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Ultrasensitive Hepatitis B Virus Detection in Blood Using Nano-Porous Silicon Oxide: Towards POC Diagnostics

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***Abstract*—**Early diagnosis of infection like Hep-B virus in blood is important for low cost medical treatment. For this purpose, it is desirable to develop a point of care device which should be able to detect trace quantities of the target molecule in blood. In this paper, we report a nanoporous silicon oxide sensor which is capable of detecting down to 1fM concentration of Hep-B surface antigen in blood without the requirement of any centrifuge or pre-concentration. This has been made possible by the presence of resonant peak in the sensitivity characteristics. This peak is observed to be dependent only on the concentration of the specific antigen and not on the interfering species in blood serum. The occurrence of opposite impedance change within the pores and at the bottom of the pore is responsible for this effect. An electronic interface has also been designed to provide a display of the virus concentration.

***Keywords*—**Impedance spectroscopy, Ultrasensitive detection in blood, Peak frequency, Electronic interface.

I. Introduction

BIOSENSORS can play an important role in areas such as diagnostic of diseases, drug detection and food quality control. Virus infections are a major cause of mortality and rapid identification of the virus has important clinical, economical and epidemiological implications. So, there is a growing need for virus sensors with improved sensitivity and dynamic range, for applications including disease diagnosis, pharmaceutical research, agriculture, and homeland security. The traditional methods for virus diagnostic are Enzyme Linked Immunosorbent Assay (ELISA) [1] and Reverse Transcriptase Polymerase Chain Reaction (RT–PCR) which are time consuming and expensive [2]. In terms of the transduction techniques used, the three main classes of biosensors are optical, electrochemical and piezoelectric. Out of the three, electrochemical methods appear to be optimum in terms of sensitivity, rapidity and portability. They can be amperometric or impedimetric, depending on whether they monitor a current as a function of potential or the resulting sensor impedance as a function of frequency. The advantage of impedimetric methods is that, unlike amperometry, they do not need enzymatic labels for detection.

Various nanostructures like nanowires, nanotubes and nanopores have been extensively explored for label free conductance type biosensors and also for detection of a single molecule in synthesized solutions. However, their major limitation is that the detection limit of biomolecules in physiological fluids like blood is only in the range of few pM [3]-[7]. There have been several attempts to push down the detection limit by performing the noise analysis of the conductance fluctuation [8], [9]. A recent paper on Plasmonic ELISA based ultrasensitive detection of disease biomarkers with the naked eye in blood down to attomolar concentration has been reported but it requires expensive plasmonic gold nanoparticles as labels [10].

In this paper we report an immunosensor on electrochemically fabricated silicon oxide nanoporous structure and show experimentally that it is capable of ultrasensitive label free detection of HBsAg in blood through antibody-antigen interactions not only by change in impedance values but also by a different mechanism of shift in peak frequency corresponding to maximum sensitivity.. This may be possible due to the presence of additional junction impedance in the conducting substrate below the nanoporous layer through which significant current flows at ac. Further this peak is only dependent on the specific antigen concentration and is not perturbed by the interfering species of blood. This enables selective detection of Hep-B virus in blood samples down to 1 fM concentration. Similar effect has been reported for food toxin detection [11] but there is no report with blood samples.

For a point of care device, a handheld electronic interface has also been designed with a sensor drive circuit and a RMS value generator circuit. It generates the different frequencies automatically, computes the sensitivity at different frequency and displays the virus concentration in a LCD after matching the peak frequency with look up table. The proposed system demonstrates a point of care device based on impedance spectroscopy for ultrasensitive Hep-B detection.

II. Materials and Methods

A. Fabrication of Nanocrystalline Porous Silicon

For fabrication of nanoporous silicon, p-type <100> silicon wafers of resistivity 10-20 Ωcm were cleaned by standard procedure and then etched anodically in a double pond electrochemical bath with an electrolytic mixture of hydrofluoric acid (48 wt%) and dimethyl sulfoxide in the ratio 1:9 by volume. Etching an area of 1.6 cm^2 was carried out under a constant current source of 2.35 mA for 30 minutes to

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obtain pores of thickness of about 100 nm and diameter of about 250 nm.

