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Full Paper

Electrochemical Determination of Human Growth Hormone (HGH) Utilizing A Pyrrole (Py) Molecularly Imprinted Polymer (MIP) On Screen-Printed Carbon Electrode (SPCE)

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Abstract- A new electrochemical biosensor based on Fe_3O_4 nanoparticles and molecularly imprinted polymer-modified screen-printed carbon electrode (SPCE) was built to record the HGH molecules. The HGH is an important metabolic hormone and is responsible for body growth, stimulating the growth and differentiation of muscle, bone, and cartilage. A stable SPCE/Fe3O4/Poly-pyrrole/MIP layer was formed by the electro-polymerization of pyrrole molecules as functional monomers and HGH as template molecules. The accuracy and sensitivity of the prepared SPCE were confirmed using the electrochemical impedance spectroscopy (EIS) technique. According to the results, the MIP showed a linear range of 1×10^{-6} to 1×10^{-11} g/cm^3 for the HGH under study conditions with a limit of detection (LOD) of 2.927×10^{-12} g/cm^3. The calibration curve showed 99.9% correlation of the data. It can be concluded that the MIP made for HGH molecules has high sensitivity and stability, low cost, short response time, and excellent reproducibility.

Keywords- Screen-printed carbon electrode (SPCE); Electrochemical impedance spectroscopy (EIS); Human Growth Hormone (HGH); Molecularly imprinted polymer; Electropolymerization

1. INTRODUCTION

Molecularly imprinted polymers (MIP) are well-known compounds as artificial receptors, with high chemical stability, easy synthesis, and low cost. The selectivity of interactions established between printed sites and target molecules in MIPs provides a remarkable substitute for the natural receptors [1,2]. Molecularly imprinting electrochemical sensors are used to rapidly estimate and identify small hazardous molecules to bio-macromolecules. They are highly sensitive diagnostic elements in chemo/biosensors based on direct analytical techniques for high sensitivity and good selectivity in scientific research aims [3]. These chemo-sensing devices offer advantages compared with traditional methodologies in terms of design, simplicity, and low cost. Other advantages are their robustness, easy miniaturization, and suitability for small-volume analysis [4,5].

Screen-printed electrodes (SPEs) are used as the sensing portable device for on-site and point-of-care detection [5]. Screen-printing technology is applied to produce the necessary electrodes involved: reference, working, and auxiliary electrodes. The SPEs are electrochemical transducers that have a very low cost and permit the miniaturization of sensors as a chip [6]. Hence, electrochemical sensors based on SPE are becoming of increasing interest for providing compact, portable, and sensitive systems for rapid detection [7].

Pyrrole is one of the main and widely utilized monomers in the production of conductive polymers. It is also used for the development and advancement of various electrochemical sensors and biosensors with different template molecules [8].

Human growth hormone (HGH) has been known as an important metabolic hormone and is responsible for body growth. Its ability to stimulate the growth and differentiation of muscle, bone, and cartilage is well known. In addition to the pituitary gland, HGH is synthesized within many other tissues, including the hematopoietic system [9]. HGH is secreted higher at night than during the day and mediated by a decrease in tonic hypothalamic somatostatin secretion [10]. Many factors including age, sex hormones, and nutritional status can affect the quality and quantity of its secretion [11,12].

Up to now, many analytical techniques such as gas chromatography with mass spectrometry, RIA (Radioimmunoassay), FRET (Fluorescence Resonance Energy Transfer), and ELISA (Enzyme-linked immunosorbent assay) are used to measure hormones [13]. However, they mostly suffer from restrictions such as long operational steps, requirements of complex apparatuses, experienced operators, radioisotopes, and so on [14]. Therefore, several research has been devoted to the development of methods that can be miniaturized and used cheaply for rapid tests. For this purpose, electrochemical sensors based on molecular imprinting techniques have recently been considered by researchers [15-19]. Fe_3O_4 nanoparticles have gained enthusiastic attention from many researchers due to their unique properties in the nanoscale, such as a large surface area, high surface energy, good biocompatibility, high absorption, and the transfer of electrons. In particular, due to their non-

toxicity property, and high chemical stability, and ease of produce on a large scale. Fe_3O_4 nanoparticles have great potential to achieve convenient properties for biomedical applications due to their biocompatibility, it is known that the human body contains around 3 g Fe within the proteins like ferritin, hemosiderin, transferritin, and hemoglobin [20-22].

In our previous work, a new molecularly imprinted electrochemical was constructed for the detection of Human Growth Hormone (HGH) [28]. The next step in that project was to miniaturize and implement it on the SPCE, but due to the fact that the reference electrode used on the SPCE was made of silver and on the other hand relatively concentrated sulfuric acid was used to polymerize aniline, this caused the destruction of the SPCE then It was not possible continued the work with aniline. Therefore, we decided to use another monomer whose polymerization conditions are compatible with the SPCE. Therefore, in this work, Human Growth Hormone and Pyrrole were considered as the template and monomer molecules, respectively. To the best of our knowledge, this work is the first report on the construction of an electrochemical sensor based on molecularly imprinted polymers on Fe_3O_4 modified SPCE for HGH detection.

2. EXPERIMENTAL SECTION

2.1. Chemical reagents

Somatropin (CinnatropinTM) was obtained from CinnaGen Co. (Tehran, Iran). Magnetite (Fe_3O_4) NPs were synthesized by alkaline hydrolysis of iron (II), iron (III) salts $(NH_4)_2Fe(SO_4)_2$ and $FeCl_3 \cdot 6H_2O$ were from (Sigma-Aldrich). Ammonia 25% (NH_3), Hydrazine hydrate ($N_2H_4.H_2O$), potassium hexacyanoferrate III $K_3[Fe(CN)_6]$, ethanol 99%(CH_3CH_2OH), sodium phosphate dibasic(Na_2HPO_4), potassium phosphate monobasic (KH_2PO_4), pyrrole(Py), sulfuric acid 99.9% (H_2SO_4), oxalic acid 99% (Oac), hydrazine monohydrate ($N_2H_4. H_2O$), potassium chloride (KCl), Bovine serum albumin (BSA), casein and urease were purchased from Sigma-Aldrich. Phosphate buffer (PB) (0.1 M, pH 7.2) was used for the dilution of samples. Millipore water purification system (18 MΩ, Milli -Q, Millipore) was utilized for the preparation of deionized or ultrapure water. The stock solution of Fe_3O_4 NPs was prepared with a concentration of 4 mg/ml.

