CHAPTER III

# EBOLAVIRUS IMMUNOSENSOR USING A QCM TRANSDUCER

## Introduction

Immunosensors to highly pathogenic diseases are receiving increased attention as the threats of bioterrorism and pandemic viruses continue to rise. Specificity of detection can be difficult as many of the threats are similarly composed of amino acids and nucleotides.[1] A variety of biological detection methodologies can be found in the literature with optical techniques (namely surface plasmon resonance)[2-5], enzyme-linked assays[6-8], and polymerase chain reaction (PCR)[9,10] as the most common. The aforementioned sensors find applications in the detection of foodborne pathogens, RNA loads, whole virus, and bacteria. However, these techniques also suffer from increased instrument costs and lengthy detection or preparation times. An emerging family of biosensors that is rapidly becoming established is the piezoelectric transducers.

Piezoelectric transducers operate by measuring the dampening of an oscillating surface from the addition of a mass load (a detailed description is in Chapter 2). Piezoeletric sensors are highly competitive against the current instruments. QCM and SPR are often compared because they both work as mass sensors. SPR measures the change of the incident angle from a laser source as the antigen binds and leaves the surface. A profile is obtained that can be transformed into kinetic information for the forward and reverse rate constants. This measurement is akin to QCM's frequency change under a mass load. Both methods follow a principle of wave propagation, with

slightly different penetration depths into the solution. SPR tends to have a slightly higher penetration depth, but since protein immunosensor assemblies often do not exceed 150 nm, either method is highly valid.[11]

The techniques have been directly compared in the literature from both Spangler *et al.*[12] and Köβlinger *et al.*[11] Because the two techniques are so similar they must be carefully analyzed for their detection method (frequency vs. angle change). Both SPR and QCM work with flow systems and have easily functionalized gold surfaces. This makes both easily amenable to immunosensor assemblies. Spangler uses the biosensors for the detection *Escherichia coli* as a comparison of their viability to food-borne pathogen detection. SPR and QCM both detect the E. coli using a $GM_1$ modified surface. However, they both have considerations in their usages.

The cost of an SPR system is often a severe drawback compared to QCM. While both techniques use gold surfaces, SPR needs a more homogeneous layer for the laser to detect properly.[12] QCM, because of its aggregate measurement, can work with a heterogeneous sensing layer and not lose sensitivity. Both techniques have some degree of response to ambient temperature. This is more so to SPR because of the optics in the detection scheme. However, SPR does typically have a lower limit of detection because high frequency QCM crystals (15 MHz and higher) are less rugged and therefore, less employed. Because ebolavirus requires portable, cost effective measures, it is hoped that it can join the family of available ebola biosensors.

In terms of methodologies Ebolavirus assays, only a handful of techniques are developed for its detection. Immunofluorescence (IF), ELISA, RT-PCR, and virus isolation are the most widely used methodologies for Ebolavirus.[13,14] Figure 28 shows

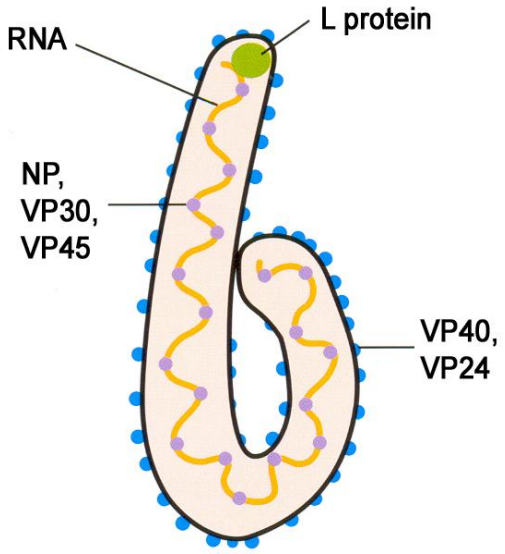


Figure 28. Cartoon representation of ebolavirus and its 7 proteins.[15]

the various proteins of ebolavirus and their relative location.[15] ELISA assays tested a series of monoclonal antibodies specific to the NP of ebolavirus, using concentrations of approximately $10^6$ at the lowest.[16] Of these, only RT-PCR detects in less than 30 minutes and is a direct measurement of Ebolavirus RNA, with a minimum concentration of $10^4$ focus forming units (FFU).[17,18] The ELISA method works based off of optical detection of the viral proteins from specific antibodies.[16] The IF method uses cells producing the nucleoprotein to recognize Ebola IgG antibodies.[14] The actual detection is rapid, but relies on the production of antibodies from the host and the technique has only been used on samples from past outbreaks. The viral isolation techniques are the most sensitive

methods, however, the requirements of BSL-4 facilities in undeveloped countries makes it difficult.[13]

