	34.11±1.08	3.15	19.35±1.38	19.951**[a]	F	F
8	Sample 18a	43.05±1.86	4.31	19.35±1.38	55.242**[a]	F	F
9	Sample 18b	43.20±2.34	5.42	19.35±1.38	55.658**[a]	F	F
10	Sample 18c	33.93±1.92	5.66	19.35±1.38	18.031**[b]	F	F

11	Sample 19a	11.63±0.98	8.40	20.77±0.99	11.787**[a]	M	M
12	Sample 19b	15.09±1.06	7.05	20.77±0.99	5.695**[a]	M	M
13	Sample 20	12.64±0.99	7.80	20.77±0.99	10.536**[a]	M	M
14	Sample 21	30.75±2.26	7.33	20.77±0.99	9.454**[b]	F	F

Note: **[a] – Significant lower than baseline current indicates male fish, **[b] - Significant higher than baseline current indicates female fish, critical value, $t_4 = 2.78$ ($p = 0.05$, 95%), baseline current based on Na-phosphate buffer (0.05M, pH 7.0), F = female and M = male.

Conclusions

A novel electrochemical sensor for the evaluation of 17β-estradiol hormone based on a carbon screen printed electrode (SPE)-modified with multi-walled carbon nanotube (MWCNT) grafted with gold nanoparticles (AuNPs) and deposited with molecularly imprinted polymers was successfully fabricated. The immobilized MIP microspheres could absorb the 17β-estradiol hormone from biological samples and detected by differential pulse voltametry current measured at 0.6 V. The mono-dispered MIP microspheres allowed for the homogenous diffusion of analytes in within the electrode materials, which significantly improved the hormone sensor performance in terms of detection limit, linear response range, response time, reproducibility regenerability, and long-term stability. The analytical performance of the estradiol sensor is comparable with the conventional immunoassay kit technique for the detection of hormone applied to the determination of the gender of arowana fish in serum samples. Therefore, the proposed amperomentric sensor has potential for the evaluation of 17β-estradiol hormone.