2.2. Instruments

For the detection of HGH molecules screen-printed electrodes (DRP C-110) were purchased from Drop Sens (Spain). Screen-printed carbon electrodes (SPCEs) included a disc-shaped carbon working electrode, a carbon counter electrode, and the silver reference electrode that were connected to the electrochemical instrument using a specific connector. Square wave voltammetry (SWV) and cyclic voltammetry (CV) were manufactured by Sama Electro

Analyzer System (Esfahan, Iran), and electrochemical impedance spectroscopy (EIS) data were obtained utilizing a potentiostat Autolab PGSTAT10 (Eco Chemie B.V., Utrecht, Netherlands).

2.3. Synthesis of Fe3O4 –Nanoparticles

Fe_3O_4 nanoparticles were synthesized by a co-precipitation method [29]. Typically, 1.748 g of $(NH_4)_2Fe(SO_4)_2.6H_2O$ and 2.162 g of $FeCl_36H_2O$ (molar ratio 2:1) and 10.0 ml of hydrazine hydrate were dissolved in 50 ml of water. 20.0 ml of ammonia solution (27% w/v) was then added immediately. The mixed solution was stirred for 30 minutes at room temperature, and then was heated to 80 ℃ and kept for 1 hour [30]. The Fe_3O_4 nanoparticles were separated from the solution and washed with ultrapure water.

2.4. Solutions

Stock solutions of HGH (2.5 g/cm^3), BSA, Casein and Urease (1 g/cm^3), and PBS (1M) were prepared by dissolving the appropriate amount in PBS/ultrapure water and diluted to the required volume using PBS (0.2 M). The supporting solution (as a probe) (4 mM) $[Fe(CN)6]^{3-/4-}$ with (0.1 M) KCl was also prepared.

2.5. Preparation of modified screen-printed Carbon electrode (SPCE)

Prior to electrode modification, the SPCEs were washed with ultrapure water followed by electrochemical cleaning with CV at a potential range of -0.3 to +0.3 V for about 20 cycles at a scan rate of 0.1 V/S in 0.05 M H_2SO_4 [31]. Then 10 mm^3 of Fe3O4 nanoparticles (4 g/cm^3) were dropped onto the surface of the working electrode of SPCEs and dried in ambient air.

2.6. Synthesis of Molecular Imprinted Polymers (MIPs)

MIPs were prepared as follows, 100 µl of Human Growth Hormone was dissolved in 86 mm^3 PBS, 14 mm^3 of Pyrrole was then added to the solution. The mixture was stirred for 10 seconds leading to the pre-assembling solution. This was followed by drooping 70 mm^3 of the prepared solution into modified SPCE for 30 min in a moisture chamber at ambient temperature. The pre-assembling between monomer and template molecules was completed in this way. The electro-polymerization was carried out using the cyclic voltammetry technique at a scan rate of 0.05 V/S between -0.8 to +0.8 V for 4 cycles. To remove HGH molecules, 70 mm^3 of H_2SO_4 (1 M) was dropped onto the synthesized sensor and incubated for 1 h in the moisture chamber. The same manner was also performed to prepare a non-imprinted polymer (NIP) excepting the presence of Human Growth Hormone molecules.

2.7. Electrochemical measurements

The electrochemical analysis technique was done as follows, 4 mM $[Fe(CN)_6]^{3-/4-}$ with 0.1 M KCl (70 mm^3) was dropped onto the SPCE as a supporting solution. In the next step, CV measurements were recorded at a potential range (-0.2 to +0.6 V). The electro-polymerization of SPCEs/Fe_3O_4@Ppy/MIP was performed in a potential range of -0.8 to +0.8 V with a scan rate of 0.05 V/S for 4 cycles. SWV measurements were done in a potential range of -0.2 to +0.6 V, pulse amplitude of 0.05 V with a frequency of 3 Hz. Electrochemical Impedance Spectroscopy (EIS) was performed at a potential of 0.2 V within a frequency of 0.1 to 100 KHz in 4 mM $[Fe(CN)_6]^{3-/4-}$ with 0.1 M KCl solution. Each of the following post-polymerization treatments was done in a similar manner on NIP SPCEs as a control in order to confirm the success of the inferential procedure tested.

3. RESULTS AND DISCUSSION

3.1. Synthesis of SPCE@Fe_3O_4/MIPs NPs

Electrochemical measurements were carried out according to the following manner: The whole process was done in five steps: (1) the working electrode of SPCEs was cleaned and activated by electro-polymerization in the solution of PBS (0.2 M), (2) the Fe_3O_4 nanoparticles were dropped onto a disc-shaped section of SPCE (working electrode) to prepare easily imprinted polymers, (3) electro-polymerization of pyrrole(Py) and HGH molecules on modified SPCE (1.25 g/cm^3 of HGH was prepared in 0.2 M PBS (pH 6.92) and then 4 mM Py was added to the mixture. After 30 minutes of incubation, the cyclic voltammetry was applied from -0.8 to +0.8 V at a scan rate of 0.05 V s^{-1} for 4 cycles.), (4) removal of the target molecule (HGH) by H_2SO_4 solution (1 M) and (5) rebinding of different concentration of HGH molecules and subsequently the square wave voltammograms were recorded from -0.2 to +0.6 V, in 4 mM solution of $[Fe(CN)_6]^{3-/4-}$ with 0.1 M KCl [32,33].

3.2. Characterization of MIPs/Ppyr/Fe_3O_4/SPCE

The morphology of synthesized Fe_3O_4 nanoparticles and MIP/ Fe_3O_4 were evaluated by scanning electron microscopy (SEM) and field emission scanning electron microscopy (FE-SEM) which was presented in Figure 1. As seen, the MIP (Figure 1B) has a large size, around 97.3 nm, while Fe_3O_4 NPs nanoparticles are about 37 nm in size, this difference in size indicates the formation of polymer on nanoparticles. Characterization of the existence of functional groups on the surface of the synthesized MIP/Fe_3O_4 NPs was done utilizing FTIR between the frequency ranges of 4000–400 cm^{-1}. In the FTIR spectrum of MIP/Fe_3O_4 NPs, the peaks at 1400 cm refer to the fundamental vibration of the pyrrole rings, the bands observed at 1152, 1091 cm^{-1} relate to the =C–H in-plane deformation vibration, the peak at 527 cm^{-1} corresponds

to the Fe –O stretching vibration of Fe_3O_4, and the peak at 1637 cm^{-1} is attributed to C= O/C–N stretching vibration of amide.

