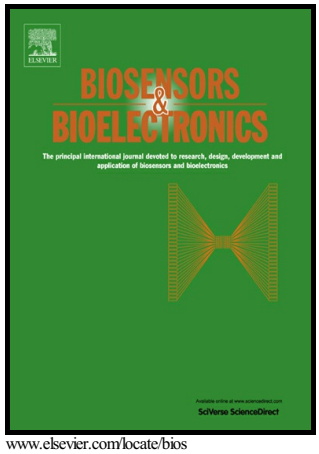


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# Multiplexed detection of DOCK8, PGM3 and STAT3 proteins for the diagnosis of Hyper-Immunoglobulin E syndrome using gold nanoparticles-based immunosensor array platform

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**Abstract**

Multiplexed biosensors hold great promise for early diagnosis of diseases where the detection of multiple biomarkers is required. Hyper Immunoglobulin E syndromes (HIES) are rare primary immunodeficiency disorders associated with mutations either in the signal transducer and activator of transcription 3 (*STAT3*), dedicator of cytokinesis 8 (*DOCK8*) or phosphoglucomutase 3 (*PGM3*) genes. Yet, the diagnosis of HIES is challenged by the complexity of the existing laboratory assays. Here, we report for the first time the development of a multiplexed electrochemical immunosensor for the simultaneous detection of DOCK8, STAT3 and PGM3 proteins. The immunosensor was constructed on carbon array electrodes that were first modified by electrodeposition of gold nanoparticles (AuNPs). The array electrodes were then used to immobilize specific antibodies for the three proteins after the functionalization of the electrodes with cysteamine/glutaraldehyde linkers. The simultaneous detection of the DOCK8, PGM3 and STAT3 proteins was successfully realized by the immunosensor with respective limits of detections of 3.1, 2.2 and 3.5 pg/ml. The immunosensor has shown good sensitivity as well as selectivity against other proteins such as cystic fibrosis transmembrane conductance regulator (CFTR) and Duchenne Muscular Dystrophy (DMD). Moreover, the immunosensor was successfully applied in human serum samples showing capability to distinguish the HIES from the control samples.

**Keywords:** biosensor, Dedicator of cytokinesis 8 (DOCK8), Signal transducer and activator of transcription 3 (STAT3), phosphoglucomutase 3 (PGM3), multiplex detection

## 1. Introduction

Inherited hyper Immunoglobulin E syndromes (HIES) are a group of rare primary immunological disorders (Freeman and Holland 2008). HIES is characterized by elevated levels of serum immunoglobulin E (IgE), eczema, recurrent lung infections, recurrent staphylococcal skin abscesses and eosinophilia. (Renner et al.)

Two common types of HIES are identified; the autosomal dominantly inherited type (AD-HIES) which was known as Job Syndrome and the autosomal recessive type (AR-HIES). Mutations in signal transducer and activator of transcription 3 (*STAT3*), dedicator of cytokinesis 8 (*DOCK8*), and phosphoglucomutase 3 (*PGM3*) genes have been identified in most patients with HIES. *STAT3* mutations are associated with AD-HIES (Chandesris et al. 2012), while *PGM3* and *DOCK8* mutations were identified with AR-HIES (Engelhardt et al. 2009; Sassi et al. 2014).

Unlike the recessive type, the AD-HIES manifests in patients in the form of skeletal, dental, and connective tissue abnormalities. STAT3 is an important transcriptional factor necessary for different cellular crucial activities. STAT3 consists of twenty-four exons and maps to chromosome 17q12. Most patients with AD-HIES have heterozygous mutations in the DNA binding and Src homology 2 (SH2) domains of STAT3, all of which are either heterozygous missense or in-frame mutations that indicates a dominant negative effect that causes loss-of-function (Chandesris et al. 2012; Holland et al. 2007; Minegishi et al. 2007; Vogel et al. 2015).

Autosomal recessive loss-of-function mutations in DOCK8 in patients with AR-HIES lead to loss of protein expression (Aydin et al. 2015; Biggs et al. 2017; Engelhardt et al. 2009). Furthermore, B cells that are lacking DOCK8 will fail to activate STAT3 when they are

stimulated by Toll-like receptor 9 (TLR9) which results in reduced immunoglobulin production and proliferation (Jabara et al. 2012). DOCK8 deficiency presents with eczema and recurrent sinopulmonary infections in infants. For PGM3, both heterozygous and homozygous mutations have been found to cause phenotypic features of HIES like eczema and recurrent sinopulmonary infections (Sassi et al. 2014; Stray-Pedersen et al. 2014). High IgE as well as eosinophilia and cytopenias are common features in PGM3 deficiency.

High serum IgE levels are the most important findings in patients with HIES. However, in adulthood, serum IgE can decrease and does not consistently correlate with the disease activity (Freeman and Olivier). Thus, serum IgE cannot give an accurate indication of the severity of the disease. Therefore, accurate newborn screening is essential for disease management and patient prognosis.

