Amperometric sensing of HIF1α expressed in cancer cells and the effect of hypoxic mimicking agents

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Hypoxia inducible factor 1 alpha (HIF1α) overexpression was detected in cancerous cells using an amperometric immunosensor with a nano-bioconjugate. The sensor probe was fabricated by covalently immobilizing the antibody (anti-HIF1α) onto a composite layer of functionalized conducting polymer [2,2:5,2-terthiophene-3-(p-benzoic acid)] (pTTBA) formed on a layer of gold nanoparticles (AuNPs). A nano-bioconjugate with hydrazine and a secondary antibody of HIF1α (sec-Ab2) attached on AuNPs reveals the immunoreaction at the sensor probe through the catalytic reduction of H2O2 by hydrazine at –0.35 V vs. Ag/AgCl. Morphology and performance of the sensor probe were characterized using FE-SEM, XPS, EIS, and cyclic voltammetry. The calibration plot at optimized experimental conditions shows a dynamic range of 25–350 pM/mL with a detection limit of 5.35 ± 0.02 pM/mL. The reliability of the sensor was evaluated using non-cancerous Vero and cancerous MCF-7 cell lysates, where the HIF1α expression was compared with three cancerous cell lines MCF-7, PC-3, and A549. Furthermore, the sensor probe confirms the stable expression of HIF1α in the A549 lung cancer cells when exposing them to hypoxic mimicking agents Co, Ni, and Mn ions. Of these, Co ions show the highest stabilization effect on HIF1α followed by Ni and Mn ions, respectively.

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1. Introduction

Hypoxia Inducible Factor 1 alpha (HIF1α) is a tumor promoting transcription factor overexpressed in cancer cells. (Semenza et al., 2003; Schofield et al., 2004). The elevated HIF1α expression, stabilization, and transcriptional activation are linked with a number of cancers such as breast, ovarian, prostate, and lungs (Galanis et al., 2008). In normoxic conditions, HIF1α is hydroxylated on specific proline or asparagine residues by specific enzymes. However, under hypoxic conditions, the expression of HIF1α is induced by the activation of numerous cell signaling pathways, which facilitates the primary transcriptional adaption and initiates the development of the neovascularization. There are various factors responsible for enhancing HIF1α expression, including desferrioxamine (Wang and Semenza, 1993), growth factors (Maxwell et al., 2001), nitric oxide (Metzen et al., 2003), and transition metal ions (Galanis et al., 2009). Some metal ions are considered as hypoxic mimicking agents in cancer cells, due to their stabilization effect on HIF1α. The effect is produced by the interference of HIF1α regulators, either by inactivation of essential regulatory enzymes like prolyl and asparaginyl hydroxylase or by inhibiting ascorbate uptake and production of reactive oxygen species (Galanis et al., 2009), which results in the elevation of HIF1α level in the cells for tumor development (Brahimi-Horn et al., 2007; Toschi et al., 2008). Metals are potentially toxic at high concentration, even though some are also essential for health. The toxic effect can arise either by dietary uptake or occupational route. These metals classified as carcinogenic and can develop different kind of cancers like lung and nasal. In this study Ni, Co, and Mn were selected because they are most commonly used in different industries where the people are maximum exposed. Clinically, HIF1α is very significant because it plays a vital role in the cancer progression and aggressiveness. HIF1α is widely considered as a sensitive, robust, and reliable biomarker to detect hypoxia in biological samples. However, the short-lived nature of HIF1α approximately 5–8 min, prevents its determination in real practices (Zhong et al., 1999; Qingdong and Max, 2006).