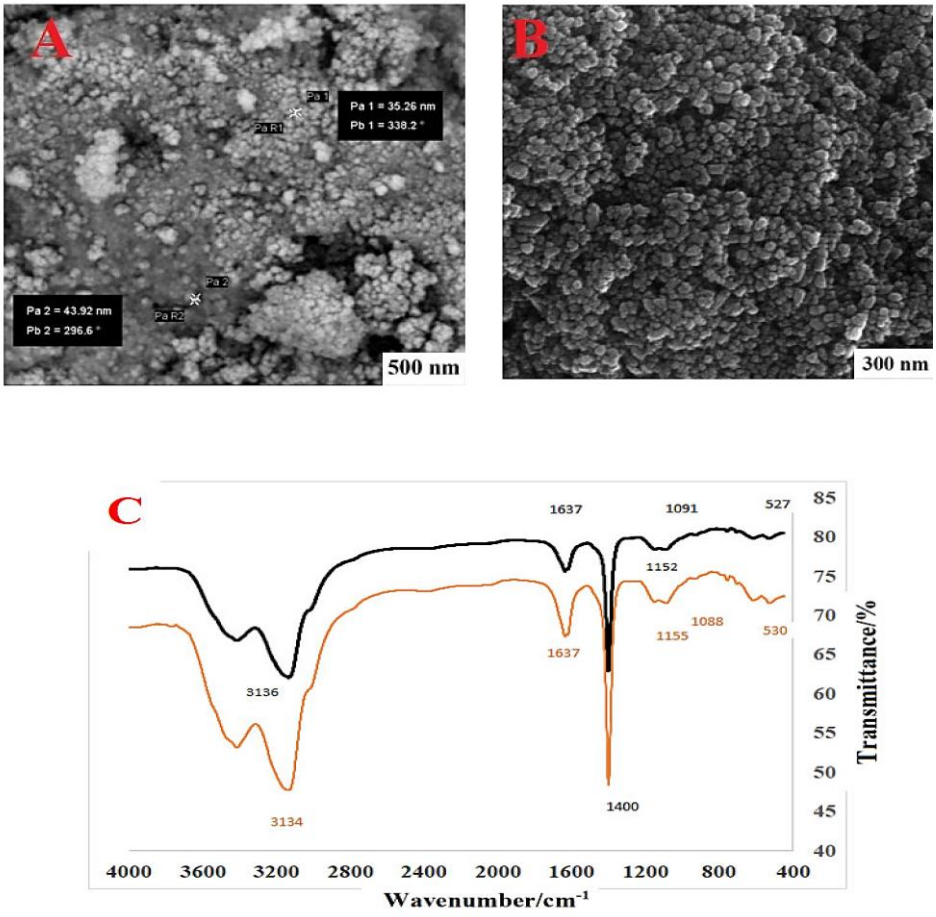

Figure 1. SEM image of (A) synthesized Fe_3O_4 nanoparticles, (B)FESEM image of Human Growth Hormone@MIPs (C) FTIR spectra of MIP/Fe3O4 NPs/SPCE and NIP/Fe_3O_4 NPs/SPCE

Moreover, it was worth noting that the weak characteristic peak of Fe_3O_4 should be due to the presence of MIP/Ppy coating layer on Fe_3O_4, which further indicated that Fe_3O_4 nanoparticles were encapsulated inside MIP/Ppy shell. FTIR results strongly confirm the formation of MIP/Fe_3O_4 NPs nanoparticles. Figure 1 C, the presence of the Human Growth Hormone molecules in the MIP/Fe_3O_4 NPs was indicated by the shifting of the peak at 3134 from 3136 cm^{-1} which is assigned to the formation of an amide bond [34]. Also, change in the peak position was found (3413 to 3416, 1618 to 1621 cm^{-1}, 1155 to 1152 cm^{-1}, and 1093 to 1088 cm^{-1}), and also the flattening of the peak between 1091 and 527 compared to the level peak in the NIP also indicates that the hormone is trapped in the polymer layer. this change indicates that the HGH has been layered to MIP/Fe_3O_4 NPs [35].

3.3. Evaluating by cyclic voltammetry technique and square wave voltammetry

CV technique was used to investigate the different steps of MIP formation, removal, and rebinding of Human Growth Hormone molecules. As evident in Figure 2, after the formation of MIP on the surface of Fe_3O_4 NPs (curve 4) and after template molecule rebinding (curve 3) due to coating the surface of the SPCE and cavities of the surface with HGH molecules the lowest height of the current peak was observed. on the other hand, the bare SPCE (curve 1) and template removal (curve 2) MIPs have the highest current peak respectively. Thus, the CV is able to show slight changes in electrochemical activity, conductivity, and any variables that can be detected by electrochemical probes. In addition, SPCE surface coating changes the $[Fe(CN)_6]^{-3/-4}$ redox potential [36].

The SWV plot of the peak current of the probe confirms that after electro-polymerization (curve 4) and after HGH molecule rebinding (curve 3), MIP/Fe_3O_4/SPCE has the lowest height (insert Figure 2). This, obviously, is due to a decrease in the number of available cavities for probe molecules. The stepwise of MIP/fabrication was investigated by CV.