The definitive diagnosis of HIES is confirmed by mutation analysis of *STAT3*, *PGM3*, *DOCK8* and most recently *IL6ST* genes (Schimke et al. 2010; Schwerd et al. 2017). Other laboratory techniques may contribute to HIES diagnosis. For example, abnormal N- and O-linked serum glycans profiling may be useful in the diagnosis of PGM3 deficiency (Freeman and Olivier). In addition, quantitative measurement of DOCK8, PGM3 and STAT3 protein levels can also be used for diagnosis. For instance, immunoblotting techniques were used to detect the DOCK8 deficiency in cell lysate. Flow cytometry based biomarkers associated with DOCK8 deficiency but not atopic dermatitis have also been identified (Janssen et al. 2014). Flow cytometry may be used to detect DOCK8 protein in CD34+ BM cells and peripheral blood (Pai et al.). Finally, immunoassays such as ELISA for STAT3, PGM3 and DOCK8 are available. However, these methods are time consuming and needs specialized laboratories, and thus, not suitable for point-of-care testing.

To help achieve timely clinical diagnosis, multiple analysis of several biomarkers provide more accurate information than results from any single biomarker. Therefore, multiplex assays of several biomarkers using one analytical device hold significant potential for early detection including newborn screening, by simplifying the diagnostic process and providing more information in less time (Munge et al. 2016; Yáñez-Sedeño et al. 2017). Electrochemical immunosensors are an interesting alternative to conventional detection methods because of their fast response, simplicity, low cost, high sensitivity, less sample volume needed for testing and capability of multiplexing, and therefore, can be used in point-of-care testing.

Here, we report the fabrication of an array immunosensor for the multiplexed and simultaneous detection of DOCK8, PGM3 and STAT3 proteins. Array of carbon electrodes are used on which gold nanoparticles (AuNPs) were electrodeposited. Gold nanoparticles is used because of their enhanced electron transfer rate and catalytic activity. Moreover, AuNPs provide higher surface area enabling simple immobilization of higher amount biomolecules which enhances the sensor signal. The antibodies for the three proteins were immobilized to fabricate the sensor. This multiplexed immunosensor holds great potential for the early diagnosis of HIES.



## 2. Experimental

### *2.1. Materials and reagents*

ELISA kits for STAT3, DOCK8, and PGM3 were purchased from Mybiosource (San Diego, CA, USA). Potassium ferrocyanide ($K_4Fe(CN)_6$), potassium ferricyanide ($K_3Fe(CN)_6$), Phosphate buffered saline (PBS), glutaraldehyde, cysteamine hydrochloride, ethanol amine, were obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human cystic fibrosis

transmembrane conductance regulator (CFTR) protein was purchased from Abcam (MA, USA). The Duchenne Muscular Dystrophy (DMD) peptide was obtained from Pepmic (Jiangsu, China). PBS (10 mM, pH 8.5) was used for the preparation of antibodies solutions for the immobilization step. PBS (pH 7.4) was used to prepare the STAT3, DOCK8, and PGM3 protein standard dilutions and solutions and washing the immunosensors. Deionized water was used to prepare all solutions.

*2.2.Instrumentation*

Electrochemical measurements were done in 5 mM PBS (pH 7.4) containing 5.0 mM $[Fe(CN)_6]^{3/-4}$ redox pair in 0.1 M PBS buffer solution using square wave voltammetry (SWV). AUTOLAB pgSTAT302N (Metrohm, Netherlands) potentiostat was used. The parameters used for the SWV measurements are: amplitude 20 mV; interval time 0.04 s; step potential −5 mV; scan rate 125 $mVs^{-1}$, and frequency 25 Hz. The potentiostat was connected to a computer and run by Nova 1.11 software. The immunosensor was fabricated on disposable electrical printed (DEP) microarray electrodes purchased from BioDevice Technology (Nomi, Japan). Each electrode consists of eight individually addressable carbon-working electrodes, a central silver/silver chloride reference electrode and a ring-shaped carbon auxiliary electrode. A sensor connector (BioDevice Technology) is used to connect the DEP electrodes to the Autolab potentiostat.

*2.3.Methods*

*2.3.1. Functionalizing of the Au electrode surface and the immobilization of antibodies:*

Eight carbon working electrodes of an array chip are modified with gold nanoparticles by electrodeposition. The electrodeposition of the gold was performed by covering the sensor with a solution of 150 µl of 6 mM $HAuCL_4$ in 0.1 M $KNO_3$, then twenty CV scans were performed

from -0.2 to -1.2 V at 50 mV/s until a golden surface is obtained. The electrodes were then incubated for 2 h. in cysteamine hydrochloride at room temperature to form self-assembled monolayers, and then the electrodes were washed with distilled water. The surface was then activated by incubating the electrodes with 2.5% glutaraldehyde solution in 200 mM of phosphate buffered saline (PBS pH 7.4) for 1 h. After activation, the electrodes were washed with PBS to remove excess glutaraldehyde. The modified electrodes were then incubated with 10 µg/ml solutions of STAT3, PGM3, and DOCK8 antibodies prepared in PBS (pH 8.5). The antibodies were immobilized on different electrodes on the same chip for 1 h. in water-saturated atmosphere. Electrodes were then washed with PBS (pH 7.4) to remove unreacted excess antibodies. Electrodes were then blocked using 0.1 M ethanolamine for 30 min., then washed with PBS (pH 7.4). The immunosensor was then used for the detection of STAT3, PGM3, and DOCK8 or stored at 4 $^{o}$C in water-saturated atmosphere.