Over the past few years, various methods have been developed for the determination of HIF1α, including immunohistochemistry (Bernhardt et al., 2007), ELISA (Fornento et al., 2005), and western blotting (Vordermark et al., 2003). However, most of these analytical techniques are invasive, require enzyme linked antibody which is unstable, costly, and time consuming. All the above mentioned shortcomings make the existing techniques inefficient for the sensitive determination and early diagnosis of the respective cancers. Therefore, there is an increasing demand for the

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development of an easy, robust, and reliable analytical method to determine the HIF1α in terms of routine assessment, early clinical diagnosis, and point of care applications. To fulfill these critical demands, the preparation of an electrochemical immunosensor composed of a primary antibody and a nanobioconjugate composed of a non-enzymatic catalyst along with a sec-Ab2, attached onto a suitable surface is considered to be greatly promising method in clinical analysis. Hence, the electrochemical sensor for the determination of HIF1α expressed in the cancer cells was developed and the effect of hypoxic mimicking agents in mammalian cancer cells was evaluated for the first time.

For a few decades, conductive polymers (CP) have extensively been studied due to the wide potential applications (Nalwa, 1997; Skotheim and Reynolds, 2006). Of these, some polymers were used for the robust sensor probe substrate, which are highly sensitive, remarkable electronic properties, non-toxic, and ultraselectivity for clinical diagnostics. Moreover, the stable covalent immobilization of biomolecules onto the CP layer is a great advantage, by using functional groups such as -COOH, or -NH2. Thus, some immunosensor have been developed using a CPs (Kim et al., 2013; Lee et al., 2001). In this study, a functionalized conductive polymer (pTTBA) was electrochemically polymerized and composed with AuNPs, then the carboxylic acid groups of the polymer were activated to immobilize the biomolecules (anti-HIF1α). For signal generation in immunoreactions, various signal indicators have been used for sensing devices to generate either a fluorescence or an electrochemical signal (Hermanson, 2013). In case of most electrochemical immunosensors, enzymes have been used to generate signal, which limit the lifetime of the sensor system. Hence, it is worth to explore the stable molecules for electrochemical signal generation. One of the candidate is hydrazine which is a small, stable, and inexpensive molecule possessing strong catalytic activity to reduce hydrogen peroxide (Shankaran et al., 2002; Chandra et al., 2015; Mir et al., 2015).

In the present study, an amperometric immunosensor was developed based on anti-HIF1α chemically bonded on CP, and a nanobioconjugate in a sandwich immunosensing approach for the first time. The sensor probe was characterized by field emission scanning electron microscopy (FE-SEM), x-ray photoelectron spectroscopy (XPS), electrochemical impedance spectroscopy (EIS), and cyclic voltammetry (CV). The applicability of the sensor was examined by detecting HIF1α in various cancer cells including MCF-7, PC-3 and A549 as breast, prostate, and adenocarcinoma of human alveolar basal epithelial cell lysates, respectively. Finally, the A549 cell lysates were examined after co-culturing the cells with hypoxic mimicking agents CoCl2, NiSO4, and MnCl2 to determine their stabilization effect on HIF1α.

2. Experimental

2.1. Materials

The monomer [2,2':5,2'-terthiophene-3,3'-[p-benzoic acid)] (TTBA) was freshly synthesized according to a previously reported method (Kim et al., 2012). Mouse monoclonal anti-human HIF1α IgG purchased from Alpha diagnostic international. HIF1α was purchased from ATGen Co., Ltd. Vero, MCF-7, PC-3, and A549 cell samples were obtained from the Korean cell line bank (South Korea). Bovine serum albumin (BSA), hydrazine sulfate (Hyd), 1-ethyl-3-[3-(dimethylamino)propyl]-carbohdiimide (EDC), N-hydroxysuccinimide ester (NHS), potassium ferricyanide(III), potassium hexacyanoferrate(II) trihydrate, gold(III) chloride trihydrate, sodium dodecyl sulfate SDS, tris(hydroxymethyl) aminomethane, sodium chloride, fetal bovine serum (FBS), trypsin-EDTA, penicillin/streptomycin, Hank's balance salt (HBS) solution, cobalt (II) chloride hexahydrate(CoCl2), nickel(II) sulfate hexahydrate (NiSO4·6H2O), manganese(II) chloride tetrahydrate (MnCl2), and H2O2 (33%) were purchased from Sigma (USA). Tetra-butylammonium perchlorate (TBAP) electrochemical grade, was obtained from Fluka and purified using the conventional method followed by drying under vacuum at 10−5 Torr (Zhu et al., 2012). Phosphate-buffered saline (PBS) solution was prepared with 0.01 M disodium hydrogen phosphate (Sigma), 0.01 M sodium dihydrogen phosphate (Sigma), and 0.9% sodium chloride (Sigma). All remaining chemicals were of extra pure analytical grade and used without further purification. Deionized water (18 MΩ cm) from a Direct-Q system (Millipore, Billerica, MA) was used to prepare all aqueous solutions.