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List of Figures

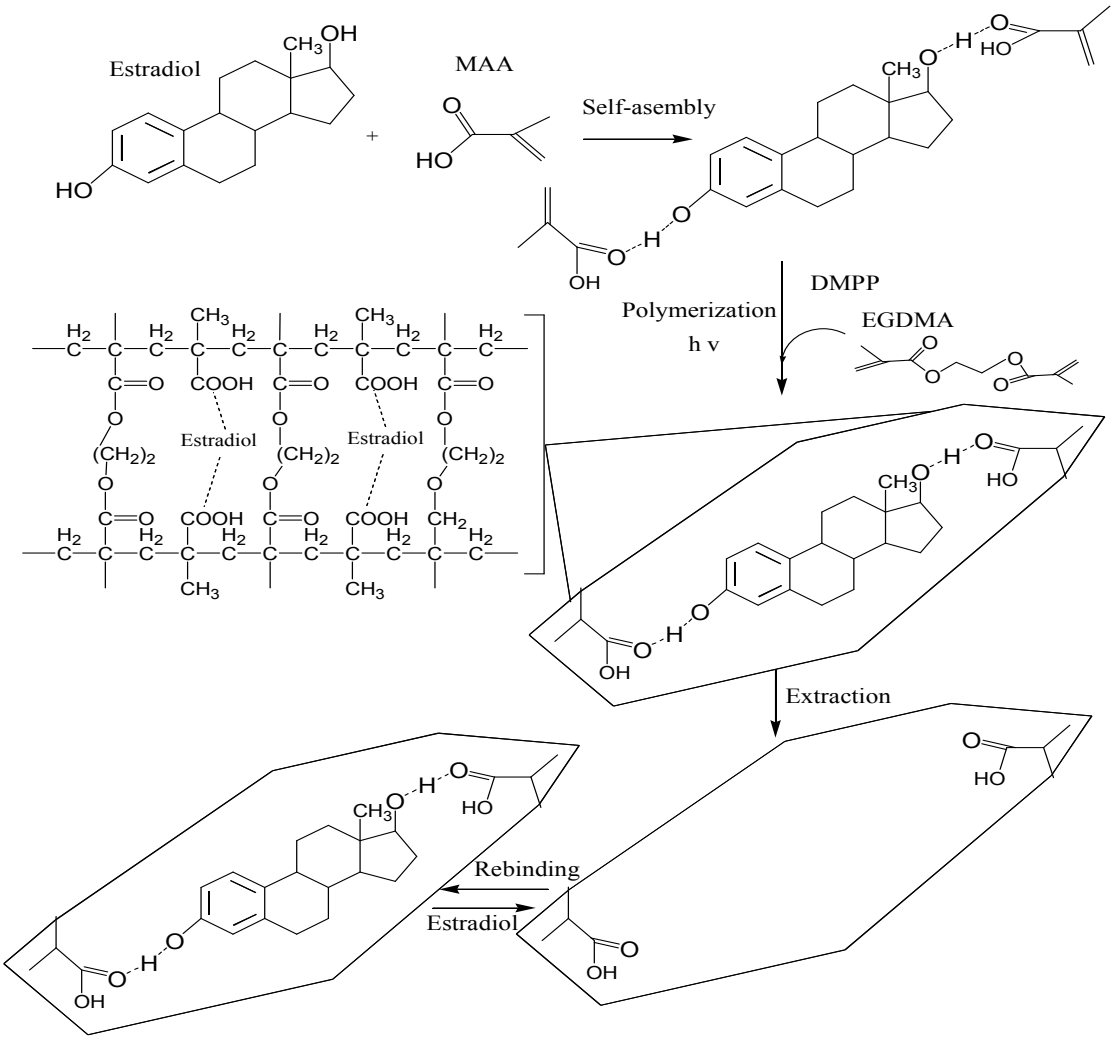

Fig. 1 Schematic illustration of preparing MIPs for the 17β-estradiol molecules.

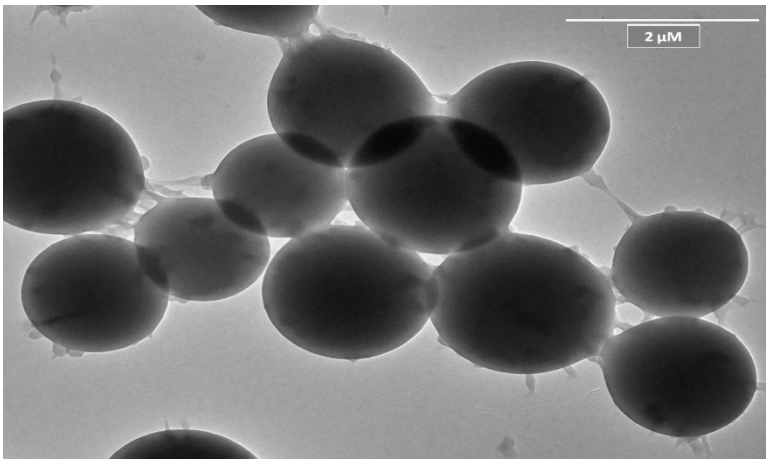

Fig.2 Morphology of molecularly imprinted polymeric microspheres captured using SEM.

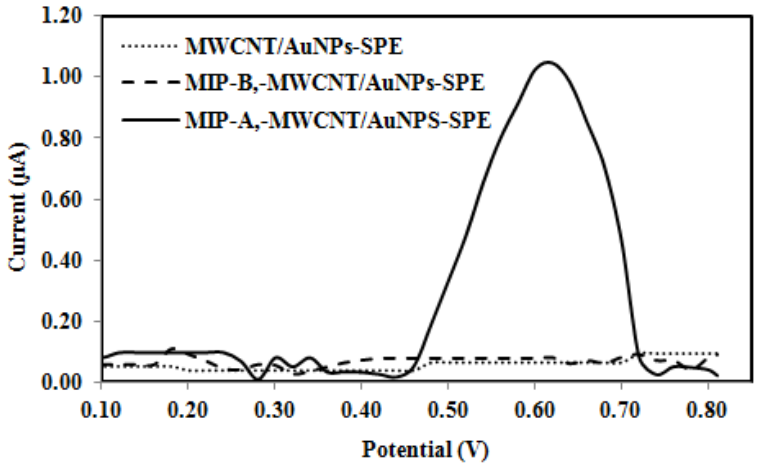

Fig. 3 Different pulse voltammograms of the MWCNT/AuNPS-SPE, MIP-B-MWCNT/AuNPs-functionalized SPE, and MIP-A-MWCNT/AuNPs-SPE measured in 0.05 M K-phosphate buffer solution (pH 7.0).