*2.3.2. Immunosensor detection experiment*

The detection experiment was performed by incubation of each modified electrode on the chip (immunosensor) with certain concentration of its specific protein, mixtures of proteins, or serum sample for 45 min at room temperature. After incubation, the electrodes were washed with PBS buffer, pH 7.4 and subjected to SWV measurement in ferro/ferricyanide redox solution as explained above. For the cross reactivity experiments, the immunosensors were incubated with either 1 ng/ml of CFTR, DMD, PGM3, STAT3 or 10 ng/ml DOCK8, and then signal was measured. The immunosensor signal was calculated as the percentage decrease in the reduction peak current of the redox molecules after protein binding (($i^{o}$-i)/$i^{o}$ %). $i^{o}$ is the peak current at zero concentration of the analyte and i is the current measured after each incubation.

*2.3.3. Application of the multiplexed immunosensor in serum samples*

Patients with confirmed genetic defects for DOCK8, PGM3, or STAT3 and healthy controls were included in this study. The blood samples were collected from subjects who attended the Allergy/Immunology clinics at KFSHRC. Samples from patients with DOCK8 (n=4), PGM3 (n=1), and STAT3 (n=1) deficiency, and healthy controls (n=9) were collected in this study. All patients signed an informed consent form by the declaration of the ethics committee and answered a baseline questionnaire regarding the symptoms of their diseases and allergies. The institutional review board at King Faisal Specialist Hospital & Research Center (KFSHRC), (RAC No. 2160 015) approved all research involving patient materials. The serum samples were diluted 1:10 with PBS buffer, pH 7.4 and incubated on the multiplexed immunosensor surface for 30 min, and then the immunosensors were measured after washing with PBS buffer. For comparison, we have also analyzed the same serum samples using commercial ELISA kits.

## 3. Results and discussion

*3.1.Immunosensor fabrication*

The multiplexed immunosensor was fabricated on commercial disposable array carbon electrodes as shown in scheme 1. First, the carbon electrodes were modified by deposition of AuNPs via the electroreduction of gold chloride salt in $KNO_3$ solution by CV sweepings between -0.2 and -1.4 V for 20 cycles. The reduction of the salt results in the formation of dense layer of AuNPs on the carbon surface, which was easily seen with the naked eye. Scanning electron microscopy images taken for the electrodes before and after AuNPs deposition are shown in Scheme 1C. The carbon surface showed overlapping sheets of carbon, whereas after deposition,

the gold particles are formed with an average diameter of 50 nm. The deposition of gold provides higher conductivity and larger surface area for immobilization of the antibodies (Eissa and Zourob 2017a). Second, the AuNPs- modified electrode was functionalized by self-assembly monolayer formation of cysteamine. Then, glutaraldehyde was used as a cross linker in which one of the aldehyde groups binds to the amine group from the cystamine forming imine bond and the other aldehyde group binds to the amino group of the antibody on the other side. The three antibodies for the DOCK8, PGM3 and STAT3 proteins were immobilized on different electrodes on the same chip in order to enable their simultaneous detection.

### *3.2.Electrochemical characterization of the multiplexed immunosensor fabrication steps on the carbon array electrodes*

The steps of the immunosensor design are characterized using SWV in ferro/ferricyanide redox couple. The SWV was measured after each step. As shown in Figure 1, the bare carbon electrode showed a well-defined cathodic peak for the redox couple reduction at pH 7.4. After the AuNPs deposition, an enhanced peak current was obtained indicating the higher surface area of the AuNP-modified electrode compared to the carbon electrode underneath (Eissa and Zourob 2017a, b). However, after the cysteamine incubation, further significant increase in the peak current was observed confirming the successful formation of self-assembled monolayer of cysteamine on gold (Eissa and Zourob 2017b). This enhancement in the current is attributed to the positive charge of the terminal amine groups at pH 7.4 which attracts the redox anions and therefore, accelerates the electron transfer process (Eissa et al. 2015). On the other hand, after the activation of the cysteamine with glutaraldehyde and the immobilization of the antibodies, a decrease in the current was seen in all the three cases. The current decrease was not the same for

the three antibodies because of the difference in the size, charge and the conformation of each antibody.

*3.3.Binding experiment of the immunosensor with the proteins and binding time optimization*

Figure 2A, B and C shows the binding of the immunosensor with 10 ng/ml of DOCK8, PGM3 and STAT3 proteins in PBS buffer pH 7.4. As shown in the voltammograms, significant decrease in the reduction peak current was observed after binding of the sensor with the three proteins. This is due to the blocking effect of the DOCK8, STAT3 and PGM3 proteins because of their bulky sizes which retard the access of the redox molecules to the electrode surface and thus, decrease the electron transfer efficiency.