2.2. Apparatus

A three electrodes system was used as follow; a glassy carbon electrode (GC), a Ag/AgCl (in saturated KCl), and a platinum (Pt) wire as working, reference, and counter electrode, respectively. CV was performed using a KS-TP1 potentiostat/galvanostat, Kosentech (South Korea). Impedance spectra were recorded with PARSTAT (EG&G Princeton Applied Research). FE-SEM images were obtained using a Cambridge Stereoscan 240 XPS experiments were performed using a VG Scientific ESCA Lab 250 XPS spectrometer coupled with a monochromatic Al Kα source with charge compensation at the Korea Basic Science Institute (Busan, South Korea).

2.3. Fabrication of the sensing surface

Prior to modification, the GC electrode (area 0.07 cm2) was polished to a mirror finish with alumina slurry 0.3 and 0.05 μm respectively, thereafter rinsed with double distilled water and ethanol to remove any adsorbed alumina particles on the GC surface. AuNPs were electrochemically deposited by linear sweep voltammetry (LSV) three times, in the potential range from +1.4 to +0.5 V in a 0.5 M H2SO4 solution containing AuNPs at scan rate of 100 mV/s. 1.0 mM TTBA monomer solution was prepared in CH3Cl2 containing 0.1 M TBAP and sonicated for 5 min. Then TTBA was electropolymerized onto AuNPs surface by performing CV three times in the potential range between 0.0 and +1.4 V at scan rate of 100 mV/s followed by immediate washing with CH3Cl2 to remove any physically adsorbed or excess of monomer. As a result, a layer of the conducting polymer was formed on to the surface of AuNPs/GC. To activate the carboxylic acid groups on pTTBA layer, the pTTBA/AuNPs/GC modified electrode was immersed in 0.1 M PBS containing 10.0 mM EDC and 10.0 mM NHS for 6 h. Thereafter, the electrode was rinsed with PBS to remove any excess of EDC/ NHS and allowed to dry at room temperature. Appropriate amount of anti-HIF1α solution (5 μL) was drop casted on the pTTBA/ AuNPs/GC modified surface and dried at room temperature. Through this step, anti-HIF1α was immobilized by formation of covalent bond between the activated carboxylic acid groups (-COOH) of pTTBA and amine groups (-NH2) of the antibody. After immobilization the modified electrode was immersed in 2% BSA for 1 h at 4 °C to block unreacted sites and finally, it was used as a sensor probe to detect HIF1α.

2.4. Preparation of Hyd/AuNPs/sec-Ab2 nano-bioconjugate

Use of a bioconjugate is one of the methods to detect electrochemically inactive species. The bioconjugate usually consists of a redox indicator and a detector molecule, both of which are attached to a suitable surface. Herein, hydrazine was used as a redox indicator to produce electrochemical signal and sec-Ab2 was used to detect the HIF1α on to the probe surface, and AuNPs were used
as a substrate for their attachment. The nano-bioconjugate was prepared, first by synthesizing citrate capped AuNPs according to a literature (Jana et al., 2001). Concisely, at first 50 mL of 0.01 wt% HAuCl₄ dissolved in H₂O and mixed with 1 mL of 38.8 mM trisodium citrate. After 1 min, 0.5 mL of a freshly prepared NaBH₄ solution was added drop wise under gentle stirring. During the addition of NaBH₄ the color of the resulting solution turned to pink-violet, indicating the formation of AuNPs. 10 mM of hydrazine sulfate (hyd) solution was prepared in water and 250 μL of sec-Ab₂ at 100 μL/mL was mixed in 0.1 M PBS solution at pH 7.4. The above three solutions were mixed in 1:1:1 ratio (v/v/v) and kept overnight at 4 °C. The electrostatic interactions between hydrazine and AuNPs enabled its attachments onto AuNPs surface, similarly, the electronic attraction between the negatively charged AuNPs and abundant positively charged sites of sec-Ab₂ facilitated its immobilization (Hermanson et al., 2013; Zhu et al., 2012). Finally, the resulting nano-bioconjugate (Hyd/AuNPs/sec-Ab₂) was stored at 4 °C for further use.

