Bio-impedance Sensing Device (BISD) for Detection of Human CD4$^+$ Cells

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ABSTRACT

The aim of this work is to develop a small on-chip bio-impedance sensing device (BISD) to rapidly detect and quantify cells with a specific phenotype in a heterogeneous population of cells (e.g., human CD4$^+$ cells in blood, for monitoring HIV-infected individuals) using a minimal sample volume and minimal preparation. The transducers, gold/titanium microelectrodes (100x100μm and 80x80μm), have been fabricated on glass substrates. The microelectrode surface is non-covalently modified sequentially with protein G', human albumin, monoclonal anti-human CD4 antibody, and mouse IgG. The anti-human CD4 antibody binds CD4$^+$ cells present in human blood. The basic function of the microelectrodes was characterized using electrochemical cyclic voltammetry before and after protein G' deposition. The binding of biomolecules, protein G' and antibodies, as well as cells was detected by precisely measuring the electrical current, as a function of frequency (1-8 kHz), that flowed between the microelectrode and the much larger reference electrode. This current was measured using a specially designed very low-noise amplifier based on instrument-grade operational amplifiers. This measurement was plotted as impedance, using the constant-amplitude voltage applied between the two electrodes. We have conducted a series of AC impedance and output voltage measurements with various sizes of gold microelectrodes with adsorbed protein layers, as well as with the CD4$^+$ cells. When a sample of human peripheral blood mononuclear cells (PBMCs) was incubated on the biosensor, an increase in impedance was observed; this increase was due to the presence of CD4$^+$ cells, which cause a decrease in the current flow. Incubation of the captured cells with FITC-labeled anti-human CD4 antibody verified that all captured cells were CD4$^+$, demonstrating the selectivity of the BISD system. The BISD system is a promising tool for the detection of CD4$^+$ cells in HIV-infected individuals, and for the detection and quantification of antigen:antibody or receptor:ligand interactions.

Keywords: microelectrode, CD4 cell biosensor, impedance, nanofabrication, human blood

1 INTRODUCTION

Impedance measurement is not new to biological applications, as early as 1898. Stewart presented a paper at a meeting of the British Medical Association at Edinburgh, later published in the Journal of Experimental Medicine [1]. He observed that the electrical conductivity of culture media containing living bacteria changed during the growth of organisms. The electrical response curves that he presented were very similar to curves that are obtained from currently available impedance systems. The significant difference is that, today, impedance can constitute a rapid measure of microbial proliferation, whereas Stewart measured changes in conductance over periods of 30 days.

Following this pioneering work, some attempts to develop impedance systems [2-4] were made during the first part of 20th century. However, it was not until the mid 1970s that the technique began to receive the attention that it merits. This coincided with the introduction of dedicated impedance systems [5-8], notably by the Torry Research Station [9]. It was the work of the Torry group that paved the way for impedance microbiology, along with their use of the Malthus and Bactometer systems. Significant progress has been made since that time [10-14]. The effects of electric fields on cells have been extensively studied, since widespread applications exist in cell biology, for the monitoring of cell migration and morphological changes in real time on gold surfaces [15]. The main outcomes of the earlier studies were limited to the determination of the preliminary electrical response of a cell. It is now possible to make sensor devices that are targeted to particular cell types in blood and that can be diagnostic or prognostic for diseases such as cancer or AIDS. At least 40-100 million individuals are thought to have been infected with the human immunodeficiency virus (HIV) throughout the World, and more than one million of them are currently living in the United States. HIV was first identified in 1983, and in 1984 it was shown to be the cause of AIDS [16-18]. HIV infection is characterized by the depletion of the CD4$^+$ helper/inducer subset of T-lymphocytes, leading to severe immunosuppression, neurologic disease, and opportunistic infections and neoplasms [19].

Dysfunction of CD4$^+$ T cells may be the result of direct infection with HIV, but it also may be caused indirectly by exposure of infected cells to various viral proteins. Because CD4$^+$ T cells play an important role in immune responses, it is not surprising that many of the immune defects that are observed during AIDS are secondary to the progressive decline in the number and function of CD4$^+$ T cells [20]. Although flow cytometry is currently used to quantify CD4$^+$ cells, it is expensive and time consuming, and it requires considerable expertise. To overcome this problem,

we are developing a micro-biosensor to quantify $CD4^+$ cells. It is a small, portable bio-impedance sensing device (BISD) based on gold surface coated with protein G' and mouse anti-human CD4 antibodies to capture $CD4^+$ cells from heparinized whole blood on microelectrodes.