It is essential to study the binding time of the proteins on the multiplexed immunosensor in order to obtain the maximum binding signal. The binding time was optimized by incubating the immunosensor with DOCK8, STAT3 or PGM3 proteins for different periods (from 5 to 45 min). The SWV signal was recorded after each incubation time. The sensor response was then calculated as $(i^o-i)/i^o$ %. Figure 2D, E and F shows gradual increase in the immunosensor response for the three proteins with increasing the incubation time. The optimum sensor response for DOCK8 and PGM3 was obtained at 30 min. However, for STAT3, the response signal was increased up to 45 min after which no further enhancement in the signal was obtained.

*3.4.Dose response of the AuNPs-based multiplexed voltammetric immunosensor*

In order to determine the analytical range and the limit of detection (LOD) of the multiplexed immunosensor for the detection of the three proteins (DOCK8, STAT3 and PGM3), the immunosensor was incubated with the three proteins at different concentrations. The SWV

measurements were recorded for the sensors after binding with the proteins. As shown in Figure 3A, B and C, the SWV signals are gradually decreasing with increasing the concentration of the protein incubated on the sensor surface in all the three cases. Figure 3D,E and F shows the calibration curves for the three proteins exhibiting good linear response. The linear response range for the DOCK8 and STAT3 was ranging from 1 pg/ml to 100 ng/ml. However, for the PGM3, the calibration curve showed a linear response in the range from 1 pg/ml to 10 ng/ml. The linear regression equations for the three proteins are: $(i^o\text{-}i)/i^o$ %= 19.98+ 3.94 log C [ng/mL], R = 0.992 for DOCK8, $(i^o\text{-}i)/i^o$ % = 31.2+ 5.84 log C [ng/mL], R = 0.984 for PGM3 and $(i^o\text{-}i)/i^o$ % = 20.5+ 3.36 log C [ng/mL], R = 0.99 for STAT3.

The multiplexed immunosensor showed a low detection limit of 3.1, 2.2 and 3.5 pg/ml for DOCK8, PGM3 and STAT3, respectively. The LOD was calculated based on 3 σ/b, where σ is the standard deviation of blank and b is the slope of the straight line of the calibration curve. These low LODs indicate high sensitivity of our multiplexed immunosensor for the three proteins which is lower than the commercial ELISA. The detection range of the DOCK8, PGM3 and STAT3 ELISA kits is from 0.156 to 10 ng/ml. The low sensitivity of our assay is very important as it will enable the detection of these proteins in HIES patients who may have low residual expression of these proteins. It should be also noted that our method is much faster and simpler than ELISA as it enable a direct detection of the three protein in 30 min, unlike ELISA which needs several incubation and washing steps and laboratory to perform.

The reproducibility of the immunosensor was also investigated by performing triplicate experiments for each protein. The relative standard deviations of the measurements ranged from 3.0-7.1% indicating very good reproducibility of the sensor performance.

*3.5.Studying the selectivity of the multiplexed immunosensor*

The selectivity of the multiplexed immunosensor was studied against other unrelated blood proteins such as DMD and CFTR in order to confirm the specificity of the sensor response. The immunosensor was therefore incubated individually with DOCK8, STAT3, PGM3, DMD or CFTR, and then the response was recorded after washing with PBS buffer. As shown in Figure 4A, the immunosensor response for the specific (HIES related) proteins was significantly higher than the non-specific proteins indicating appropriate selectivity of the method. Moreover, the simultaneous detection of the three proteins was also confirmed by mixing three as well as two specific proteins. As shown in Figure 4B when the three proteins were mixed, high responses were obtained for all three electrodes on the sensor, whereas only two protein specific high signals were obtained when two proteins were mixed implying the feasibility of applying our sensor for multiplexed detection of these proteins.

*3.6.Application of the multiplexed immunosensor in serum samples*

In order to test the applicability of our multiplexed immunosensor in biological samples for the diagnosis of HIES, serum samples from patients with confirmed diagnoses of DOCK8, STAT3 and PGM3 deficiency were analyzed with the immunosensor and ELISA. Control samples of healthy individuals were also tested for comparison. As shown in Figure 5A, B and C, the samples which have mutations in the STAT3, DOCK8 and PGM3 genes exhibited significantly lower protein levels than the control samples which were in agreement with the ELISA results.

These results indicate the potential of using our multiplexed immunosensor to differentiate between HIES patients and healthy individuals.