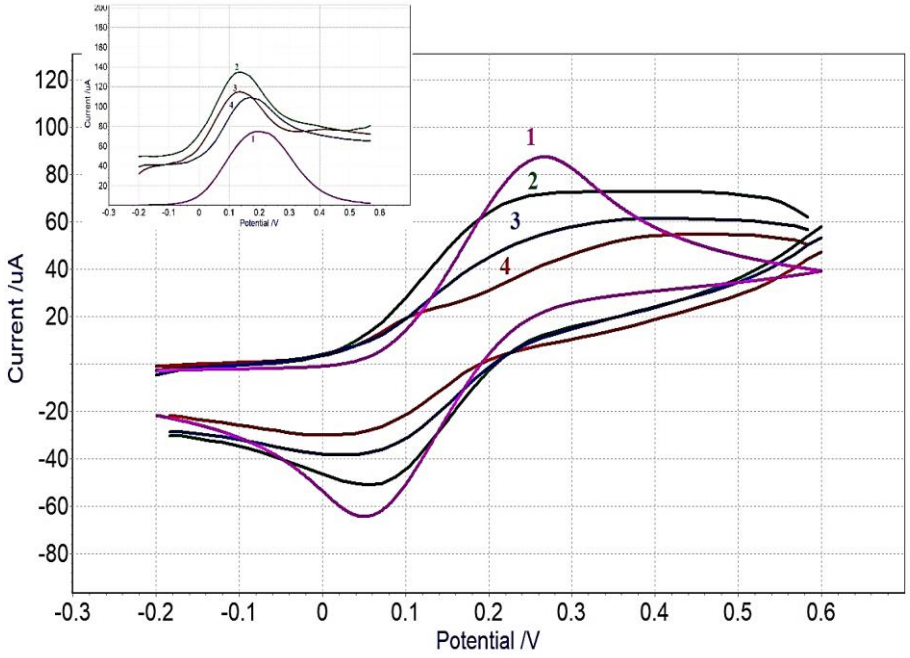

Figure 2. The CV and SWV (inset Figure) voltammograms of; (1) bare SPCE, (2) template removing MIPs/Ppyr/Fe_3O_4/SPCE, (3) template rebinding of MIPs/Ppyr/Fe_3O_4/SPCE and (4) MIPs/Ppyr/Fe_3O_4/SPCE, (insert Figure) SWV profile of the sensor; (1) bare SPCE; (2) electro-polymerized probe after Human Growth Hormone molecules removal (3) electro-polymerized probe after rebinding with 100 µg/L template and (4) MIPs/Ppyr/Fe_3O_4/SPCE probe; experiments were done in $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ solution (5 mM) prepared in KCl (0.1 M).

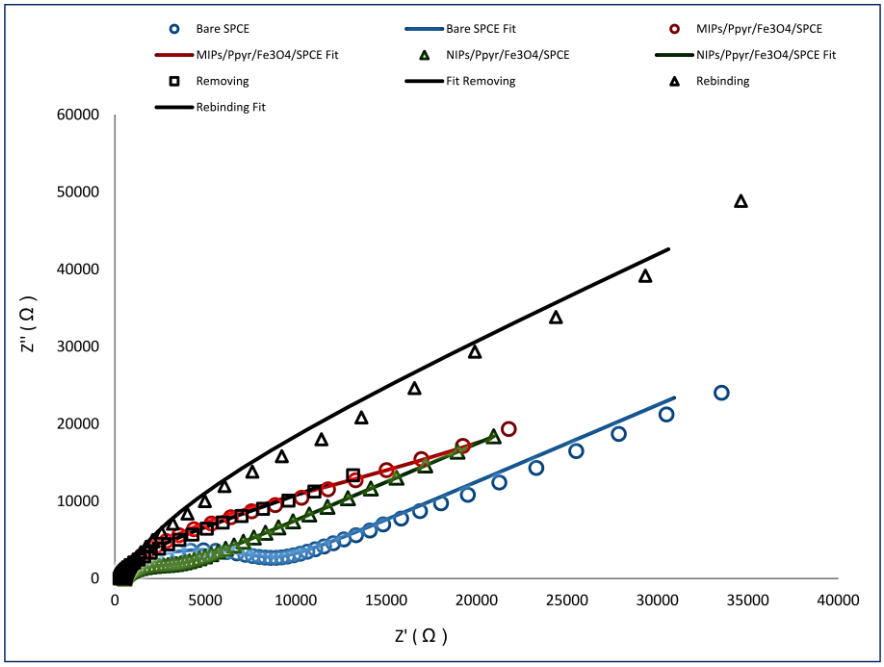

Figure 3. The Nyquist plots of Bare SPCE, MIPs/Ppyr/Fe_3O_4/SPCE, NIPs/Ppyr/Fe_3O_4/SPCE, MIPs/Ppyr/Fe_3O_4/SPCE template removal and MIPs/Ppyr/Fe_3O_4/SPCE template rebinding

3.4. Evaluating by Electrochemical Impedance Spectroscopy (EIS) Technique

EIS is currently a common technique for investigating electron transfer kinetics at the electrode surface. EIS typically includes a semicircular portion at higher frequencies and a linear portion at lower frequencies related to the finite electron transfer process and the diffusion process [24,37]. Semicircle diameter demonstrates load transfer resistance (R_{ct}) $[Fe(CN)_6]^{-3/-4}$ probe redox.

Table 1. Results from the fitting of the impedance test results

Samples	R_s ($\Omega.cm^2$)	Q ($S.s^n/cm^2$)	n	R_{ct} ($\Omega.cm^2$)	C_{dl} ($\mu F/cm^2$)	W ($S.s^{0.5}/cm^2$)
Bare SPCE	509.7	0.00000159	0.9532	7241	2.53	0.0001208
MIPs/Ppyr/Fe3O4/SPCE	512.3	0.0001307	0.9081	13680	345.34	0.0001744
NIPs/Ppyr/Fe3O4/SPCE	509.8	0.00001742	0.9021	4117	43.62	0.000162
MIPs template Rebinding	511.9	0.0001431	0.9244	14740	319.83	0.0001832
MIPs template Removing	510.5	0.0002283	0.9088	9866	581.17	0.0002064

As is clearly shown in Figure 3 After electro-polymerization, the MIP/SPCE has the highest R_{ct} (13680 Ω.cm^2) than the Bare SPCE and NIP, due to the presence of the HGH molecules and the formation of the thick films. However, R_{ct} of NIPs/Ppyr/Fe_3O_4/SPCE (4117 Ω.cm^2), despite the formation of electro-polymerization films, in comparison with the Bare SPCE (7241 Ω.cm^2), show the lowest R_{ct} [38]. This is due to the fact that poly-pyrrole films formed are uniform, free of the HGH molecules together with very high conductivity.