2.5. Preparation of mammalian cell lysates and hypoxic treatment

Normal cells (Vero) and Cancer cells (MCF-7, PC-3, and A549) were cultured in a DME medium containing 10% heat inactivated fetal bovine serum, 100 units/mL of penicillin and 100 units/mL of streptomycin in a T75 culture flask at constant 37 °C under 5% CO₂ and 95% humidity in a CO₂ incubator. The media was replaced once in every two days until 95% confluence was obtained and then sub cultured. To detect HIF1α, cells were lysed according to Cold spring harbor protocol (Ji, 2010). Briefly, lysis solution was prepared as (150 mM NaCl, 0.1% SDS and 50 mM Tris–HCl). First, the cells were removed from culture conditions following trypsinization and suspended in 0.1 M PBS, then cell lysis solution was added directly into the suspension on ice cubed container. The resulting cell suspension was centrifuged and supernatant removed carefully without disturbing the pellet. For hypoxic treatment, four samples of A549 cells of each metal were prepared and incubated for 48 h in a humidified incubator containing 5% CO₂. Fresh solutions of CoCl₂ (0.2 mM), NiSO₄ (1 mM) and MnCl₂ (0.1 mM) were prepared and dissolved into cell culture media after 48 h and the cell samples were lysed after every 6 h and similar procedure was followed for control sample. The exposure of the given metals to cells and the concentrations used in this work are based on the previous cell survival data for A549 (Li et al., 2006; Salnikow et al., 2003). A549 cell line was selected because the wide usage of metals in various industries and lungs considered as a prime potential target for metal's toxicity.

2.6. Electrochemical detection of HIF1α

The immobilized anti-HIF1α sensor was incubated in Tris–HCl buffer solution containing HIF1α for 30 min to allow the antibody-antigen immune complex formation. Afterwards, the modified electrode was washed with 0.1 M PBS and immersed into the nano-bioconjugate solution for 30 min at 37 °C; similar immune complex was formed due to explicit binding of sec-Ab₂ on the nano-bioconjugate to HIF1α captured on the modified electrode surface. Finally, the electrode was washed with distilled water and tested in a 0.1 M PBS, already purged with high purity nitrogen gas for 20 min. 10.0 mM H₂O₂ was added into the measuring solution to produce the catalytic response. CV was performed by cycling the potential between 0.0 and −0.6 V at a scan rate of 50 mV/s.

3. Results and discussion

The proposed amperometric sensor was fabricated by covalent attachment of anti-HIF1α onto a layer composed of functionalized conducting polymer, where hydrazine in the nano-bioconjugate was used to generate electrochemical signal. Fabrication steps are shown in Scheme 1. The layer by layer modifications of the sensor was characterized using FE-SEM, XPS, EIS, and CV. Finally, the sensor was evaluated by applying it for the analysis of HIF1α in biological system.

3.1. Surface characterization of the sensor probe

The surface morphology of the sensor was characterized by FE-SEM and XPS analysis. Supplementary material (Fig. S1) shows FE-SEM images of (a) AuNPs, (b) pTTBA embedded on AuNPs, (c) attachment of anti-HIF1α on pTTBA/AuNPs layer, (d) captured HIF1α on the sensor probe layer, and (e) attachment of a nano-bioconjugate. The size of the AuNPs deposited on GC was approximately 40–65 nm as shown in (Fig. S1(a)). The morphology of the electrode surface after TTBA polymerization appeared as a film over the deposited AuNPs (Fig. S1(b)). However the attachment of Anti-HIF1α onto pTTBA layer formed small clusters, which was due to the interaction between –COOH of pTTBA and –NH₂ terminal of the antibody (Fig. S1(c)). The morphology of the product between Anti-HIF1α and HIF1α depicts regular and nicely shaped dendrite like structure, which indicates the formation of antibody-antigen immune complex (Fig. S1 (d)). The nano-bioconjugate appeared as small rough structures with visible AuNPs (Fig. S1(e)).

