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Short communication

# A cytokine immunosensor for Multiple Sclerosis detection based upon label-free electrochemical impedance spectroscopy using electroplated printed circuit board electrodes

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ABSTRACT

A biosensor for the serum cytokine, Interleukin-12 (IL-12), based upon a label-free electrochemical impedance spectroscopy (EIS) monitoring approach is described. Overexpression of IL-12 has been correlated to the diagnosis of Multiple Sclerosis (MS). An immunosensor has been fabricated by electroplating gold onto a disposable printed circuit board (PCB) electrode and immobilizing anti-IL-12 monoclonal antibodies (MAb) onto the surface of the electrode. This approach yields a robust sensor that facilitates reproducible mass fabrication and easy alteration of the electrode shape. Results indicate that this novel PCB sensor can detect IL-12 at physiological levels, <100 fM with *f*-values of 0.05 (typically <0.0001) in a label-free and rapid manner. A linear (with respect to log concentration) detectable range was achieved. Detection in a complex biological solution is also explored; however, significant loss of dynamic range is noted in the 100% complex solution. The cost effective approach described here can be used potentially for diagnosis of diseases (like MS) with known biomarkers in body fluids and for monitoring physiological levels of biomolecules with healthcare, food, and environmental relevance.



## 1. Introduction

Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system marked by progressive demyelination of neural axons. The disease causes slow damage to the nervous system, leading to inhibited muscle coordination, loss of visual sensation, and other effects on the nervous system. MS affects over 400,000 people in the United States and 2.5 million world wide, making it a pressing issue in healthcare (National MS Society). Therapeutic treatments can modify the course of the disease and relieve symptoms if used at an early stage of MS. Early action requires early diagnosis (Santos et al., 2006). Diagnosing MS begins with observation of overt MS symptoms and tests of motor function like the Multiple Sclerosis Functional Composite (Cutter et al., 1999; McDonald et al., 2001), which are documented to have poor reliability (Hindson et al., 2005). More accurate diagnosis using techniques such as magnetic resonance imaging (Barkhof et al., 1997), radioimmunoassay, or western blotting for protein detection (Galboiz and Miller, 2002) have prohibitive costs or time requirements resulting in substantial delay between the onset of MS and the diagnosis of the disease.

Inflammatory cytokines such as the interleukins, are relevant biomarkers for MS which reflect on the disease's state. The concentrations of a pro-inflammatory cytokine Interleukin-12 (IL-12) have been found to vary in MS with normal serum ranges of 0–5.0 pg/ml and elevated ranges from 5.5 to 18.6 pg/ml for MS patients (Hafler and Weiner, 1995). A clinical sensor for measuring IL-12 and other MS biomarker levels would aid in accurate diagnosis of the disease in a timely manner, improving the possibility of successful intervention. Such a sensor must be reliable, rapid, low cost, and sensitive to fM changes in biomarker concentrations in physiological solutions such as blood, serum, urine, sweat, and cerebrospinal fluid.

Electrochemical biosensors are an excellent candidate for meeting the needs of a clinical sensor for protein biomarker detection. Such sensors pair the sensitivity of electrochemistry and selectivity

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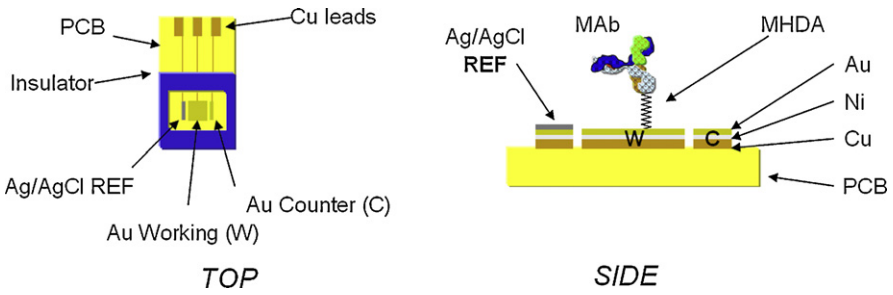


**Scheme 1.** Basic schematic of device fabrication. (Left) standard three electrodes (reference, working, counter) for electrochemical detection. Formation of 100 μl well achieved with insulator layer also used to protect underlying copper on PCB. (Right) side view showing PCB with electroplated copper (Cu), nickel (Ni), gold (Au), and Ag/AgCl (on reference only) with covalently immobilized monoclonal antibody.