Equivalence of measured samples with electrochemical equivalent circuits was done by Zsimps software. The results of the fitting of this equivalent circuit are shown on the Nyquist cures in Figures 3 and Table 1. As can be seen from Figure 3, the results of electrochemical modeling have been able to match the results of the impedance test. monomers create poly-pyrrole (Ppyr) molecules in the presence of HGH, when the template molecule is harvested from the MIP, R_{ct} (9866 Ω.cm^2) decreases. By removal of the template, cavities are created on the MIP surface, that are available for probe molecules $[Fe(CN)_6]^{-3/-4}$, thus increasing the conductivity of MIP/SPCE. Instead, when Human Growth Hormone molecules rebound to these cavities, the electrode surface is unavailable for probe molecules $[Fe(CN)_6]^{-3/-4}$. As a result, the sensor conductivity decreases, and the R_{ct} (14740 Ω.cm^2) index increases. Table 1 shows the Bare SPCE, MIP/Ppyr/Fe_3O_4/SPCE, NIP, template removal, and template rebound impedance data.

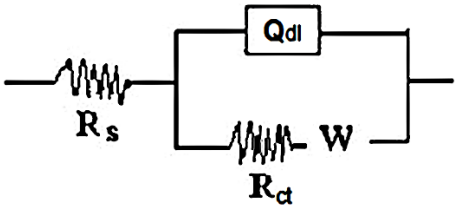

Figure 4. The equivalent circuit is used to model impedance test results

Table 2. The results obtained from EIS experiments. Template rebinding to MIP/Ppyr/Fe_3O_4/SPCE Cavities with Specific Concentrations of the Human Growth Hormone

Sensors	The Concentration of human growth hormone for rebinding to cavities	R_s (Ω.cm^2)	Q (S.s n/cm^2)	n	R_{ct} (Ω.cm^2)	C_{dl} (μF/cm^2)
1	1.25 (mg/ cm^3)	768.5	0.00008712	0.9081	14510	231.44
2	1 (mg/ cm^3)	753.1	0.0001162	0.9102	13390	299.67
3	100 (μg/ cm^3)	752.5	0.0001452	0.9203	12310	334.22
4	100 (ng/ cm^3)	749.2	0.0002074	0.9201	8616	464.95

In the equivalent circuit shown in Figure 4, R_s are the soluble resistance and R_{ct}, $Q_{dl,}$ and W are the charge transfer resistance, dual layer capacitor admittance, and Warberg element, respectively [39,40].

3.5. Optimization of variables

The variable examined in the assembly of the human Growth Hormone imprinted film were the concentration of Human Growth Hormone at the imprinted stage and the time required for the protein incubation. The concentration of HGH as a variable was selected between 1×10^{-6} g/cm^3 and 1×10^{-3} g/cm^3 for a constant concentration of pyr (always equal to 1.25×10^{-3} g/cm^3). The time of the HGH molecule incubation varied between 10 and 30 min.

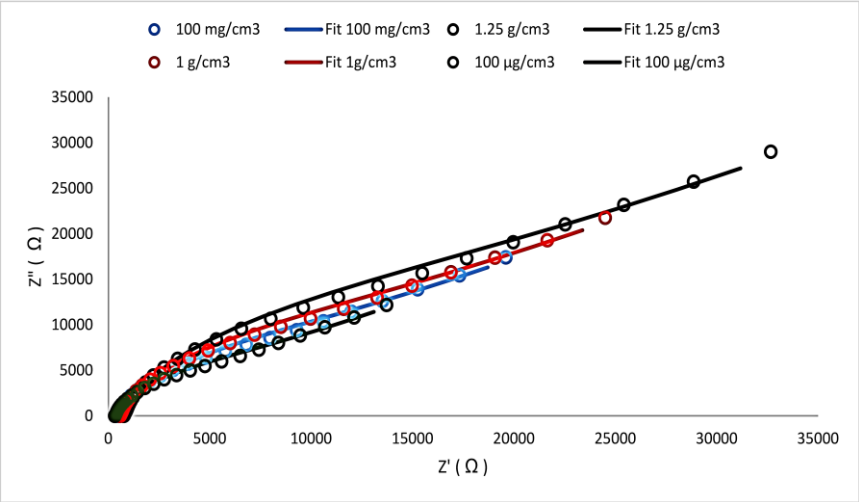

Figure 5. The Nyquist plots of the formed MIPs; the MIPs constructed with template molecule concentrations of 1.25 g/cm^3, 1 g/cm^3, 100 mg/cm^3 and 100 µg/cm^3

The change in HGH concentration was evaluated by EIS. EIS monitors the resistance change transfer (R_{ct}) of a redox system. For upper concentrations of HGH, the increase at R_{ct} was amplified, as HGH is not a conductor when it was entrapped within the polymer network. In order to construct the MIP, amounts of the template molecules in the range of 1×10^{-6} to 1×10^{-3} g/cm^3 were selected. As shown in Figure 5, with increasing the number of HGH molecules in the polymerization solution, the R_{ct} also increases. In the experiments performed and the impedance results, the optimal amount of hormone for the production of MIP, 1.25 g/cm^3 was selected with R_{ct} (14510 Ω.cm^2). However, it should be noted that in the amounts of hormone used (more than 1.25 g/cm^3), the equilibrium of monomer/template becomes highly shifted towards the template. As a result, the structure of formed MIP/Ppyr/Fe_3O_4/SPCE is weakened and will not have sufficient strength. As the selected amount of pyrrole was 3 mM, the best result was obtained according to the continuation of experiments with 1.25 g/cm^3 HGH. The R_{ct} values and other EIS data are listed in Table 2.

The incubation time of the HGH at the rebinding stage was tested between 5 and 30 minutes [41]. As Figure 6 shows, 30 min incubation caused the highest amount of R_{ct} (12100 Ω.cm^2), and 5 min the lowest amount of R_{ct} (4840 Ω.cm^2). The incubation time of 30 min with R_{ct} 12100 Ω.cm^2 indicates that the hormone molecules are properly inserted into the cavities and prevented the probe molecules from penetrating into the MIP cavities.