For further confirmation of the electrode surface modification, XPS data was obtained for (I) pTTBA/AuNPs/GC, (II) Anti-HIF1α–pTTBA/AuNPs/GC, (III) HIF1α/Anti-HIF1α–pTTBA/ AuNPs/GC, and (IV) nano-bioconjugate/HIF1α/Anti-HIF1α–pTTBA/AuNPs/GC (Fig. 1 (A)). All XPS spectra were taken after 20 s of Ar ion gas etching and calibrated using the C1s peak at 284.6 eV as an internal standard. As shown in the survey spectra (Fig. 1(A) (a)), the O1s, C1s, and Au4f peaks were observed for all the surfaces at 531.9, 284.2, and 86.3/83.7 eV, respectively. However, the N 1s peak was absent for

![Scheme 1. Schematic representation for the fabrication of sensor probe.](image)
Figure 1. (A) a) XPS survey spectra for (I) pTTBA/AuNPs/GC, (II) Anti-HIF1α-pTTBA/AuNPs/GC, (III) HIF1α/Anti-HIF1α-pTTBA/AuNPs/GC, and (IV) nano-bioconjugate/HIF1α/Anti-HIF1α-pTTBA/AuNPs/GC, (B) S2p peak for (I) AuNPs/GC (II) pTTBA/AuNPs/GC, (c) deconvoluted C 1s peak and (d) N 1s peak for (I) pTTBA/AuNPs/GC and (II) Anti-HIF1α-pTTBA/AuNPs/GC. (B) EIS of (a) Anti-HIF1α-pTTBA/AuNPs/GC, (b) HIF1α/Anti-HIF1α-pTTBA/AuNPs/GC, and (c) nano-bioconjugate/HIF1α/Anti-HIF1α-pTTBA/AuNPs/GC.

The pTTBA/AuNPs layer (Fig. 1A (a (I))) and was observed after the attachment of Anti-HIF1α at 399.9 eV confirming the immobilization of Anti-HIF1α on the electrode surface as shown in Fig. 1A (a (II)). The N 1s peak was also observed in the spectra for the nano-bioconjugate, indicating the attachment of the Anti-HIF1α onto the probe (Fig. 1A (a (IV))). A peak at 163.7 eV was observed for the polymer modified surface, corresponding to the S2p electron of sulfur present in the TTBA monomer. The deconvoluted S2p spectrum exhibited a peak at 163.7 eV corresponding to the C–S bond at pTTBA/AuNPs/GC layer as shown in Fig. 1A (b (II)), whereas this peak was absent at the AuNPs/GC layer (Fig. 1A (b (I))). The deconvoluted C 1s spectrum of pTTBA/AuNPs/GC exhibited two peaks at 284.7 and 286.7 eV that correspond to the C–C, C–H, and/or C–S, and C=O, C=O bonds, as shown in Fig. 1A (c (I)). However, an additional peak was observed in Fig. 1A (c (II)) for Anti-HIF1α-pTTBA/AuNPs/GC surface at 285.5 eV, corresponding to the C–N bond indicating the formation of an amide bond. In addition the deconvoluted N 1s spectrum of Anti-HIF1α-pTTBA/AuNPs/GC shows a peak at 399.8 eV, which was absent on pTTBA/AuNPs/GC layer (Fig. 1A (d (I))), confirming the C–N bond formation between the polymer and the anti-HIF1α antibody as shown in Fig. 1A (d (II)) (Noh et al., 2012). These results adequately validated the successful formation of pTTBA onto the AuNPs/GC layer and immobilization of Anti-HIF1α onto the pTTBA/AuNPs composite (Mir et al., 2015; Chandra et al., 2012).