When the virus is proliferating in the body, there are multiple targets in the blood for an immunosensor to measure. The first target is the whole virus, which contains 7 proteins for function.[19] From there antibodies are developed against the NP, GP, and VP40. Ideally, the superficial proteins allow binding to an antibody based sensor. This limits the choice proteins to the GP and VP40, which both have developed antibodies. Antibodies to GP and VP40 are also useful as neutralizing antibodies because the two proteins are responsible for steps in viral entry, cell binding and viral insertion. The QCM is used in this chapter to look at the detection of the whole pathogen against a NIH derived mAb, EB-MAB1, and two GP specific antibodies, 15H10 and 6D11, previously used in chapter 2.

## Experimental

### *Materials*

Protein A and G were purchased from Pierce Biotechnologies. A three-channel Research Quartz Crystal Microbalance, 5MHz Ti/Au, and 9MHz Ti/Au were purchased from Maxtek, Inc. A four channel pump system and microbore santoprene tubing (1/16 in. I.D.) were purchased from Masterflex®. Inactivated, whole Zaire strain Ebolavirus (Death Certificate DTC # 0128) and anti-Ebolavirus monoclonal antibody (EB-MAB1) were purchased from the Department of Defense Critical Reagents Program through BEI Resources. Anti-Mouse IgG antibody was purchased from Sigma-Aldrich. $NaH_2PO_4$,

$NaH_2PO_4$, solid NaCl, 30% $H_2O_2$, concentrated sulfuric acid, acetic acid, and sodium acetate were all purchased from Fisher as ACS grade unless specified. 70% isopropyl alcohol (IPA) was made from ACS grade IPA from Sigma-Aldrich. A 5000 ppm bleach/water mixture was made from Clorox® bleach from the Clorox Company. Water was supplied from a ~18 MΩ NanoPure Diamond (Barnstead) with UV source for sterilization.

*Crystal Cleaning*

Crystals were cleaned using standard procedures.[20] Briefly, a mixture of concentrated $H_2SO_4$ :30% $H_2O_2$ was mixed in a 3:1 ratio. While the solution was still hot, it was applied dropwise onto the crystal surface, left for one minute, washed with DI water and ethanol, and blown dry with nitrogen. The procedure was repeated four times, or more as needed. The crystals were then cleaned using UV light from a UVO-cleaner for 10 minutes.

*Stock sample preparation*

Stocks of protein A and G were made by dissolving the lyophilized solid (5 mg) in 1 mL PBS. Then, solutions of 500 μL protein A or G were made at a concentration of 4.5 μM in a 50/50 mix of PBS/acetate buffer. Antibodies (15H10, 6D11, and EB-MAB1) were diluted from stocks to make 500 μL aliquots at a concentration of 133 nM (20 μg/mL, MW of 150 kDa) in PBS. BSA solid was dissolved in PBS to a final concentration of 1 mg/mL. Ebolavirus was diluted from a 1.7 x $10^7$ PFU stock to 2.55, 1.7, and 0.85 x$10^5$ PFU.

*Immunosensor Fabrication*

QCM crystals were secured in the Maxtek flow cell with the working electrode (large size) facing the air, and the semi-circle electrodes making contact on the POGO® pins. The flow cell holder was then tightened and the buffer was pumped at 30 μL/min. The buffer was flowed for an hour to reduce drift and adjust the capacitance to null. After an hour, the data logger was started. Buffer was run for 10 minutes to establish a baseline, and then the inlet was changed to the protein A solution. Next, the inlet was moved back to buffer in order to wash away excess protein A and create a new baseline. BSA was flowed for 10 minutes after buffer to block any bare gold surface and prevent non-specific adsorption. Again, buffer was pumped after BSA, followed by the antibody (15H10, 6D11, or EB-MAB1). The antibody was followed by buffer and then by the antigen itself. To obtain a final mass measurement, the run was finished by flowing buffer.