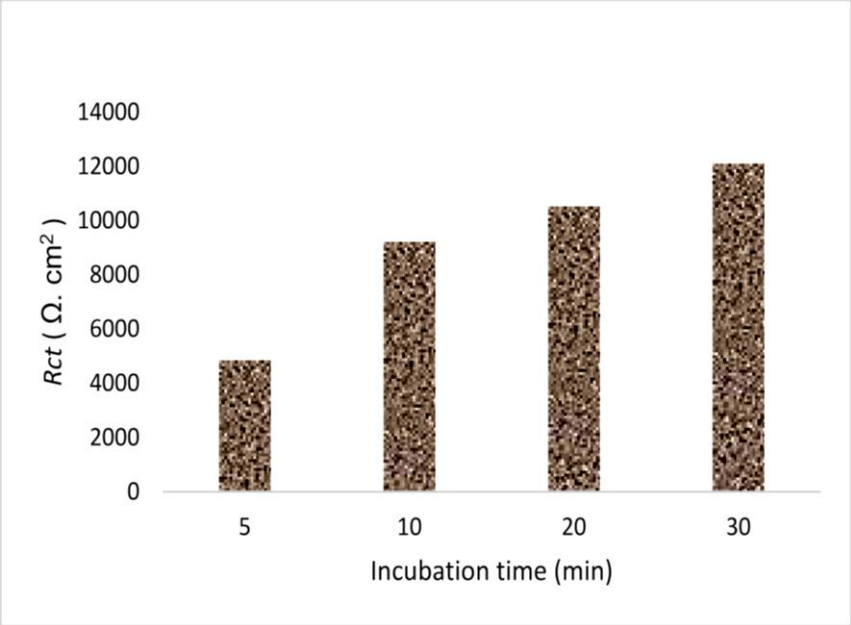

Figure 6. The incubation time of the template rebinding stage with concentrations of 0.1 mg/cm^3

3.6. Calibration curve

To obtain the calibration curve, different concentrations of the human growth hormone in the range of 1×10^{-6} to 1×10^{-11} g/cm^3 were prepared in series using the stock solution. After the MIP/Ppyr/Fe_3O_4/SPCE was made, the template molecule was removed from the MIP/Ppyr/Fe_3O_4/SPCE using 1 M sulfuric acid to create specific cavities for the HGH molecule. The electrode was then rinsed with ultra-pure water followed by dropping 60 µl of 1×10^{-6} g/cm^3 HGH solution on to carbon disk (working electrode) of SPCE and incubated for 30 minutes under a damp chamber to template rebinding (Figure 7). At the later stage, R_{ct} was obtained using the EIS technique (Figure 8). This was done exactly for other concentrations to obtain the data needed to draw the calibration curve. Therefore, a calibration curve was obtained using the EIS data. The MIP/Ppyr/Fe_3O_4/SPCE exhibited a linear relationship between the changes of Rct and the logarithms of human growth hormone concentrations with a linear range from 1×10^{-6} to 1×10^{-11} g/l (R=0.9996). The linear regression equation was as follows; R_{ct} =31.414-1.939 Log C (g/l) and the limit of detection (LOD), was found to be

2.927×10^{-12} g/cm^3. The calibration curve showed a 99.9% correlation of the data (Figure 7). Small LOD indicates the identification of HGH at very low concentrations.

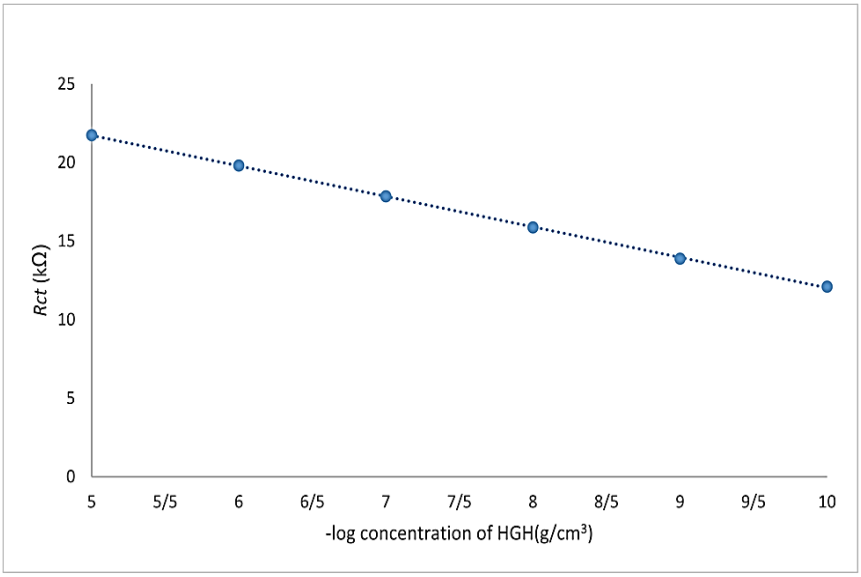

Figure 7. The calibration curve of different concentrations for Human Growth Hormone in the range of 1×10^{-6} to 1×10^{-11} g/cm^3, showing 99.9% correlation of data

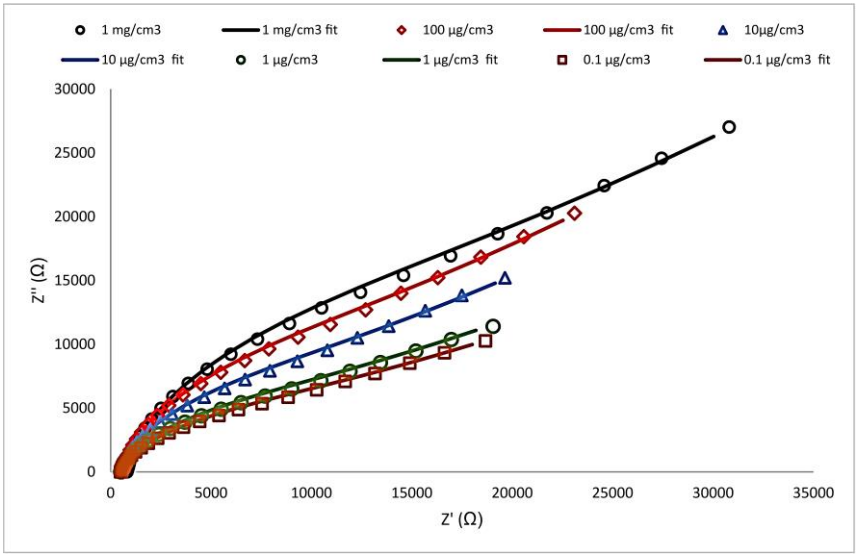

Figure 8. The Nyquist plot of the different concentrations of the Human Growth Hormone at 1×10^{-6} to 1×10^{-11} g/cm^3 range

As EIS spectrum data show, the electron transfer resistance (R_{ct}) at the concentration of 1×10^{-6} g/cm^3 Human Growth Hormone has the highest value (R_{ct} = 19810 Ω.cm^2), which decreases by reducing the concentration of HGH molecules in the rebinding step (1×10^{-11} g/cm^3), R_{ct} = 12100 Ω.cm^2.