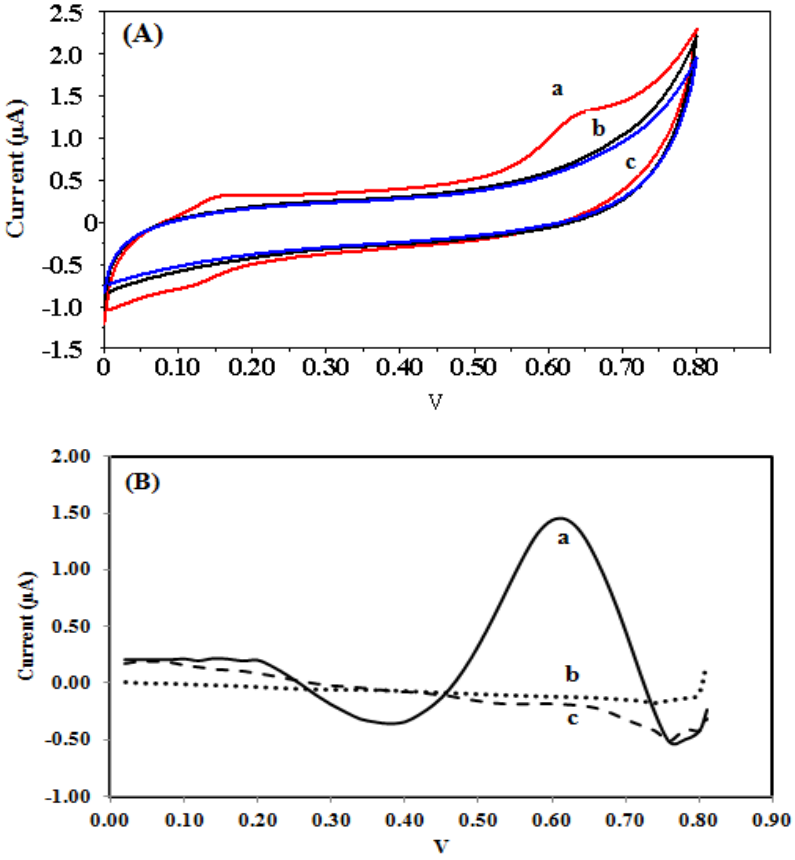

Fig. 4 Cyclic voltammograms (A) and differential pulse voltammograms (B) current response of MIP-B-MWCNT/AuNPS-SPE (a), MWCNT/AuNPs-SPE (b) and bare SPE (c) incubated in 1.0×10^{-5} M of 17β-estradiol in acetonitrile solution. The sensor response was measured in 0.05 M K-phosphate buffer at pH 7.0.