3.2. Electrochemical properties of the sensor probe

To examine the surface conditions of the sensor probe, EIS was performed with [Fe(CN)6]3–/4– and the frequency was scanned from 100 kHz to 100 mHz at an open circuit voltage. Fig. 1B (a–c) shows the EIS spectra obtained for (a) Anti-HIF1α-pTTBA/AuNPs/GC, (b) HIF1α/Anti-HIF1α-pTTBA/AuNPs/GC, and (c) nano-bioconjugate/HIF1α/Anti-HIF1α-pTTBA/AuNPs/GC. Each parameter value was obtained by fitting the experimental results to a simple equivalent Randle circuit using the Zview2 impedance software, the circuit is shown in the inset of (Fig. 1B) where, Rs is the solution resistance, Rp1 and Rp2 are the polarization resistances, W represents Warburg impedance (ZW), and Qdl & Qf denote the constant phase elements (CPE), representing double-layer capacitance and film capacitance, respectively. As depicted the immobilized anti-HIF1α layer Fig. 1B (a), it showed a semicircle curve with a simulated Rp value of 2.21 kΩ. The plot for the captured HIF1α layer Fig. 1B (b) exhibited a dramatic increase in the diameter of the semicircle compared to plot (a) with an increase in the Rp value from 2.21 to 4.87 kΩ, indicating an increase in the interfacial electron transfer resistance. This was due to the formation of immune complex, which confirms the attachment of HIF1α onto the electrode surface. For the nano-bioconjugate layer (plot c), the plot showed the largest semicircle and the Rp value was estimated to be 6.34 kΩ which is the highest electron transfer resistance value as compared to the other two layers. Variations in the interfacial impedance properties between modified layers clearly demonstrate the capture of HIF1α and the successful attachment of the nano-bioconjugate to the sensor probe.

To validate the applicability of the sensor, CVs were recorded in 0.1 M PBS containing 10 mM H2O2. As shown in Fig. 2A, a series of CVs recorded for (a) HIF1α/Anti-HIF1α-pTTBA/AuNPs/GC, (b) nano-bioconjugate/HIF1α/Anti-HIF1α-pTTBA/AuNPs/GC where, the nano-bioconjugate was prepared without and (c) with hydrazine moiety. As expected, CV (a) and (b) exhibited no reduction peak of H2O2 due to the absence of any electroactive species. However, CV (c) showed a clear reduction peak at around −0.35 V vs. Ag/AgCl. The reduction peak is due to the catalytic activity of the hydrazine in the nano-bioconjugate towards H2O2 reduction.

As shown in Fig. S2, CVs were recorded to confirm the attachment of hydrazine onto the nano-bioconjugate, between −0.2 and +0.3 V in a 0.1 M PBS for nano-bioconjugate/HIF1α/Anti-HIF1α-pTTBA/AuNPs/GC where the nano-bioconjugate was formed (a) without and (b) with hydrazine moiety. As expected, a pair of redox peaks of hydrazine were observed at 0.08/−0.06 V for the nano-bioconjugate with hydrazine (curve b). These results indicate that the sensor probe is capable of binding with HIF1α and the AuNPs/Hyd/sep-Ab2 nano-bioconjugate was successfully prepared. These results are in a good agreement with the EIS and XPS results.

3.3. Optimization of the analytical parameters and calibration plot

Experimental parameters such as temperature, pH, immune reaction time, and anti-body concentration were optimized using CV in 0.1 M PBS containing 10 mM H2O2 as shown in Fig. S3. The effect of temperature on the response was studied in a temperature range from 20 to 45 °C (Fig. S3 A). The current increased gradually as the temperature increased from 20 to 35 °C and dropped beyond 35 °C, therefore, 35 °C is selected as an optimum temperature. The influence of pH on the response current was examined under different pH from 6.5 to 8.5 (Fig. S3 B). A steady enhancement in the current was observed from pH 6 and reached maximum at pH 7.5. However, a sharp decrease in the current was
noticed beyond pH 7.5 therefore, 7.5 is chosen as the optimum pH. To find the optimum immune reaction time, the sensors were incubated for different incubation times from 5 to 35 min (Fig. S2 C). The results showed that as the incubation time increased the response also increased linearly reaching a maximum at 25 min, and there was no enhancement in signal response after 25 min. Hence, 25 min is selected as the optimum immune reaction time. To investigate the optimum concentration of the antibody to be immobilized on the sensor surface, 5 μL of anti-HIF1α with various concentrations ranging from 50 to 400 nM/mL was drop casted on to the probe surface (Fig. S2 D). It was noticed that the current also increased proportionally as the concentration increased from 50 to 250 nM. However, beyond 250 nM, the current decreased until 350 nM/mL and remained unchanged after that, therefore, 250 nM/mL is selected as an optimum anti-HIF1α concentration. All optimized parameters are selected for further experiments.