Table 3. The EIS spectrum data for different concentrations of the Human Growth Hormone at 1×10^{-6} to 1×10^{-11} g/cm^3 range

Samples	R_s (Ω.cm^2)	Q (S.s n/cm^2)	n	R_{ct} (Ω.cm^2)	C_{dl} (μF/cm^2)	W (S.s 0.5/cm^2)
a (1×10^{-7} g/cm^3)	532.5	0.0001278	0.9262	19810	286.00	0.000112
b(1×10^{-8} g/cm^3)	530.5	0.0001421	0.926	17850	317.00	0.000125
c(1×10^{-9} g/cm^3)	531.9	0.0001504	0.926	16860	333.75	0.000132
d(1×10^{-10} g/cm^3)	530.2	0.0001827	0.9261	13880	399.21	0.00016
e(1×10^{-11} g/cm^3)	532.1	0.0002096	0.9261	12100	453.36	0.000184

Table (3) of EIS data clearly indicates that increasing Human Growth Hormone concentration reduces the double layer capacitor (C_{dl}) values, but increases the R_{ct} values. These changes are clearly visible in Figure 8.

3.7. Selectivity and stability and comparison of constructed SPCE/Fe_3O_4/MIPs

In order to confirm the selectivity of the prepared MIPs, bovine serum albumin (BSA), casein, and Urease were dissolved in 0.2 M PBS to obtain a 1×10^{-8} g/cm^3 protein solution. The same experiments were performed on the template molecule for these proteins at exactly equal concentrations.

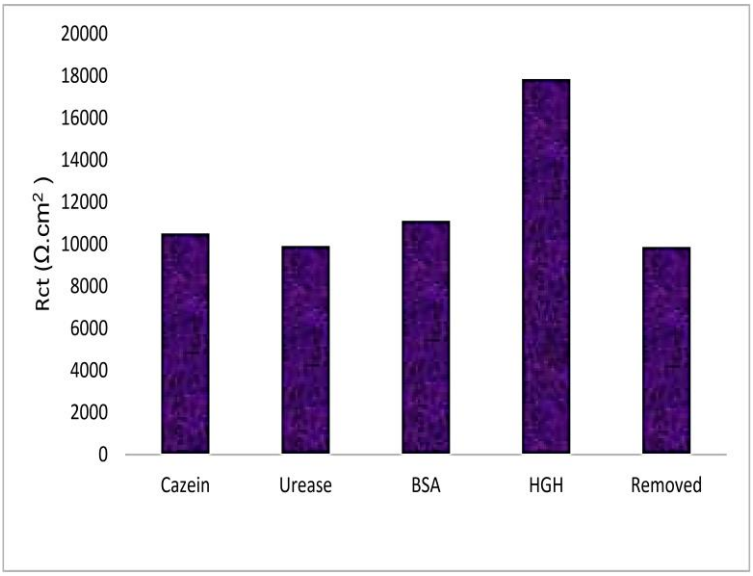

Figure 9. Selectivity of HGH molecule as compared to casein, BSA, and urease proteins with equal concentrations

As shown in Figure 9, the HGH molecules rebound to the cavities of the template molecule with high selectivity (R_{ct}=17850 Ω.cm^2), while the BSA, casein, and urease molecules are

randomly and not selectively attached to the MIPs surface. The R_{ct} values of the used proteins in the experiment confirmed this result very well. The R_{ct}s of BSA (11110 Ω.cm^2), casein (10510 Ω.cm^2), and urease (9917 Ω.cm^2) with R_{ct} of template removal (9866 Ω.cm^2) show the RSD=4.89% that there is a significant relationship between rebinding of these molecules. This could be explained as R_{ct} being close to R_{ct} template removal then the rebinding of these molecules to the special site is almost negligible.