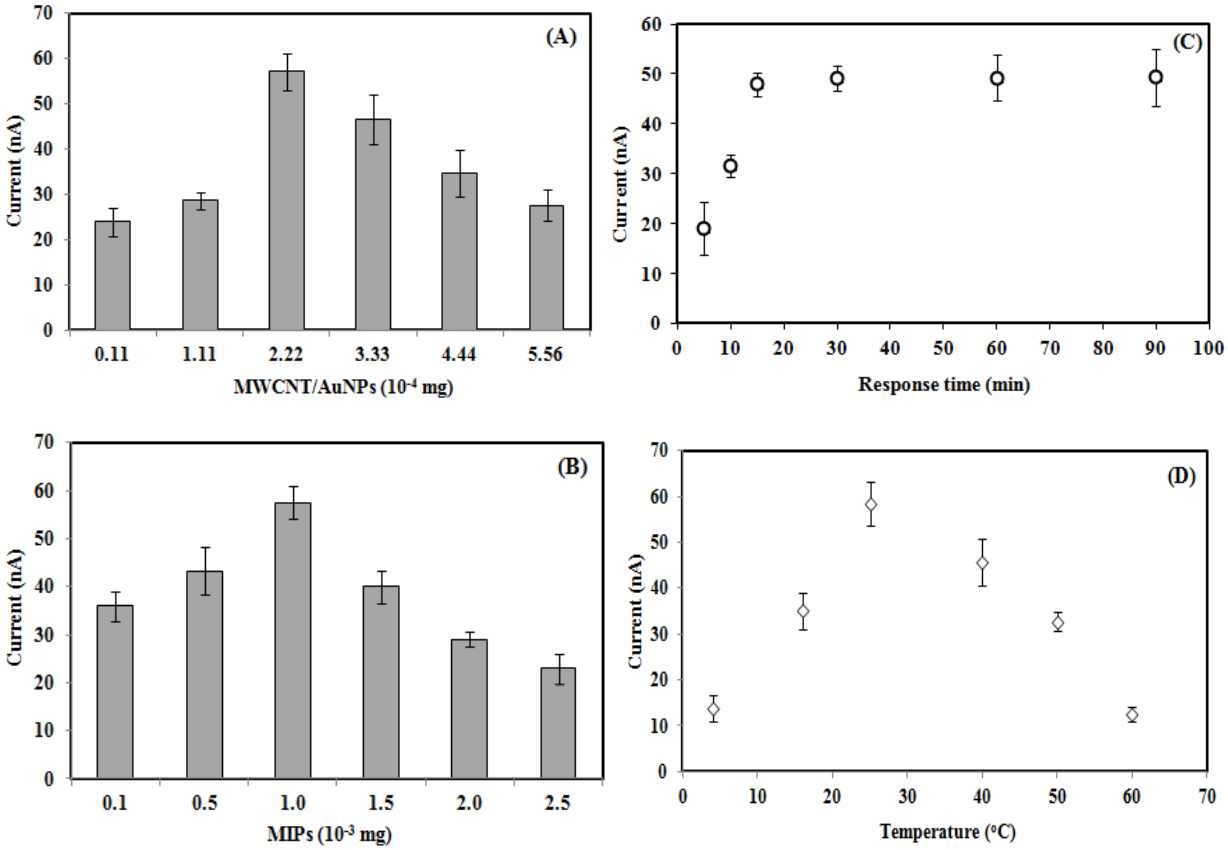

Fig. 5 Effect of the MWCNT/AuNPs loading (A), MIPs quantities (B), response time (C) and temperature (D) toward the DPV current hormone sensor in the presence of 1.0×10^{-5} M 17β-estradiol.

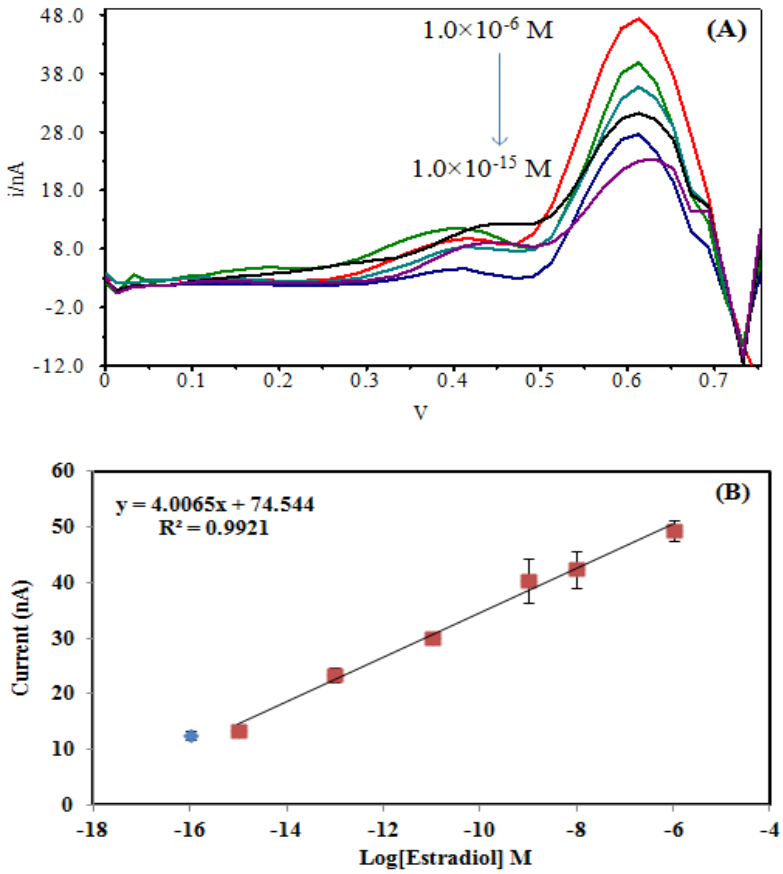

Fig. 6 The differential pulse voltammograms (A) and calibration curve (B) of the electrochemical sensor obtained from different 17β-estradiol concentrations at the potential of 0.6 V with incubation times of 15 min.