B. Thermal Oxidation and Metallization

Dry thermal oxidation has been done in an oxidation furnace at 1000 ºC for 3 hours and 90 minutes to obtain oxide thickness of 0.05 µm.

For metallization, metal electrodes of high temperature silver paste have been fabricated using screen printing technique and then cured at 750ºC for 1 minute. After that, gold metal is evaporated on silver.

C. Surface Functionalization and Antibody Immobilization

For surface functionalization [12], the oxidized samples were treated with sulphochromic acid for 10 minutes followed by treatment with hydroxylated silane (MTS) on dancing shaker for 1 hour. Next, samples were incubated at 95ºC and then treated with cross-linker 2 mM GMBS. Finally anti-HBsAg antibody was immobilized.

D. Blood Sample Preparation

The target protein used is HBsAg (1 mg/ml) which is purchased from US Biological, USA. For calibrated measurements, serial dilutions of this HBsAg solution were made with PBS to yield HBsAg solution of concentrations are 1fg/ml and 1pg/ml. The blood sample is kept for some time to separate the serum. Then different concentration of Hep-B surface antigen has been spiked in serum sample in the range of 1fg/ml to 1pg/ml.

E. Signal Processing

The basic requirements of the signal processing unit are:

- Automatic generation of different frequencies of simulation waveform in the range of 125Hz to 52 KHz.
- User selectable switches to indicate the control and test reading.
- LCD interface for display of virus concentration.

In the signal generation part a signal generator chip ICL8038 by Intersil Corporation has been introduced. It has a wide frequency range from 0.001Hz to 300 KHz and operates with a ±5V power source. The frequency values between 125 Hz - 52 KHz are generated by tuning the capacitance values with five MOS switches of Texas Instruments- TPS2023. A voltage divider circuit has been introduced at the output of the signal generator chip to control the amplitude of sinusoidal wave within 50mV.

The signal generator is followed by a sensor drive circuit. The sensor drive circuit has been designed with an operational amplifier in non-inverting mode. The operational amplifier used is low noise, low offset and low power OP07. The bandwidth, noise and offset voltage are 600 kHz, 600nV and around 75µV respectively which are sufficient for our scheme. The passive components used are low temperature coefficient precision metal film resistors and polyester capacitors to ensure stability of the waveforms. The output of the sensor drive circuit is sinusoidal in nature and hence before directly interfacing with the built-in ADC of the microcontroller, the output is first interfaced with a true RMS generating chip AD737. But the output voltage of AD737 is negative so an inverting amplifier is used.