Under optimized parameters, chronoamperometric experiments were performed to detect HIF1α in varied concentrations in a 0.1 M PBS containing 10 mM H2O2 (inset of Fig. 2(B)). Results indicated that as the HIF1α concentration increased, the response to the reduction current of H2O2 catalyzed by hydrazine was also enhanced; this is due to the increased formation of antibody-antigen complexes consisting terminal hydrazine. A gradual increase in the amperometric response was observed as HIF1α concentration varied from 25 to 350 pM/mL. The corresponding calibration plot is shown in (Fig. 2(B)), which exhibited a good linearity with a dynamic range from 25 to 350 pM/mL of HIF1α. The linear dependencies of HIF1α analysis yielded an equation of Ip (μA) = (0.295 ± 0.004) + (0.014 ± 0.004) [HIF1α] (pM/mL) with a correlation coefficient of 0.997. The sensitivity of the sensor (the slope of this plot) is 0.014 ± 0.004 μA mL pM⁻¹. The detection limit of HIF1α is determined to be 5.35 ± 0.02 pM/mL based on five measurements for the standard deviation of the blank noise (95% confidence level, signal noise ratio k = 3). The Detection Limit (DL) was assessed by linear regression of the calibration curve applying the following equation

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DL = 3σ/m
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The following abbreviations are used for the variables of the equation: σ-standard deviation of the response in the absence of analyte and m-slope of the calibration curve. To the best of our knowledge there is no published data about HIF1α electrochemical detection hence this can be considered as lowest detection limit.

3.4. Interference study, reproducibility, and stability

To evaluate the selectivity of the sensor, the interfering effect was studied against the responses of the sensor in the presence of 400 nM/mL concentration of interfering agents as shown in Fig. 3. At optimized conditions, the interference effects of arginine, human IgG, and serine were investigated by recording the current of each of the interfering agent when incubated individually. The results showed that when the sensor was incubated with arginine and serine, no significant effect on current was observed. However, when it was incubated with human IgG protein, the sensor probe showed increased response in comparison with other two proteins, this could be because, the anti-HIF1α protein belongs to the same class of proteins as anti-human IgG antibody. Furthermore, when the modified electrode was incubated with a mixture solution containing arginine, human IgG and serine, each at 400 nM/mL concentration and 250 nM/mL of HIF1α, the current was slightly higher compared when HIF1α existed alone, this might be due to the interference effect of IgG. These results confirmed that the sensor exhibited sufficient selectivity for the determination of HIF1α due to the highly specific antibody-antigen interaction. Further, the relative standard deviations (RSDs) value was calculated and found to be 2.54% when 5 different electrodes were used to detect HIF1α and the result showed good electrode to electrode reproducibility.
Finally, to evaluate intraday stability of the sensor, CVs were recorded for the same concentration of A549 cells (1 × 10^6 cells/mL) at regular intervals (5 days) for a month. When the sensor was stored at 4°C for a period of 30 days, the sensor retained more than 93% of its initial response. The results demonstrated that the proposed sensor possesses an acceptable level of stability which is due to stable immobilization of the anti-HIF1α on to the CP layer as shown in Fig. S5.