of antibodies with low cost fabrication and robust designs. Previous efforts in this area have yielded a variety of immunosensors, but these sensors have been limited in sensitivity with limits of detection of 1 ng/ml with differential pulse voltammetry (Shen and He, 2007) or 50 pg/ml with a complicated electrode arrangement with two electrodes off sensor and 20 min incubation with target prior to detection (Zhang et al., 2007). Electrochemical impedance spectroscopy (EIS) is a sensitive, label-free approach that can be used to interrogate biomolecular interactions (Tahir and Alocilja, 2003; Davis et al., 2005). Previously, we reported the development of a label-free EIS immunosensor for detection of IL-12 on gold-sputtered silver ribbon (La Belle et al., 2007a). Within the previous report, a limit of detection of 1.0 pg/ml IL-12 was achieved, although the system demonstrated significant nonlinearities over the dynamic range. In addition, our metal ribbon electrode design lacked robustness and rigidity affecting its stability and reproducibility.

Here, we present a gold electrode EIS biosensor using scalable fabrication techniques (Approach 1) designed as an immunosensor for label-free, low level detection of IL-12 at physiological concentrations and beyond. Using PCB design paired with electroplating, an optimized design is readily achieved that specifically addresses the limitations of our previous sensor design and promises future utility as a clinical sensor. This new design allows for a rigid substrate for electrodes to be fabricated upon, rapid change out of design (from concept to sensor in under 25 min), and by controlling electroplating time and current, final impedance of the sensor can be optimized. Using IL-12, an improved, exponential model with an increased dynamic range over our previous work is demonstrated. Furthermore, this new approach's performance in a complex solution of fetal bovine serum (FBS) is also explored—a critical next step in clinical sensor development.

## 2. Experimental details

### 2.1. Electrode fabrication

The PCB electrodes used here for impedance spectroscopy measurements were designed to incorporate a standard three-electrode system comprising of a Ag/AgCl reference electrode, a gold working electrode, and a gold counter electrode (La Belle et al., 2007b). Initially, the electrodes were designed using computer aided design (CAD, Autodesk) software which allowed for the easy modification to sensor geometry. Using a single 15.2 cm × 15.2 cm pre-sensitized copper clad PCB (MG Chemicals), 50 individual sensors could be obtained at a time. For fabrication purpose, the electrode layout was first printed on a transparency and then carefully aligned to the board. The board was then put in a UV light box which held the transparency in place, and the unmasked portions of the board were exposed to UV light. Next, the PCB board was developed in a 0.25 M, pH 14 sodium hydroxide (MG Chemicals) solution for 2 min while the photoresist was gently removed. The PCB was rinsed thoroughly with distilled water and then placed in an etching tank containing a 1.1 M, pH 1.0 solution of ammonium persulfate (MG Chemicals) heated to 40 °C and agitated by bubbling air through it. The board remained in the solution for about 8–10 min until all of the exposed copper had been removed. After rinsing, the PCB board was cut into individual sensors and the remaining photoresist was removed from the surface of the electrodes using acetone. As seen in Scheme 1, a rubber insulator (Plasti Dip Intl) was used to create wells on the sensor surface capable of holding a sample volume of 100 μl.

For electroplating, the PCB sensors were first cleaned in a degreaser solution of sodium perborate (Caswell) at 98 °C for 2.5 min under slight agitation. Electroplating of nickel was done in sets of 5 sensors, with a 380 ml solution of nickel sulfate (Caswell) at pH 4.5 at 43.3 °C. The plating current density was approximately 10 mA/cm$^2$, and the chips were plated until the total charge transfer was approximately 6.3 C in an attempt to deliver 0.5 mA per lead of the three leads per 5 sensors simultaneously plated, actual output was 0.42 C for a given amount of time that resulted in 6.3 C. The electrodes were immediately rinsed with warm distilled water and then degreaser and rinsed again. The electrodes were then electroplated in a gold sulfate (Caswell), pH 9.6 solution at 60 °C. The plating current density was 0.8 mA/cm$^2$, and the chips were plated until the total charge transfer was ~0.15 C. Silver nitrate solution (Caswell) was brush plated onto the reference electrode using a marker tip held at a fixed potential of 1.7 V for 30 s and then chlorinated with a bleach solution for 2 min. The electrodes were immediately stored in sterile pH 7.4, 1× phosphate buffer saline (PBS) at room temperature (RT) to minimize the possibility of surface contamination.

