Development of an Electrochemical Immunosensing Platform for Detection of Avian Influenza A Viruses Using a Hybrid Nanocomposite Scaffold

by

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ABSTRACT

DEVELOPMENT OF AN ELECTROCHEMICAL IMMUNOSENSING PLATFORM FOR DETECTION OF AVIAN INFLUENZA A VIRUSES USING A HYBRID NANOCOMPOSITE SCAFFOLD

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University of Guelph, 2018

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This thesis is an investigation of a sandwich-based electrochemical immunosensor for detection of avian influenza virus (AIV) strains H5N1, H4N6, and H9N2. This sensor was developed using gold-graphene nanocomposites, immobilized viral antibodies, and cadmium telluride (CdTe) quantum dot electrochemical tagging. The nanocomposites were formed by the simultaneous reduction of a gold salt and graphene using hydroquinone as the reducing agent, thus producing non-spherical gold nanoparticles on graphene sheets. Viral antibodies were immobilized on nanocomposites and CdTe quantum dots through N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide and N-hydroxysuccinimide chemistry. Cyclic voltammetry studies were used to validate the detection of H5 surface protein and H4N6 inactivated virus. The immunosensor detected H5 protein in phosphate buffer solution (pH 7.4) with a limit of detection (LOD) of 10 fg/mL and a linear detection range was established for 10 ng/mL to 10 pg/mL. The biosensor detected H4N6 and H9N2 viruses in three parts diluted whole chicken blood with a LOD of 1.28x10^-7 hemagglutinating units (HAU). Commercial ELISA testing for H4N6 showed a limit of detection of 0.128 HAU. The sensor showed 10^6-fold increased detection of H4N6 virus in blood in comparison to its commercial ELISA kit counterpart. The developed immunosensor may effectively change the way avian influenza is detected, monitored, and controlled; transforming time-consuming reactive methods, into rapid predictive technology.
ACKNOWLEDGEMENTS

I would like to thank Dr. Suresh Neethirajan, Dr. Manjusri Misra, Dr. Gordon Hayward, and Dr. Karen Gordon for their unwavering guidance and moral support throughout this thesis project. I would also like to thank my collaborator, Dr. Éva Nagy, from the department of pathobiology, OVC, for her generous donation of influenza virus cultures. I would like to send an extreme thank-you the postdoctoral fellows, more specifically Dr. Rohit Chand, for sharing his technical experience, knowledge, and friendship. Without your help and support, I would have gotten lost in the thick of it. This project would have been impossible without the financial backing of the Natural Sciences and Engineering Research Council of Canada and the Ontario Ministry of Agriculture, Food and Rural Affairs. Thanks to Ryan Berthelot, Abdulmonem Murayyan, Patrick Egan, and the other bionano group members for their friendship both inside and outside of lab. Lastly, I would like to thank my family, especially my uncle Ben, for always believing in me and encouraging me to pursue graduate studies.
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**LIST OF ABBREVIATIONS**

AIV: Avian Influenza Virus

HA: Hemagglutinin

NA: Neuraminidase

RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

ELISA: Enzyme Linked Immunosorbent Assay

NAHLN: National Animal Health Laboratory Network

EDC: N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide
NHS: N-hydroxysuccinimide

CdTe: Cadmium Telluride

LPAI: Low Pathogenic Avian Influenza

HPAI: High Pathogenic Avian Influenza

Arg: Arginine

RBS: Receptor Binding Site

3’ – SLN: 3’-Sialyl-N-acetyllactosamine

Gly: Glysine

Asn: Asparagine

NS1: Non-Structural Influenza Protein 1

NVSL: National Veterinary Services Laboratory

2D: 2 – Dimensional

LBL: Layer-By-Layer

LOD: Limit of Detection

Au: Gold

SERS: SurfaceEnhanced Raman Scattering

UV – VIS: Ultraviolet – Visible Spectrum

TEM: Transmission Electron Microscopy

SEM: Scanning Electron Microscopy

PBS: Phosphate Buffer Solution

EDX: Energy Dispersive X-ray

CV: Cyclic Voltammetry

HAU: Hemagglutinin Units

PG: Peptidoglycan

EIS: Electrochemical Impedance spectroscopy
CA: Chronoamperometry,
DPV: Differential Pulse Voltammetry
SWV: Square Wave Voltammetry
LSV: Linear Sweep Voltammetry
SPGE: Screen-Printed Gold Electrode
RGO: Reduced Graphene Oxide
Ab: Antibody
Ab\textsubscript{1}: Primary Antibody
Ab\textsubscript{2}: Secondary Antibody
Ag: Antigen
SPCE: Screen-Printed Carbon Electrode
NP: Nanoparticles
PL: L-polylysine
ALP: Alkaline Phosphatase
GCE: Glassy Carbon Electrode
BSA: Bovine Serum Albumin
pZnO: Porous Zinc Oxide
scFV: Single Chain Variable Fragments
NR: Nanorods
G: Graphene
MAb: Monoclonal Antibody
PAb: Polyclonal Antibody
PFU: Plaque Forming Units
1. INTRODUCTION

To meet the growing demands for animal protein, global poultry production will double in the next 25 years. The global poultry industry has been deeply impacted by outbreaks of avian influenza virus (AIV) since the late 1990’s. The Canadian poultry industry has also had its share of major losses due to AIV, most notably was the 2004 outbreak in British Columbia, which resulted in culling 19 million birds. Recent AIV outbreaks in British Columbia and Ontario in 2015 also have caused economic losses to the Canadian poultry industry. Aside from the significant impact of AIV on animal health, some of these viruses have an impact on public health. AIV causes three- to five-million people to fall severely ill, resulting in 250,000 to 500,000 fatal cases annually in developing countries (World Health Organization, 2017a).