Our work suggests that the BISD has considerable potential as a biosensor that forms the basis of a rapid, sensitive system for monitoring and quantifying $CD4^+$ cells. Initial results in our laboratory are promising. A further advantage of the BISD is the ability to collect data directly onto a PC, thereby allowing easy analysis. In summary, our BISD is a rapid, economical, and flexible tool, for use in detection of specific particles or cell types.

2 DEVICE DESIGN AND FABRICATION

A three-electrode device was designed and fabricated at the Cornell Nanoscale Facility. Four-inch glass wafers were prepared in a hexamethyldisilazane (HMDS) vapor prime oven for 34 min to improve the adhesion of the resist to the glass surface. Wafers were then spun with microposit S1818 photoresist (Shipley Company, Marlborough, MA) at 4000 rpm for 40 sec and soft-baked for 60 sec at 115ºC.

The EV620 contact aligner was used to expose the resist for 7 sec. A dummy wafer was also used to check alignment prior to exposure of the glass wafers. The wafers were then put in a vapor priming oven and subjected to an ammonia bake. Following the image-reversal bake, the wafers were flood-exposed in the HTG System III contact aligner for 30 sec, and they were then developed in MF 321 developer (tetramethylammonium hydroxide) for 1-1.25 minutes, rinsed, and dried.

Titanium (150 nm) and gold (1200 nm) films were deposited on the wafers, using a CVC SC4500 combination thermal/E-gun evaporator via E-beam for the titanium and thermal for the gold. After deposition, the wafers were left in a microposit remover 1165 (N-methyl-2-pyrrolidine, Shipley company) bath. After 4 hr, the wafers were rinsed with a water spray and dipped in acetone, to remove any residual gold and/or resist.

Polyamide was spun on the patterned glass wafer and soft-baked for 1 min. The insulating layer was patterned to expose the electrodes and contact pads. Finally, wafers were baked for 8 hr in a 450PB high temperature vacuum oven using a programmable temperature controller and nitrogen flow cycles for curing of polyimide films. Details of the dimensions of the electrodes are shown (see Fig. 1).

3 SENSOR DEVELOPMENT AND MEASUREMENTS

3. 1 Chemicals and Instrumentation

Protein G' was obtained from Sigma (St Louis, MO). Mouse monoclonal anti-human CD4 antibody and fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 antibody was from Pharmingen USA. Human peripheral blood mononuclear cells (PBMCs) were obtained from volunteers in accordance with Institutional guidelines of the Wadsworth Center, Albany, NY. All other reagents and solvents were analytical grade.

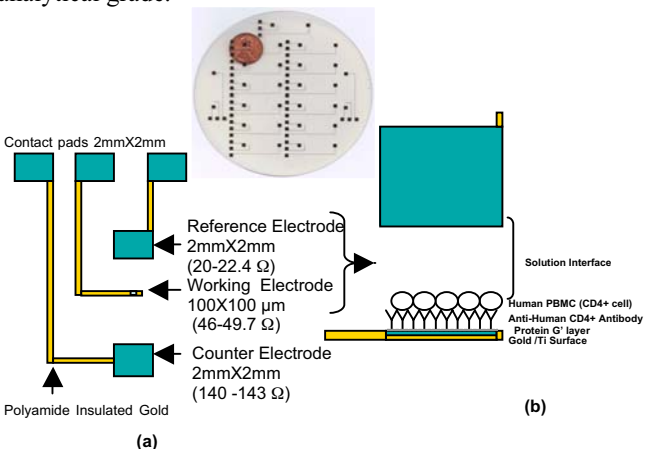

Fig. 1: BISD and its capture system(a) Electrode pattern and dimensions and (b) Modification of gold electrode surface by successive layer of protein, antibody and cells.

3.2 Procedure

The gold electrodes were first washed with nanopure water and dried under nitrogen. A PDMS well was applied on the 4 inch wafer, creating a well over the three electrode sets forming the biosensor. Prior to use, all electrodes were inspected under a microscope, and a blank impedance measurement was recorded with 1X PBS at pH 7.15. The protein was deposited on the working electrode ($0.01mm^2$) that was 400 times smaller than the gold reference electrode ($4\ mm^2$). A small volume (10 µL) containing 1 µg of protein G' was incubated on the microelectrode surface overnight at 4ºC in a water-saturated atmosphere. After incubation, the microelectrode was washed three times with 1 X PBS to remove any unbound protein. Protein G'-modified microelectrodes were then coated with human albumin as a blocking agent. The sensor's gold-protein G' electrode was further modified by incubating it in 1 µg/10µL of mouse monoclonal anti-human CD4 antibody in a wet chamber at 4ºC for 2-4 hr, washed, and again blocked with 1µg/10µL mouse IgG. The microelectrode was washed after each coating, and impedance was recorded using gold spring contacts between the sensor's external pads and the external electronics. An AC voltage with an amplitude of 50.5mV was swept over a frequency range from 1 to 8 kHz. After the impedance measurements, the working electrode was washed. The microelectrode was then incubated in lysed whole blood containing $CD4^+$ cells (10µL containing 3,000-100,000 total cells) for 1.5 hr in a wet chamber. After incubation, the working electrode was washed three times with 1X PBS to remove unbound cells. The impedance was measured again. To verify that the bound cells were $CD4^+$ cells, the electrode was incubated for 1 hr with 10µL of anti-human FITC CD4 antibody. The electrode surface was washed with PBS, and