## 4. Conclusions

In conclusion, a simple and low cost voltammetric array immunosensor was developed for the diagnosis of Hyper- Immunoglobulin E syndrome via the detection of DOCK8, PGM3 and STAT3 proteins. The multiplexed immunosensor was fabricated on gold nanoparticles-modified disposable screen printed electrodes. A label-free strategy was used for the detection by monitoring the change in the square wave voltammetry reduction peak current of a redox solution upon binding of the sensor with the specific protein. This method was more sensitive than the commercially available ELISA kits. We believe that the sensitivity of the sensor was due to the signal enhancement caused by the use of AuNP modified carbon electrodes with high surface area and fast electron transfer. Moreover, good specificity of the immunosensor was obtained against other non-specific proteins that normally exist in the serum. The multiplexed array platform was successfully applied in serum samples showing ability to discriminate the various forms of Hyper- Immunoglobulin E syndrome from severe atopic dermatitis patients who share several clinical and laboratory features. This multiplexed platform can be applied for high throughput screening by increasing the number of electrodes on the sensor which will dramatically reduce the cost of the device. Moreover, the detection of the target proteins using this platform takes less than 45 min which is significantly less than the currently used molecular and immunological assays.

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**Figure captions**

**Fig. 1.** Square wave voltammetry in ($K_3Fe(CN)_6/K_4Fe(CN)_6$) redox couple of the bare carbon electrodes (black), after AuNPs deposition using 20 CV scans (red), after cysteamine attachment (green), after immobilization of DOCK8 (purple), STAT3 (blue) and PGM3 (cyan) antibodies.

**Fig. 2.** Square wave voltammetry in ($K_3Fe(CN)_6/K_4Fe(CN)_6$) redox couple of the immunosensor before (black) and after (red) binding with 10 ng/ml of DOCK8 (A), PGM3 (B) and STAT3(C) proteins. The time optimization experiments (a plot of the sensor response versus the incubation time) for DOCK8 (D), PGM3 (E) and STAT3 (F). The error bars represent the standard deviation of the measurements.

**Fig. 3.** Square wave voltammograms of the multiplexed immunosensor before and after binding with different concentrations of HIES specific proteins, DOCK8 (A), PGM3 (B) and STAT3 (C) immunosensor. The calibration curves for DOCK8 (D), PGM3 (E) and STAT3 (F) detection (plot of logarithm of the protein concentration versus the change in the peak current before and after binding $(i^o-i)/i^o\%$. The error bars represent the standard deviations of three measurements.

**Fig. 4.** The multiplexed immunosensor response to 1 ng/mL of PGM3, STAT3, DMD, CFTR and 10 ng/ml of DOCK8 (A) and the immunosensor responses to the simultaneous detection of DOCK8, PGM3 and STAT3 (after incubation with a mixture of 1 ng/ml PGM3+STAT3+DOCK8; STAT3+DOCK8; and PGM3 alone).

**Fig. 5.** Application of the immunosensor for the detection of DOCK8, PGM3 and PGM3 in HIES patients and control serum samples. The error bars represent the deviation of the

measurements (DOCK8, n=4; controls, n=4; STAT3, n=1; controls, n=3; for the PGM3, n=1; controls, n=2).

**Scheme 1** (A) Schematic diagram of the array electrodes on which the three antibodies were immobilized. (B) Functionalization steps of the AuNPs-modified electrodes by cysteamine, glutaraldehyde and antibody immobilization. (C) Scanning electron microscopy images of the carbon electrode surface before and after AuNPs deposition.