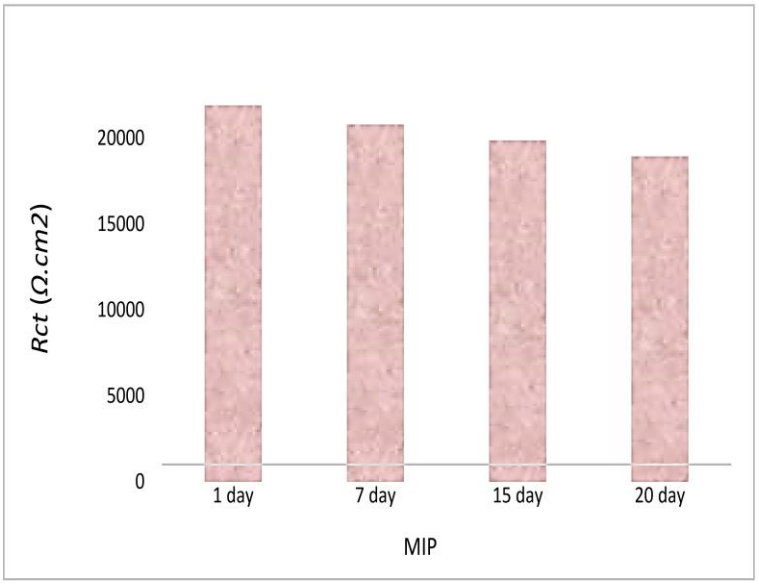

Figure 10. Stability of prepared MIP in 1st day, 7th day, 15th day and 20th day

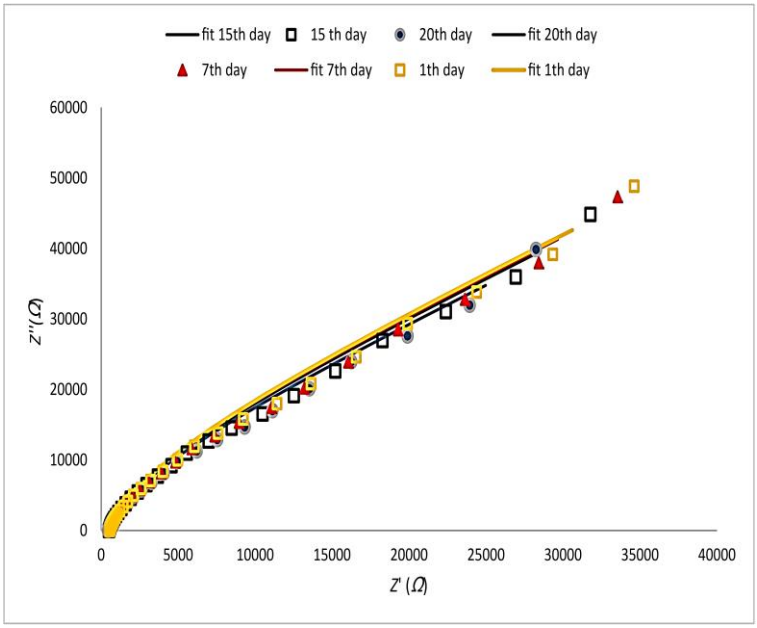

Figure 11. The Nyquist plot of stability of prepared MIP/Ppyr/Fe_3O_4/SPCE on 1st day, 7th day, 15th day, and 20th day

Figures 10 and 11 show the stability of the prepared MIP/Ppyr/Fe_3O_4/SPCE during of 1st day, 7th day, 15th day, and 20th day. For this purpose, the R_{ct} of rebinding of template molecule was determined. Table 1 shows the results obtained. After 20 days of storage of the MIP at room temperature, the value of R_{ct} reached 17932 Ω.cm2, which is 86.44%. The RSD was 5.4%. Therefore, according to the obtained results, the prepared MIP has very good stability after 20 days.

The EIS data were utilized to evaluate and determine the MIP/Ppyr/Fe_3O_4/SPCE reproducibility. Equal amounts of Human Growth Hormone were used to evaluate the measurement accuracy with four different SPCEs. The RSD was 1.95. The results are shown in Table 4. The results confirm the excellent reproducibility of the MIP/Ppyr/Fe_3O_4/SPCE with RSD=1.95.

Table 4. Results of reproducibility and stability of sensors

	Resistance charge transfer (R_{ct}) of MIP/Ppyr/Fe_3O_4/SPCE Sensors (Ω.cm ^{2})				
Items	MIP Sensor 1	MIP Sensor 2	MIP Sensor 3	MIP Sensor 4	RSD (n= 4)
Reproducibility	20500	20500	20290	21340	1.95
Stability	1 day 21900(100%)	7 days 20790(94.93%)	15 days 19890(90.68%)	20 days 18932(86.44%)	5.4

3.8. The comparison with other sensing systems

It should be noted, however, that researchers have mostly used antibody-based biosensors to detect hormones, and very few MIP methods have been used. Since immuno-biosensors are antibody-based, they are expensive to manufacture and will not be cost-effective for commercialization and clinical trials. Very few molecular imprinted polymers have been developed for the detection of human growth hormone by molecular polymer-based electro-polymerization. Therefore, the biosensor designed in this work has been compared with other proteins MIPs such as Hemoglobin (Hb), Bovine Hemoglobin (BHb), Cardiac Troponin T (cTnT), Myoglobin (Myo), a cardiac biomarker for ischemia (Table 5).

Due to the limit of detection, rapid detection, low manufacturing cost, easy production, non-toxicity and, most importantly, the portability of the designed biosensor for human growth hormone, large-scale industrial production can be planned for clinical diagnostics.

Table 5. Comparison of the construction steps of the Human Growth Hormone designed MIPs with other proteins MIPs such as Hemoglobin (Hb), bovine Hemoglobin (BHb), Cardiac troponin T (cTnT), myoglobin (Myo)

Target molecule	MIP step	Polymerization method	Assay method	LOD	Monomer	Ref.
Hb	Fe_3O_4@SiO_2MMIPNPs	Sol – gel	Amperometric (DVP)	0.0010 mg/ml	Tetraethoxysilane	[23]
BHb	Fe_3O_4@SiO_2 MIP	Self-polymerization	Amperometric (DVP)	1.184×10^{-8} g/ml	Dopamine	[24]
CTnT	MIP/MWCNTs/SPCE	Electro polymerization	Amperometric (DVP)	0.040 pg/ml	Aniline	[25]
Myo	MIP/poly(AP)/AuSPCE	Electro polymerization	Impedometric (EIS) Amperometric (SWV)	4-3.5 µg/mL	*o*-Aminophenol	[26]
BHb	MIP/ Ppyr/AuE	Electro polymerization	Impedometric (EIS), Amperometric (DVP)	--	Pyrrole	[27]
HGH	MIP/Fe_3O_4 NPs/GCE	Electro polymerization	Amperometric (SWV)	0.6×10^{-10} g/cm^3	Aniline	[28]
HGH	MIP/Fe_3O_4 NPs/SPCE	Electro polymerization	Impedometric (EIS)	2.927×10^{-12} g/cm^3	Pyrrole	This Work

4. CONCLUSION

The MIP/Ppyr/Fe_3O_4/SPCE based sensor was prepared and applied to selectively monitor levels of HGH. In summary, the sensor was made by electro-polymerization of pyrrole in the presence of Human Growth Hormone molecules. The modification of the electrode surface with Fe_3O_4 nanoparticles provided a large surface area. The MIP/Ppyr/Fe_3O_4/SPCE linear response was determined for HGH molecules in the range of 1×10^{-6} to 1×10^{-11} g/cm^3 in the buffer solution using EIS data (R_{ct}) with a limit of detection of 2.97×10^{-12} g/cm^3 with a correlation of 99.9% of the data. Based on the data obtained in this study, it could be concluded that the MIP made for Human Growth Hormone molecules has quite high sensitivity and stability, reasonable low cost, short response time and excellent reproducibility. Therefore, it is suggested that a prepared sensor could be applied to monitor levels of HGH in biological fluid.

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