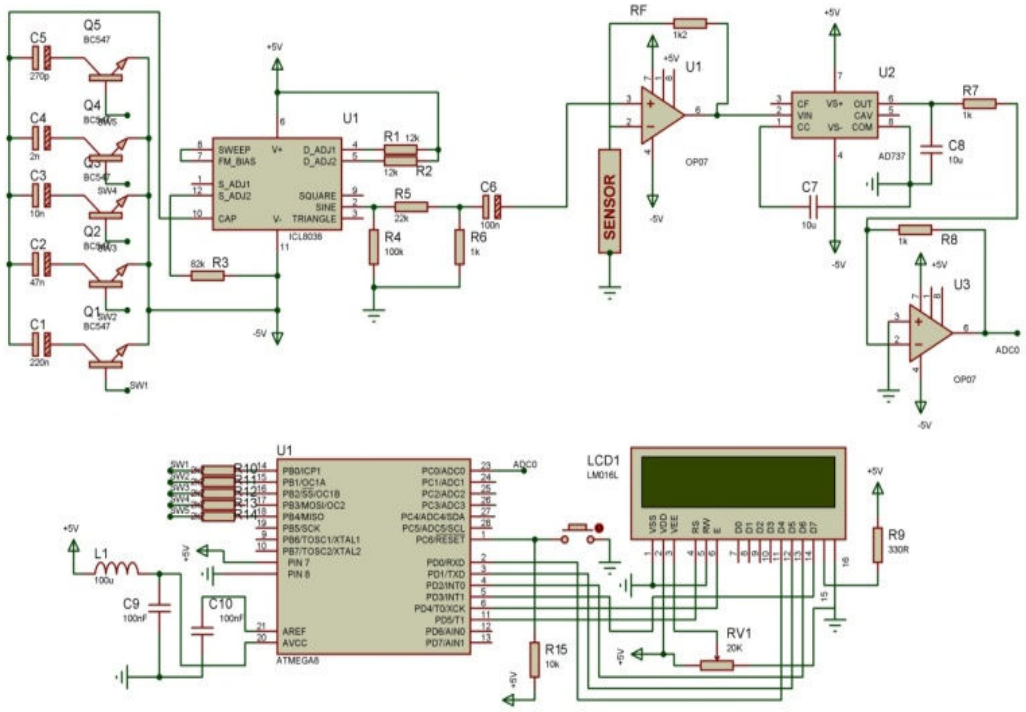

Fig. 1 Schematic of signal processing unit

The microcontroller used in this system is ATMEGA8. It is a low-power ATMEGA 8-bit AVR RISC-based microcontroller which combines 8KB of programmable flash memory, 1KB of SRAM, 512K EEPROM, and an 8 channel 10-bit A/D converter. The device supports throughput of 16 MIPS at 16 MHz and operates between 2.7-5.5 volts. The MOS switches are controlled from the microcontroller ports for tuning the capacitance values. A 16X2 alphanumeric LCD by WINSTAR has been attached with the controller ports to display the output result. The total schematic of the electronic interface is shown in Fig. 1. In the software part of the system microcontroller is programmed with an intelligent algorithm. The program is written in simple BASIC language with BASCOM AVR compiler.

III. Result and Discussion

A. Characterization

Biosensor surface morphology was characterized by scanning electron microscopy (SEM). Fig. 2 (a) shows a SEM image of oxidized nanocrystalline porous silicon. The antibody immobilization can be observed by a change in surface topology to a speckled, grainy image shown in Fig. 2 (b). Each of the speckles represents clusters of protein on the surface of the silanized nanoporous silicon. The surface topography shows good definition of antibody particle binding with sensor surface.

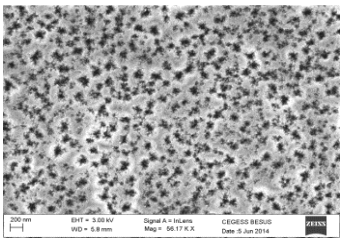

Fig. 2 (a) SEM image of oxidized nanoporous silicon

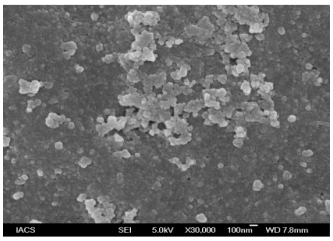

Fig. 2 (b) SEM image of antibody coated nanoporous silicon oxide

B. Impedance Measurements

After the immobilization of monoclonal antibodies for HBsAg, impedance spectroscopy measurements have been carried out with the help of LCR meter (GW INSTEK LCR 821) with a 50mV amplitude signal varying from 62.5 Hz to 100 kHz. Before measurement with every HBsAg concentration spiked in serum, a control measurement with PBS is done to establish the reference impedance. The serum containing HBsAg is then directly pipetted onto the sensor without the requirement of any pre-concentration or centrifugation. To ensure repeatability of measurements, a well like structure has been fabricated on polydimethylsiloxane (PDMS) sheets and bonded with the surface of the sensor with a plasma treatment. The solution required to fill up the well is around 30µl. The picture of the sensor is shown in Fig. 3. The variation in impedance with frequency before and after capture of HBsAg molecules with concentration of 1 fg/ml is shown in Fig. 4.