*Sterilization*

All containers, tubing, and benchtop surfaces were sterilized following the experiment. The 5000 ppm bleach/water solution was flowed through the tubing and flow cell for 20 minutes, and the bench was exposed to the solution for the same amount of time. Next, the benchtop and tubing were exposed to a 70% IPA solution for 15 minutes for additional sterilization. As a final step, the tubing and flow cell were rinsed with water to remove any residual bleach and IPA.

## Results and Discussion

The whole ebolavirus was paneled against 3 different antibodies. One antibody, EB-MAB1 is known to recognize the whole virus and the two others (15H10 and 6D11) target the different regions on the glycoprotein, part of Ebola's surface. The goal for comparing the three antibodies is to determine the most efficient sensor that can detect the virus in a minimum of time. In chapter 2 results showed that 15H10 and 6D11 do bind the glycoprotein using the QCM. However, their usage against the whole virus has never been tested.

The first antibody, EB-MAB1 is known to target Ebolavirus and, in this set of experiments, is the keystone of the best immunosensor. The initial layer of protein A bound 240 ng to the QCM, indicating approximately two monolayers. The BSA shows a very rapid on and off, translating into no actual binding event. With two monolayers of protein A already on the gold, the lack of much, if any, BSA binding is expected. Upon the addition of the monoclonal EB-MAB1, 192 ng of binding to the sensor is measured. Figure 29 shows the final step, where 30 ng of whole Ebolavirus is successfully detected from an 8.5 $x10^4$ PFU sample.

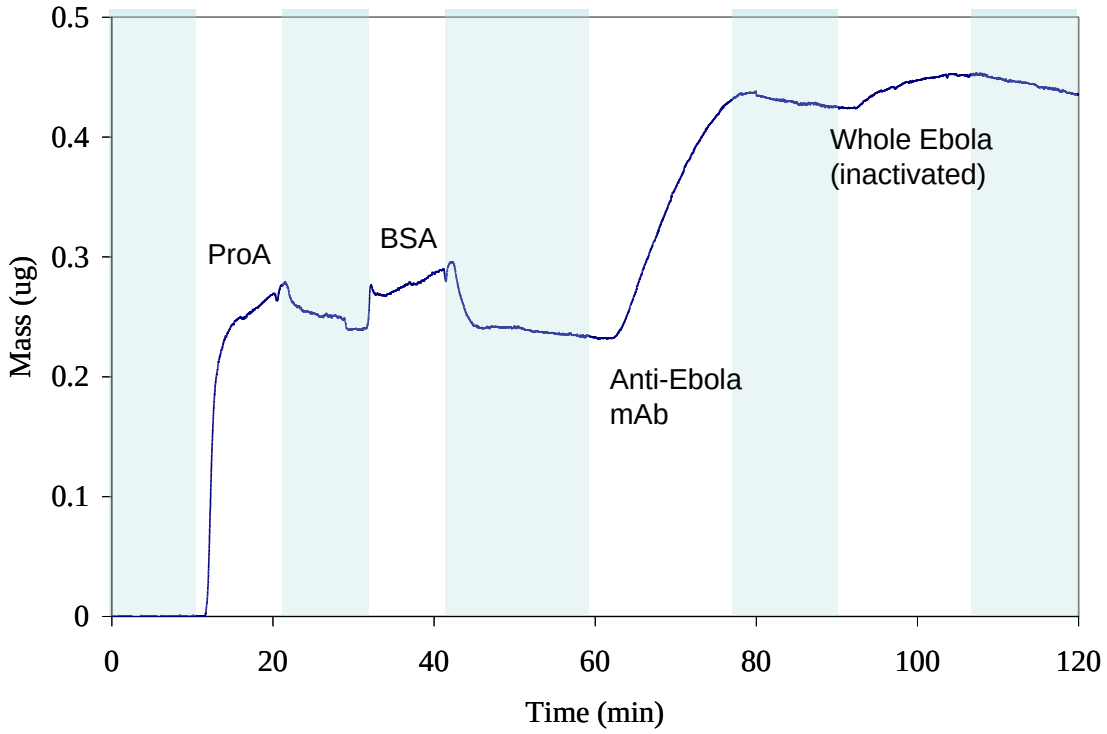


Figure 29. The QCM gravimetry shows the binding of whole ebolavirus to the EB-MAB1 antibody immunosensor. The blue regions are buffer flow only. The Ebolavirus sample was 8.5 x$10^4$ PFU.