### 2.2. Immobilization of antibody on electrodes

Previously described methods to immobilize a molecular recognition element onto a PCB electrodes were utilized (La Belle et al., 2007b). Briefly, in order to immobilize the anti-IL-12 antibody onto the surface of the electroplated PCB electrodes, 100 μl of a 25 mM 16-mercaptohexadecanoic acid (16-MHDA) (Aldrich) solution in reagent grade ethanol was placed into each sensor wells. The sensors were placed in Petri dishes filled with ethanol soaked towels to reduce solution evaporation and sealed closed with Parafilm. The sensors were allowed to set at RT for 12 h. Next, the surface was carefully rinsed with ethanol and then distilled water. A 100 μl droplet of a 40 mM N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) (Pierce biotechnology), 10 mM N-hydroxysulfosuccinimide (sulfo-NHS) (VWR international) solution was placed in each sensor well. After 1 h at RT, the sensors were rinsed in PBS buffer. Next, a 100 μl droplet of a 50 μg/ml solution of anti-IL-12 IgG (R&D systems) in PBS buffer was placed on the electrode and left at RT for 1 h. The

sensors were carefully rinsed with PBS and then a 100 μl droplet of 1 mM ethanolamine was added for 30 min at RT to block unreacted carboxyl groups of 16-MHDA and EDC/NHS. The blocked sensors were washed with PBS and stored in PBS at 4 °C until future use. Verification of the electrode functionalization and specificity testing, including antibody immobilization density calculations, is available in the supplemental material available online.

### 2.3. Impedance spectroscopy measurements

Measurements of electrochemical impedance were made using an Electrochemical Workstation from CH Instruments (CHI660C). All test solutions were prepared using a redox probe solution which contained 5 mM potassium ferricyanide and 5 mM potassium ferrocyanide in PBS. The IL-12 sample solutions were diluted in the redox probe from 0 to 100 pg/ml and stored at 4 °C until use. Each PCB sensor was connected to the impedance spectrometer and 100 μl of redox probe solution was added to the well. An AC potential with a 5 mV amplitude and +250 mV DC offset was applied between the working and reference electrodes and the measured current through the counter electrode was used to calculate the sensor impedance. The AC frequency was swept from 1 to 100,000 Hz to create an impedance spectrum for analysis. This process repeated for each concentration of IL-12. This process was also repeated using complex redox probe solutions containing 1% and 100% FBS.

In order to find the frequency at which maximum changes in impedance characteristics are seen, a plot was made of maximal percent change in impedance versus frequency for each of the concentrations of IL-12 samples tested. By doing so, an optimal frequency was identified for comparison between different PCB electrodes, controls and samples. Selection of the optimal frequency and data are included in the supplemental material available online.

## 3. Results

### 3.1. Improved disease marker detection

Determination of maximal response (impedance) was found to occur at 5 Hz ($Z_{5\,Hz}$) by calculating change in impedance over frequency for the sensors as concentration changes occurred. Data was from four sensors were gathered and background subtracted for ease of comparison with previous study over the range of 0–100 pg/ml of target molecule (Fig. 1). Scale of Au-coated-Ag ribbon data was adjusted until reaching the level of the PCB electrodes (hence error bars appear to be quite large—also scaled). $Z_{5\,Hz}$ at physiological levels of IL-12 had an $f$-value <0.0001 over

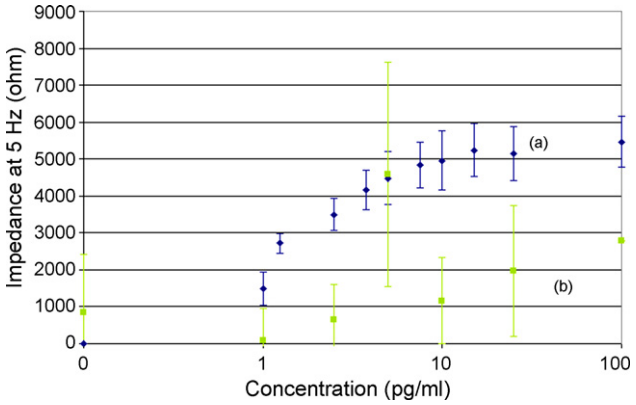


**Fig. 1.** Comparison of "normalized" results from (a) electroplated PCB electrodes ($n$ = 4) to previous (b) silver ribbon, gold-sputtered electrodes ($n$ = 4). Sample volumes were either 100 μl (a) or 400 μl (b) of the different IL-12 concentrations.