images were recorded in a fluorescence microscope. To determine whether all the cells were $CD4^+$ cells, the electrode was also stained with 5µg/mL of 4', 6-diamidino-2-phenylindole (DAPI) in milliQ water.

4 RESULTS

4.1 Protein G' Adsorption on Microelectrode

The interaction between proteins and solid surfaces occurs widely in nature. There has been extensive research into the mechanisms intrinsic to protein adsorption, particularly in the fields of biomaterials and biocompatibility. It is well known that the adsorption of proteins onto electrode surfaces alters the electrochemical reaction at the electrode surface. Cyclic voltammetry is a very power tool for measuring electrode kinetics in the solid as well as the solution phase. In this work, the adsorption and subsequent effect of protein G' on the electrochemical properties of a gold electrode were observed, using cyclic voltammetry with a 0.05 mM potassium ferricyanide/0.01 mM sodium nitrate solution. The well-defined Fe^{2+}/Fe^{3+} redox couple peaks were observed in the potential range from -1.00 to 0.5V, at a scan rate of 100 mV/sec in the absence of protein G'. The same procedure had been applied to a gold electrode that was coated with 1 µg of protein G'. The Fe^{2+}/Fe^{3+} redox couple peaks were not observed in the presence of protein G' indicating that the protein was adsorbed to the gold electrode. The adsorbed protein G' layer acted as a blocking agent that hindered the electron transfer process at the electrode/solution interface (Fig. 2). Further, we verified that the optimal size ratio between the reference electrode and the working microelectrode is in the range of 300-600:1. In the present system, the reference electrode (4 mm^2) was 400 times larger than the working electrode (0.01 mm^2). This size ratio between two electrodes has little effect on the detection of the electrode's dynamics, and small changes in electrode behavior can be monitored.

Our BISD is capable of detecting the electrical response created by either single or multiple layers of protein deposited on the working microelectrode (see Fig 3.). Further, the signal resulting from two layers is significantly greater than that from a single layer. The protein layers include antibodies that serve as a molecular capture system for cells of the human immune system, in this case $CD4^+$ cells. The increase in impedance resulting from the capture of $CD4^+$ cells was significant with respect to the impedance produced by the capture system. Fig. 3 shows the increase in impedance produced by four cells in10 µL of PBMC's from previously frozen blood bound to the microelectrode. is The impedance change is due to presence of $CD4^+$ cells hindering the current flow from the small microelectrode. The number of cells on the electrode was confirmed by fluorescent light microscopy of FITC-labeled anti human CD4 antibody and DAPI (a stain for cell nuclei). Figure 4 shows that four $CD4^+$ cells were captured, which is in good agreement with the expected ~5-6 $CD4^+$ cells based on the microelectrode size and the number of cells in the preparation.

When the molecular capture system included blocking steps, (human albumin after protein G' and mouse IgG after the anti-human CD4 antibody), the change in impedance caused by a single cell captured from lysed blood (Figs. 5a & b) was less than that for the unblocked preparation (Fig. 5c). The smaller magnitude of the impedance change was probably due to differences in the electrical characteristics of the cells and to the presence of the additional layers of protein. Figure 5d also indicates that the output voltage may be a more sensitive measure of cell binding. The differences in output voltage before and after cell binding were due to a frequency-dependent change in capacitance.

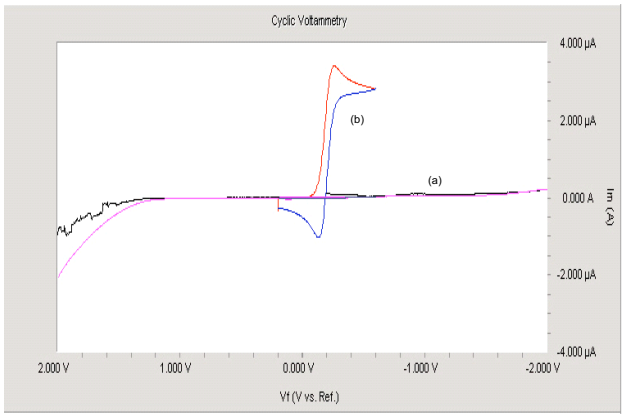

Fig. 2: Cyclic voltammogram of Fe^{2+}/Fe^{3+} redox couple in the presence (a) and absence (b) of 1µg protein G' on Microelectrode.