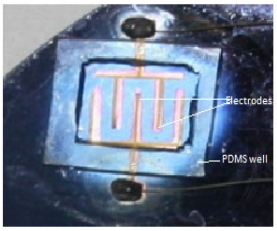

Fig. 3 Final view of the sensor

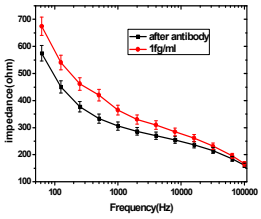

Fig. 4 Variation in impedance with frequency before and after 1fg/ml HBsAg molecules

Fig. 5 shows the percentage change in impedance commonly termed as sensitivity with frequency for different HBsAg concentration and a serum solution without HBsAg. It is observed that sensitivity is more with increasing concentration which is very obvious. But the interesting feature is that the maximum change occurs at different frequencies for different HBsAg concentration. Usually in most of the reported impedance biosensors the sensitivity gradually decreases, increases, or remains almost constant with frequency. In this case, we observe that the shift of the peak frequency is quite significant with HBsAg concentration. The percentage change in impedance is around 25% for 1 fg/ml and around 61% for 1 ng/ml which is only two and half times but the peak frequency shifts from 500Hz to 16 kHz for the same change in concentration and hence is significantly less susceptible to noise.

C. Measurement with Electronic Interface

In the signal generator different frequency values are generated by changing the capacitances. Some of the frequency values and the corresponding capacitance values are given in the Table I. Then the output sinusoidal wave has been used in the sensor drive circuit. When the signal passes through the sensor drive circuit the output signal will be phase shifted. The input and output waveform of sensor drive circuit has been shown in Fig. 6. The comparison of the peak

frequency obtained from the LCR meter and electronic interface is shown in Table II.

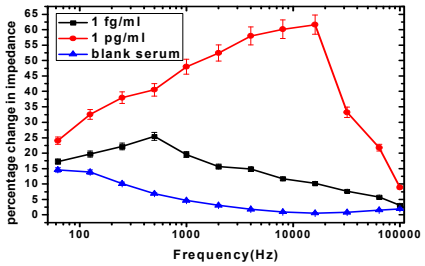

Fig. 5 Sensitivity for blank serum and different HBsAg concentration in serum

TABLE I
CAPACITANCE VALUES AND CORRESPONDING FREQUENCY VALUES
$R_1=R_2=R=12K\Omega$

Output amplitude (p-p)mV	Capacitor value	Output frequency	
		Calculated frequency	Resultant frequency
95.65	C_1=220nF	125Hz	127Hz
95.65	C_2=56nF	491Hz	530Hz
95.65	C_3=2nF	13.75Khz	10.50Khz
95.65	C_4=1.5nF	16.17Khz	16Khz
95.65	C_5=470nF	55.89Khz	52Khz

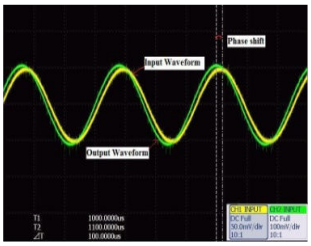

Fig. 6 (a) input and output waveform at 530 Hz

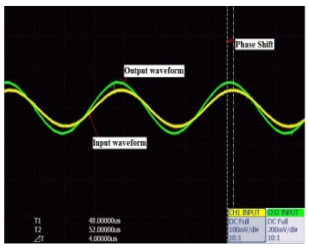

Fig. 6 (b) input and output waveform at 16.5 Khz

TABLE II
PEAK FREQUENCIES RECORDED BY LCR METER AND ELECTRONIC INTERFACE

HBsAg concentration	Peak frequency From LCR	Peak frequency from electronic interface
1fg/ml	500Hz	530Hz
10fg/ml	1Khz	1.25Khz
100fg/ml	8Khz	9.5Khz
1pg/ml	16Khz	16.5Khz

It is observed that the output of the electronic interface matches closely the LCR meter readings. Thus we have been able to realize a complete portable system to quantify Hep-B virus in blood in trace quantities without the requirement of any centrifuge or other pre-concentration techniques. The complete picture of the packaged set up is shown in Fig. 7. The sensor device along with the electronic interface has the potential for deployment in point of care diagnostics.

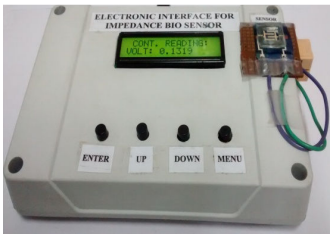

Fig. 7 Pictorial view of electronic interface

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