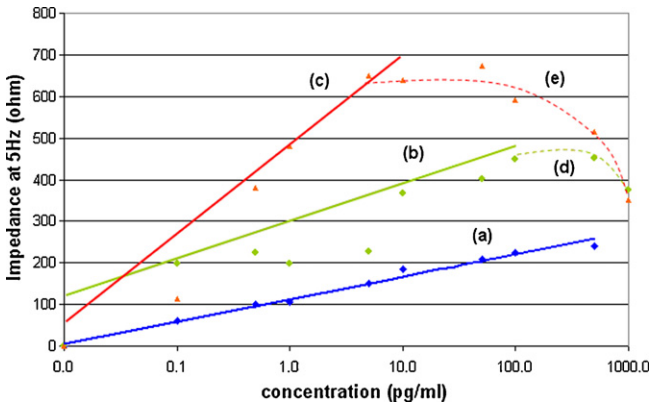


**Fig. 2.** Detection of IL-12 in 0% (a) with $R^2$ of fit at 0.99, 1% (b) with $R^2$ of fit at 0.84, and 100% (c) with $R^2$ of fit at 0.43 (to 1000 pg/ml of target) FBS solution. Data are unreplicated experiments with best-fit lines added with extended portions showing nonlinearity due to increasing complexity of solution in (d) and (e).

background but as concentrations reached maximal (calculated) detection range (250 pg) a flattening of the slope occurred thus demonstrating a larger than physiological dynamic range. In this design, the electrode has slightly improved variance than the previous design. Relative Standard Deviation (RSD) is 9.92–16.13% over 0–100 pg/ml, compared to 11.07–25.51% RSD in our previous work (La Belle et al., 2007). Also, a more linear response (with respect to log concentration) is displayed over the 0–25 pg/ml (physiological) range of IL-12 between normal levels and levels of IL-12 in patients with MS. The sensitivity enhancement designed into the PCB electrodes is clearly a marked improvement over the previous feasibility study using Ag-ribbons as substrates for the gold.

### 3.2. Complex fluid target detection

PCB electrodes were studied with targets in 0%, 1%, and 100% FBS (Fig. 2). Electrodes had been blocked with 1% ethanolamine. Results indicate that detection of targets was extremely linear in purified samples up to approximately 10 pg/ml (Fig. 2a). As the concentration of complex solutions was increased from 1% to 100% (Fig. 2b/c), the dynamic range became shortened (and very nonlinear as in Fig. 2d/e). In the purified samples, a maximum detection of ~250 pg/mL was estimated (calculations available in supplemental material available online), and the device appeared to level off between 100 and 500 pg/ml of IL-12. A slope change was noticed in the 1% FBS samples and was most likely due to background interactions of the other proteins present in the FBS. Again, maximum detection levels between 100 and 500 pg/ml. The extreme case of 100% FBS showed a marked decrease in maximum sample detection at 5 pg/ml and increase in slope. Again, this background level of protein in FBS (approximately 70 mg/ml) decreased the maximum detection. This was attributed to diffusion issues more than nonspecific interactions with the electrode surface as the controls for 0 pg/ml showed no increase (or significant variance) between 0%, 1%, and 100% FBS samples.

## 4. Conclusions

This work demonstrates the critical development of a rapid, label-free, easily intermediate precision EIS sensing technology. This technique requires, from sample to detection, only 90 s and is 1 step, add sample, unlike complicated or labeled systems such as ELISA, Radiolabeled Immunoassay, or mass spectrometry. This technique allows for a label-free, ultra-low level (<100 fM) detection of a prototypical biomarker for partial diagnosis of MS with significance of $f$-value of 0.08 (significant in model) between 3.75

and 7.5 pg/ml at maximal disease detection range. This sensor shows an overall RSD of ~9% between 0.1 and 500 pg/mL, a limit of detection of 3.5 pg/ml using the 3.3Sigma FDA method, and saturated at 250 pg/mL, making clear improvements in sensor performance. Sensor performance is also evaluated in 0%, 1%, and 100% FBS and shows that FBS increases the slope of the concentration response plot. The sensor retained full dynamic range in 1% FBS. These results demonstrate the sensors' high sensitivity and intermediate precision. Combined with a robust design and scalable fabrication techniques, this sensor offers exciting new opportunities in clinical sensor development. Future work will include improvement of sensor sensitivity and selectivity in complex solutions and expansion of the repertoire of detectable biomarkers.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.07.017.

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