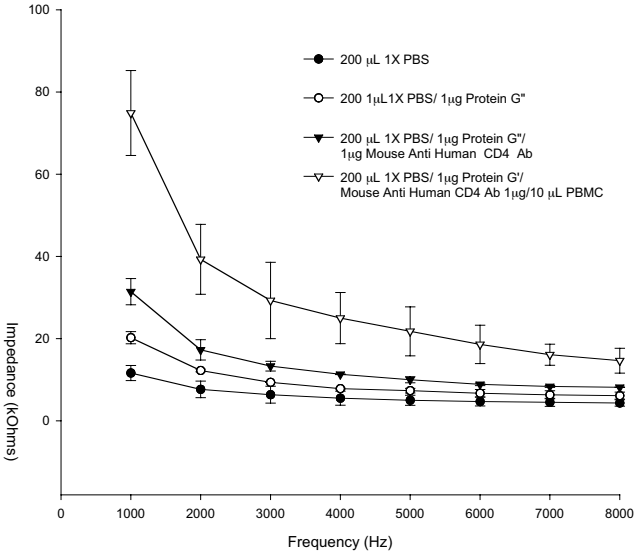

Fig. 3: Comparative impedance graph of successive layers of PBS, protein G', anti human CD4 Ab, and PBMCs on gold 100 X 100 µm electrode (no. of measurements/electrode = 3)

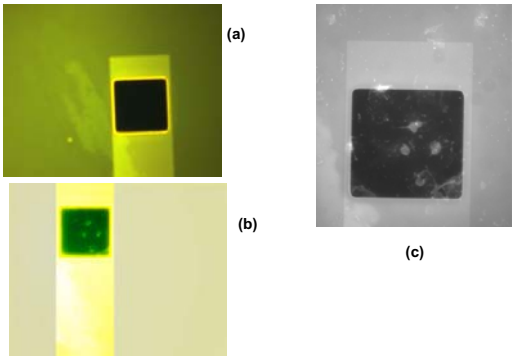

Fig. 4: Fluorescence image of blank electrode (a), FITC anti-human CD4 antibody-stained electrode showing 4 $CD4^+$ cells on the microelectrode (b), and image of DAPI stained biosensor, showing the same cells (c).

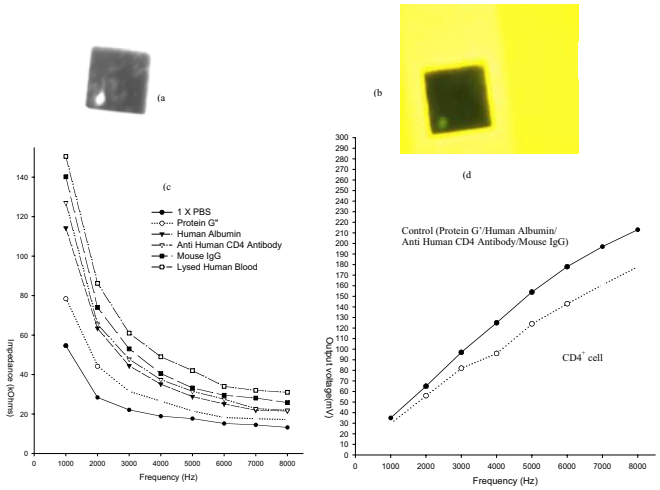

Fig. 5: Images and impedance/output voltage characteristics of a captured $CD4^+$ cell from 10μL of lysed whole blood. (a) UV image stained with DAPI, (b) fluorescence image of FITC anti-human CD4 antibody, (c) impedance of each successive layers, and (d) capacitance of modified electrode and the captured cell.

5 CONCLUSION

We are developing an on-chip BISD that makes use of a nanoscale cellular adhesion mechanism in an integrated electronic micro-system to capture $CD4^+$ cells from blood, and have demonstrated that all cells captured using CD4 antibodies were $CD4^+$. Future work will focus on quantitating the number of cells based on the electrical signal, as well as the possibility of quantitating specific protein levels in fluid samples, including serum proteins or toxic molecules such as botulinum toxin.

6 ACKNOWLEDGEMENT

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