As a control to assure specificity, a second sensor is fabricated using a standard IgG anti-mouse antibody. This sensor tests whether ebolavirus is non-specifically binding to the sensor or if it is selective for the EB-MAB1. Figure 30 shows the construction of the biosensor with an anti-mouse IgG in place of the ebolavirus specific antibody. The measurement shows 160 ng of Protein A binding to the surface followed by 175 ng of IgG anti-mouse antibody. The lack of ebolavirus binding to the anti-mouse antibody suggests the sensor is specific to EB-MAB1 and not the protein A, BSA, or paratope region of any IgG.

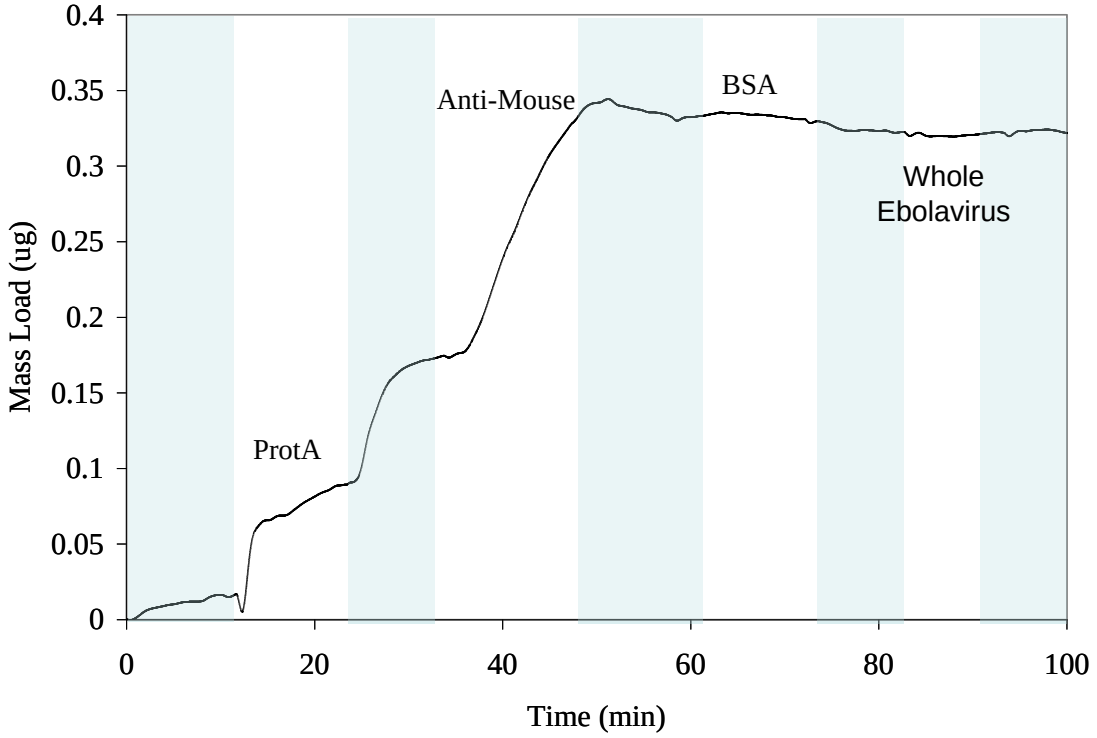


Figure 30. A control experiment showing an anti-mouse IgG based biosensor to prove ebolavirus specificity. The whole virus does not bind the sensor without the EB-MAB1 antibody.