**Fig. 1**

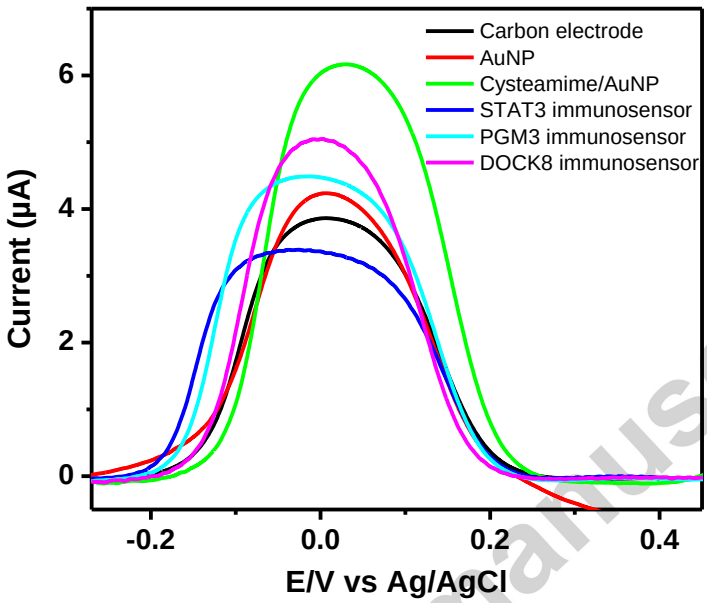


**Fig. 2**

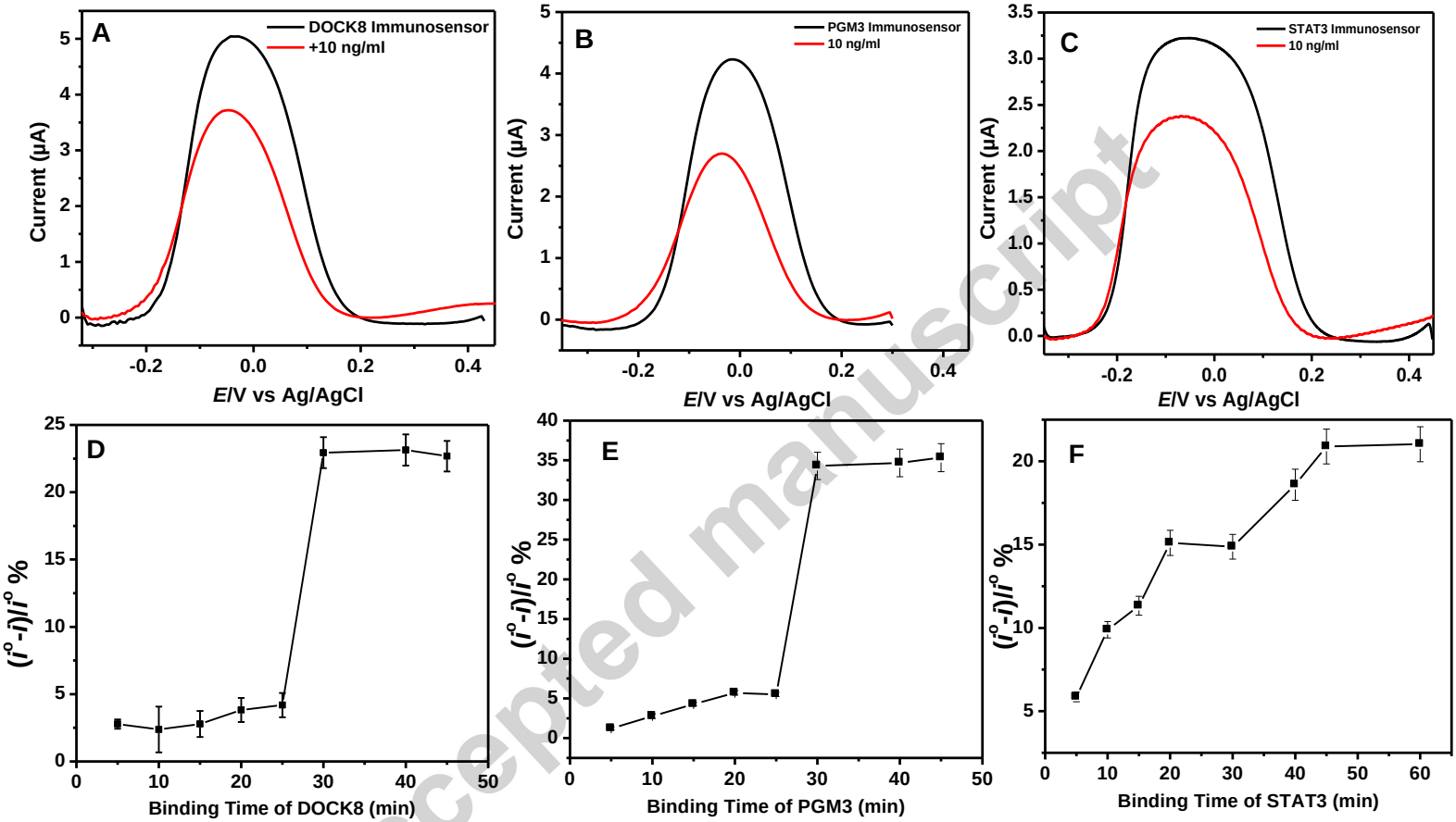


**Fig. 3**

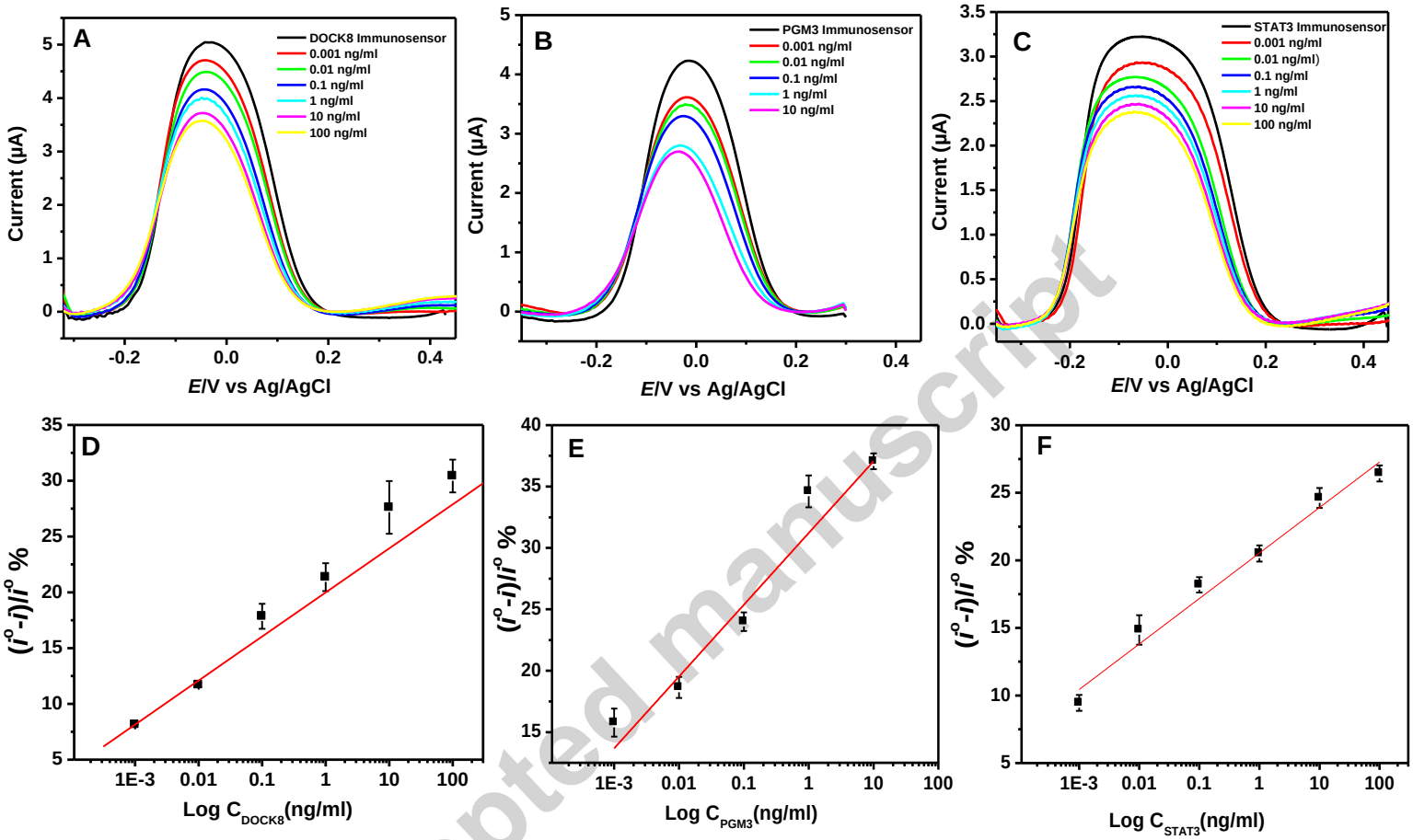


**Fig 4**

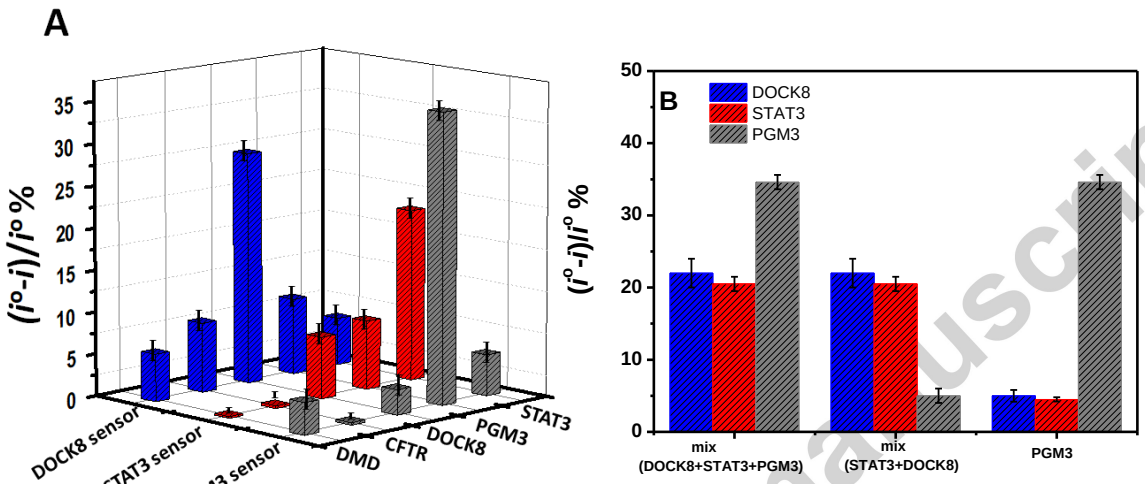
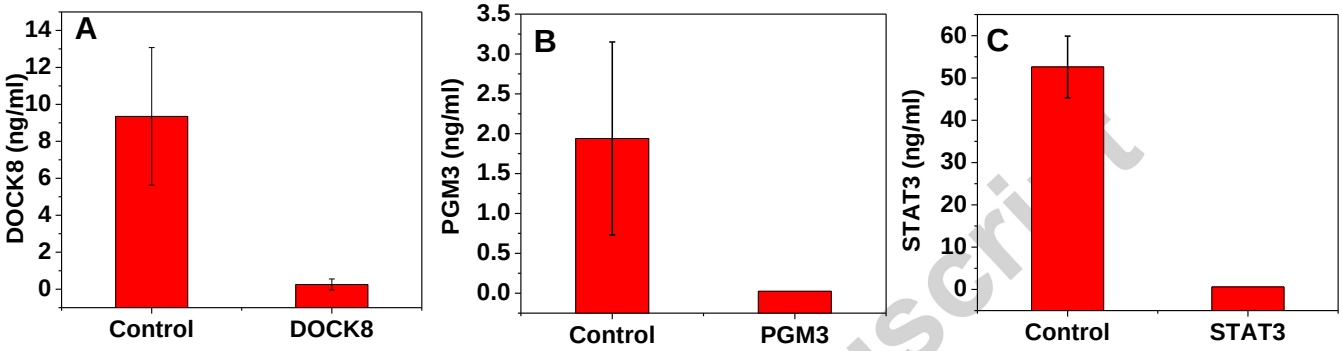
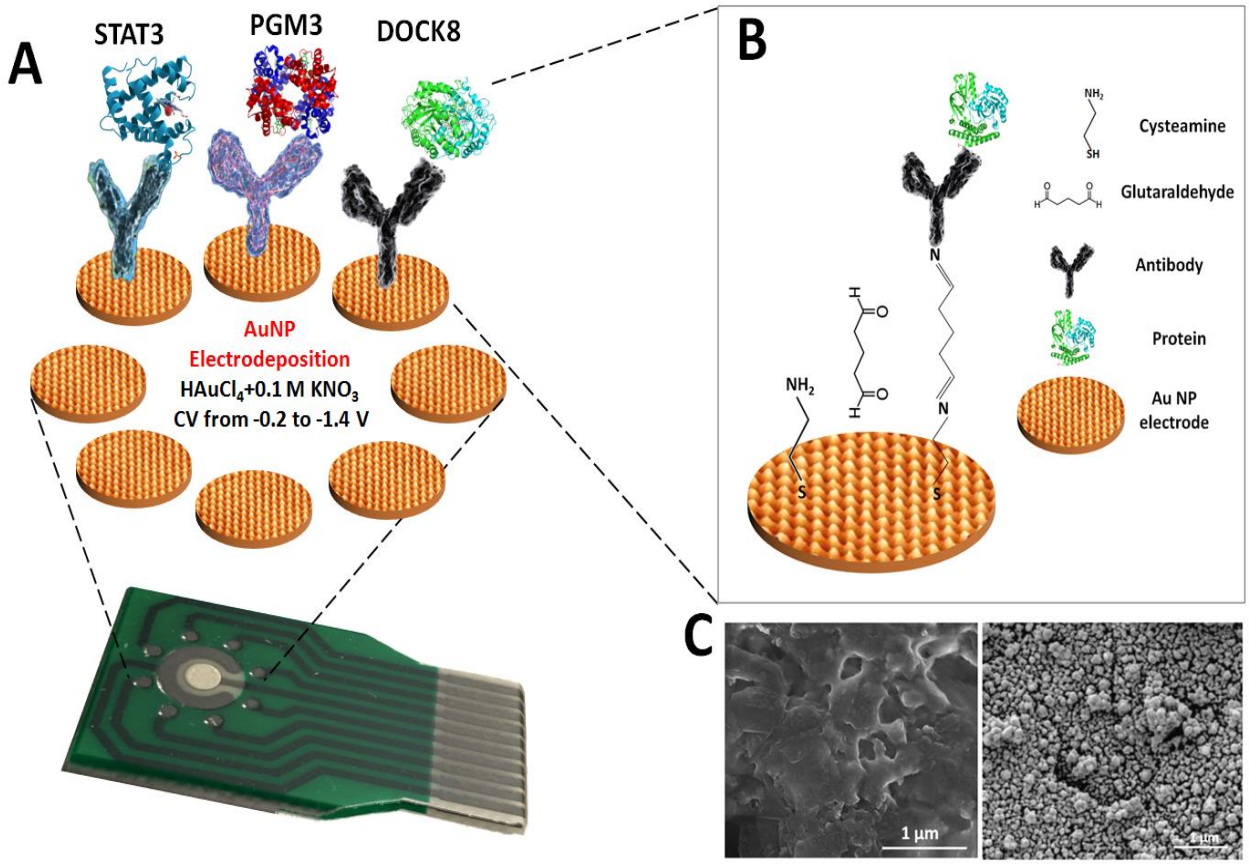


**Figure 5**



**Scheme1**



**Highlights**

- Hyper Immunoglobulin E syndromes (HIES) are rare primary immunodeficiency disorders associated in *STAT3*, *DOCK8 and PGM3*.
- Yet, the diagnosis of HIES is challenged by the complexity of the existing laboratory assays.
- Multiplexed electrochemical immunosensor for the simultaneous detection of DOCK8, STAT3 and PGM3 proteins.
- D.L.of the DOCK8, PGM3 and STAT3 proteins were 3.1, 2.2 and 3.5 pg/ml.
- The immunosensor was successfully applied in human serum samples showing capability to distinguish the HIES from the control samples.