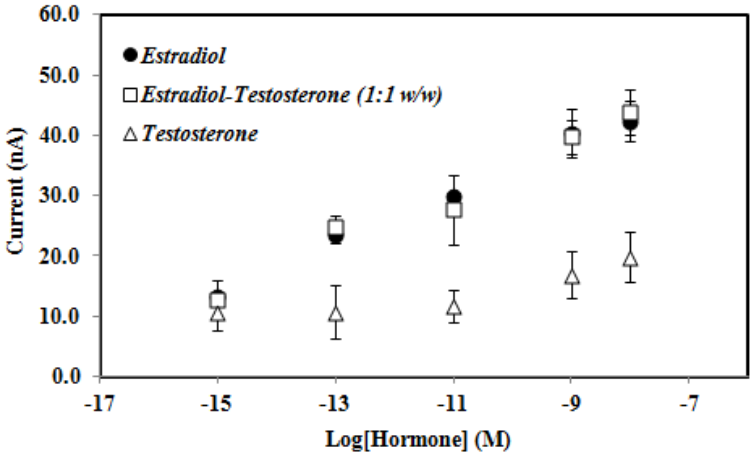

Fig. 7 Selectivity properties of the MIPs biosensor that incubated in various concentration of 11-testosterone and 17β-estradiol hormone (1.0×10^{-15} – 1.0×10^{-8} M) for 15 min.

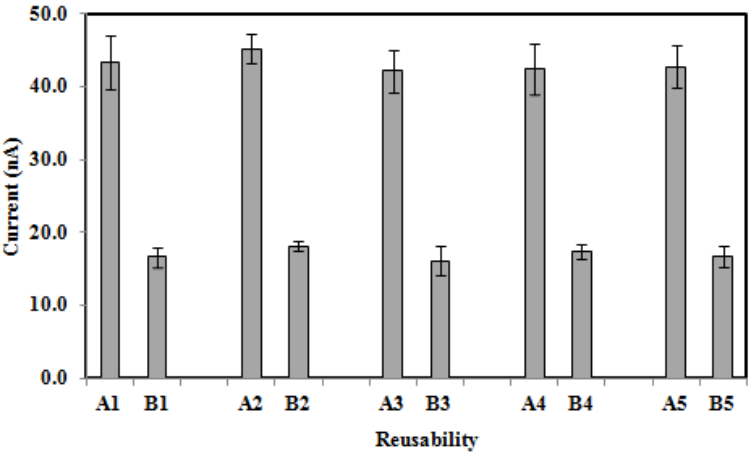

Fig. 8 The alternate reaction of MIP with (A) and without (B) of 1.0×10^{-5} M 17β-estradiol in acetonitrile as regeneration solution.

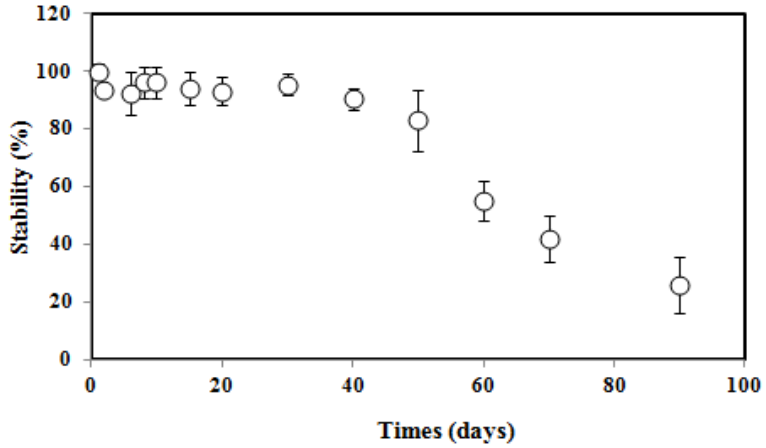

Fig. 9 The long-term stability of the MIPs biosensor response towards 1.0×10^{-6} M 17β-estradiol for 90 days at 4°C.