A third control experiment was setup to test for non-specific binding to any antigen in the system. For this study, the flu H3N2 was used as a control antigen against the already constructed sensor with the EB-MAB1 antibody present. Figure 27 shows the creation of the sensor with 84 ng of Protein A followed by 101 ng of BSA binding. The ebola antibody then bound 58 ng to the protein A layer. In the last step, H3N2 was flowed over the surface and showed no apparent binding during its flow. This indicates that EB-MAB1 is selective to ebolavirus and not another antigen. This data in conjunction with data shown in Figure 31 indicates that ebolavirus is selective to the sensor, and the sensor itself is selective to ebolavirus.

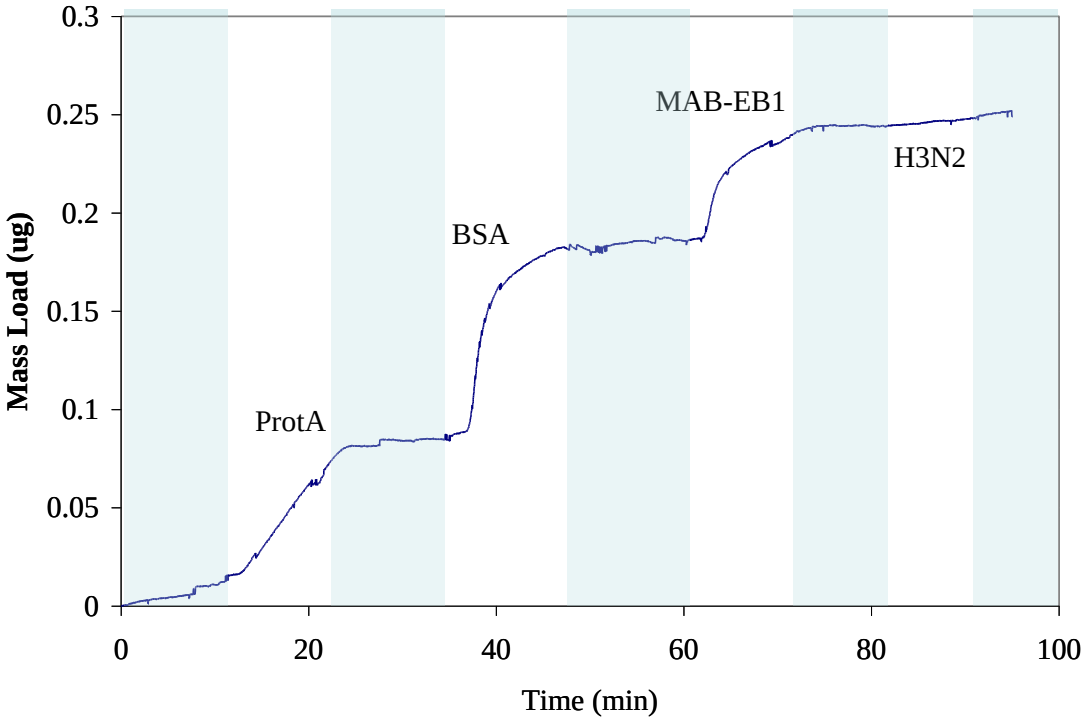


Figure 31. A second control experiment showing that the sensor does not bind another selected antigen, H3N2 flu. The EB-MAB1 shows that it has no specificity toward this antigen by the lack of H3N2 binding to the surface.

Two other antibodies, 15H10 and 6D11, are specifically targeted to the GP on ebola's surface. This superficial protein should be a feasible target for the recognition of the whole virus. However, an immunosensor fabricated with these antibodies does not work for the whole virus in the same fashion as the GP alone. This is unexpected because of the GPs location and the theoretical chance for multivalent attachments. At concentrations as high as 1.7 $x10^7$ PFU/mL, minimal binding above drift is discernable.

## Conclusions

The QCM has been successfully employed to detect whole ebolavirus using an available monoclonal antibody, at a minimum concentration of 8.5 $x10^4$ PFU/mL. This is

a lower detection limit then the previously established ELISA concentrations at $10^6$ and is comparable to RT-LAMP's $10^4$ FFU. The GP targeted antibodies, 15H10 and 6D11, were both paneled and showed less success versus the intact virus.