3.5. HIF1α detection and stabilization effects of hypoxic mimicking agents in mammalian cancer cells

To validate the applicability, the sensor probe was investigated in cancerous MCF-7 and the results were compared to that of normal Vero cells. Each cell lysate was prepared and incubated with the sensor probe, followed by the nano-bioconjugate incubation. The measurements were taken in a 0.1 M PBS (pH 7.5) containing 10 mM H2O2 with the purging of oxygen with N2 gas for 20 min. The cyclic voltammograms in Fig. 4(A) depicts incubation of the sensor probe with cell lysates of different MCF-7 cell concentrations ranging from 4 to 8 × 10^6 cells/mL. The results showed that as the number of lysed MCF-7 cells increased the current also enhanced linearly and the current responses were plotted against different number of cells to get the calibration plot as shown in Fig. S4. To validate the above results, the same experiment was performed using Vero cell lysates with the same experimental conditions to that of MCF-7 as shown in inset of Fig. 4(A). ~6.1 fold less current response was observed for Vero cell lysates as compared to MCF-7 cells. The small response current observed in normal cells is due to low expression of HIF1α in normal physiological conditions (Schofield et al., 2004). These results adequately confirm the overexpression of HIF1α in cancer cells as compared to normal cells. Furthermore, chronoamperometry was carried out to compare the HIF1α expression among MCF-7, A549, PC-3, and Vero cell lysates using same number of cells (6 × 10^6 cells/mL) as shown in (Fig. 4(B)). Results showed that the response of MCF-7 was 40% and 94% higher in comparison to A549 and PC-3 cells respectively. However, the response current of Vero cell lysate was insignificant, the higher current is due to more expression of HIF1α in MCF-7 which are captured by the sensor probe. These results are in good agreement with the previously reported works (Zhong et al., 1999; Talks et al., 2000). Finally, chronoamperometry was carried out to monitor the stabilized expression of HIF1α by chemically induced hypoxia in A549 and the effect was examined in 24 h duration as shown in Fig. 5. Each treated sample was lysed at every 6 h interval and compared with the control sample (no hypoxic treatment). The results showed good linearity in current as the pretreatment time increased from 0 to 24 h for all Co, Ni and Mn incubated cultures, owing to the enhancement in the expression of HIF1α. However, the control sample showed a stable response throughout the incubation time. Further, when the lysates were exposed to Co, Ni, and Mn ions containing solution at 24 h, a dramatic enhancement in signal was observed. The current enhancement with respect to Co, Ni, and Mn was 1703%, 114%, and 567% respectively, in comparison with control sample. In addition, the response with respect to Co at 24 h, was 589% and 11,369% higher as compared to that of Ni and Mn, respectively. The larger current response of Co to Ni could be due to the fact that Co possess higher redox activity, oxygen binding ability, and faster complex formation, which results in rapid enzyme inactivation as well as ascorbate damage, which in turn result in higher stability of HIF1α. However, in case of Mn pretreated samples response was lowest as compared to Co and Ni that could be due to different mechanism of action of Mn (Bredow et al., 2007). The obtained results suggest that Co possess highest stabilization effect on HIF1α in A549, followed by Ni and Mn ions.
Pretreated results are in a good agreement with previous reports (Li et al., 2006).

4. Conclusions

A highly sensitive amperometric immunosensor was successfully developed for the detection of HIF1α through the antibody-antigen immune complex formation, where, a reduction catalytic molecule was introduced in the nano-bioconjugate for the first time to label sec-Ab2 using AuNPs as a linker. The ultra-high sensitivity of the sensor probe enabled to detect HIF1α and differentiate cancerous from non-cancerous cells. Furthermore, it was employed to compare HIF1α expression in MCF-7, A549, and PC-3 cell lysates, where MCF-7 showed the maximum response due to highest expression of HIF1α followed by A549 and PC-3. Finally, the effect of metal ions exposure shows the stability of HIF1α in A549 cells. Conclusively, Co possess the highest stabilization effect on HIF1α, followed by Ni and Mn ions respectively. The proposed sensor probe have the potential in diverse clinical and pharmaceutical applications and can be used as a complementary tool in screening, early prognosis and diagnosis in cancer as well as in the development of anti-angiogenic drugs.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.04.068.

References