The next step for this biosensor will be ebolavirus spiked blood samples or sera. A field portable device must be able to handle whole blood or its sera from potentially infected patients, with a minimum of preparation. By using the killed form of ebolavirus, the QCM can be tested in a safe manner as a potential assay for a real world situation.

## References


(1) Ligler, F. S.; Phil, D.; Sc, D. In *Optical Chemical Sensors*; Baldini, F., Chester, A. N., Homola, J., Martellucci, S., Eds.; Springer Netherlands: Netherlands, 2006; Vol. 224, p 437-455.

(2) Salamon, Z.; Macleod, H. A.; Tollin, G. *Biochem. Biophys. Acta* **1997**, *1331*, 131-152.

(3) Salamon, Z.; Macleod, H. A.; Tollin, G. *Biochem. Biophys. Acta* **1997**, *1331*, 117-129.

(4) Schmid, A. H.; Stanca, S. E.; Thakur, M. S.; Thampi, K. R.; Suri, C. R. *Sens. Actuators, B* **2006**, *113*, 297-303.

(5) Naimushin, A. N.; Spinella, C. B.; Soelberg, S. D.; Mann, T.; Stevens, R. C.; Chinowsky, T.; Kauffman, P.; Yee, S.; Furlong, C. E. *Sens. Actuators. B* **2005**, *104*.

(6) Engvall, E.; Perlmann, P. *Immunochemistry* **1971**, *8*, 871-874.

(7) Ogasawara, Y.; Amexis, G.; Yamaguchi, H.; Kajigaya, S.; Leppla, S. H.; Young, N. S. *in vivo* **2006**, *20*.

(8) Chargeledgue, D.; Obeid, O. E.; Hsu, S.-C.; Shaw, M. D.; Denbury, A. N.; Taylor, G.; Steward, M. W. *J. Virol.* **1998**, *72*, 2040-2046.

(9) Huletsky, A.; Rossbach, V.; Gagnon, F.; Truchon, K.; Picard, F. J.; Bergeron, M. G. ₸Development of a Real-Time PCR Assay for Rapid Detection of Methicillin-Resistant *Staphylococcus aureus* Directly from Clinical Specimens Containing a Mixture of Staphylococci,₹Infectio Diagnostic Inc.

(10) Piqueur, M. A.; Verstrepen, W. A.; Bruynseels, P.; Mertens, A. H. *Virology J* **2009**, *6*.

(11) Kößlinger, C.; Uttenthaler, E.; Drost, S.; Aberl, F.; Wolf, H.; Brink, G.; Stanglmaier, A.; Sackmann, E. *Sens. Actuators, B* **1995**, *24-25*, 107-112.

(12) Spangler, B. D.; Wilkinson, E. A.; Murphy, J. T.; Tyler, B. J. *Anal. Chim. Acta* **2001**, *444*, 149-161.

(13) Saijo, M.; Niikura, M.; Ikegami, T.; Kurane, I.; Kurata, T.; Morikawa, S. *Clin. Vaccine Immunol.* **2006**, *13*, 444-451.
(14) Saijo, M.; Niikura, M.; Morikawa, S.; Kurane, I. *J. Clin. Micro.* **2001**, *39*, 776-778.
(15) National Institute of Health: 2005.
(16) Lucht, A.; Grunow, R.; Moller, P.; Feldmann, H.; Becker, S. *J. Vir. Met.* **2003**, *111*, 21-28.
(17) Leeroy, E. M.; Baize, S.; Lu, C. Y.; McCormick, J. B.; Georges, A. J.; Georges-Courbot, M.-C.; Lansoud-Soukate, J.; Hoch-Fisher, S. P. *J. Med. Vir.* **2000**, *60*, 463-467.
(18) Kurosaki, Y.; Takada, A.; Ebihara, H.; Grolla, A.; Kamo, N.; Feldmann, H.; Kawaoka, Y.; Yasuda, J. *J. Vir. Met.* **2006**, *141*, 78-83.
(19) Sanchez, A.; Rollin, P. *Vir. Res.* **2005**, *113*, 16-25.
(20) Maxtek, I., Operation and Service Manual - Research Quartz Crystal Microbalance.