Hemagglutinin (HA) and neuraminidase (NA) surface protein combinations are used to characterize influenza viruses. There are 18 HA (H1 – H18) and 11 NA (N1 – N11) subtypes, respectively. Of these subtypes, H5 and H7 are of major concern within the scientific community; as they manifest as low pathogenic infections in waterfowl, which can become highly pathogenic when introduced to domestic poultry, and possess the capability to jump species (Canadian Food Inspection Agency, 2015; Centers for Disease Control and Prevention, 2017, 2015; Health canada, 2008; Jensen et al., 2013; Olsen et al., 2006; World Health Organization, 2017b; Zhu et al., 2014). Consequently, governments and farmers alike are under immense pressure to ensure the health of poultry and poultry consumers.

Preventing the spread of avian influenza infection is the best way to keep disease outbreaks under control. Prevention starts with effective bio-surveillance through early disease diagnosis, quarantine, and testing of adjacent facilities. To date there are no pen-side or coop-side tests available for rapid diagnosis. Conventional methods of avian influenza detection include one-step reverse transcription polymerase chain reaction (RT-PCR), hemagglutinin inhibition tests,
enzyme-linked immunosorbent assay (ELISA), embryonated egg virus culturing, and chicken pathogenicity tests (Jensen et al., 2013; United States Department of Agriculture, 2015; World Organization for Animal Health, 2016).

In the United States, tests carried out by the National Animal Health Laboratory Network (NAHLN) (United States Department of Agriculture, 2015) are as follows: matrix screening for AI viruses, H5 subtype screening, H7 subtype screening, and N1 subtype screening; all of which are RT-PCR based tests (American Plant Health Inspection Service, 2008; United States Department of Agriculture, 2015). These tests are followed by three types of confirmatory tests: virus isolation tests (in embryonated eggs), genetic sequencing tests, and chicken pathogenicity tests. The test samples are usually obtained from fecal or tracheal swabs from live specimens. Typically these tests take 2-3 weeks to run, require expensive equipment, and require highly trained technicians (American Plant Health Inspection Service, 2008).

To overcome the obstacles of poor diagnostic turnaround and the need for specialized facilities, research has been moving towards virus detection on the nanoscale using point-of-care biosensors (Neethirajan et al., 2017). The major benefits of nanoscale virus detection include: a significant reduction in reaction time due to increased surface area for the reaction to take place; and a significant reduction in the costs of testing (e.g. reagent costs, personnel costs, facility costs, and transportation costs). Due to the current technology limitations, this work will focus on bridging the gap through the design of a rapid point-of-care biosensor for the detection of avian influenza A viruses.

Graphene is an abundant, inexpensive two-dimensional atomic crystal with outstanding physical properties, including extreme mechanical strength, exceptionally high electronic conductivities, superior surface area, and biocompatibility. It is an excellent substrate for biomolecule anchoring and detection due to its surface area of 2630 m$^2$/g and unique sp$^2$ (sp$^2$/sp$^3$) bonded network (Hu et al., 2015; Veerapandian and Neethirajan, 2015). In addition,
by exploiting the electrochemical properties, graphene can be functionalized easily for developing novel biosensing and transduction mechanisms. Recent graphene-based biosensing platforms developed in our lab and others (Veerapandian et al., 2016a, 2016b; Weng and Neethirajan, 2016) indicate the potential for an electrochemical nanobiosensing platform for virus detection applications.

In this thesis work, an immunosensor was designed by incorporating gold-graphene nanocomposites, antibody-antigen immunochemistry, and electrochemical quantum dot tagging. The goal of this study was to develop a sensing mechanism that was more sensitive and less time consuming than commercial ELISA. The proposed immunosensor used a thin film fabricated of gold-graphene nanocomposites on a screen-printed electrode. Virus-specific antibodies were then immobilized on the nanocomposite surface using N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) carbodiimide chemistry. Cadmium telluride (CdTe) quantum dots were conjugated with virus-specific antibodies in an in-situ manner using EDC/NHS chemistry. This mechanism is immunosensing on a screen-printed electrode, in which the magnitude of the CdTe electrochemical signal is proportional to the antigen concentration. The immunosensor was first designed for H5 viral protein as a proof-of-concept. To demonstrate the practicality of the immunosensor for real virus detection, low pathogenic H4N6 spiked in whole chicken blood was studied, by using an H4N6 antibody as the bioreceptor. To demonstrate the versatility of the mechanism, low pathogenic H9N2 spiked in whole chicken blood was studied, by using an H9N2 antibody as the bioreceptor element.
2. LITERATURE REVIEW

2.1 Avian Influenza A Viruses

There is consensus that waterfowl are the natural reservoirs of all influenza viruses (Health canada, 2008; Krauss et al., 2004; Olsen et al., 2006; World Health Organization, 2017b). Furthermore, hemagglutinin (HA) and neuraminidase (NA) surface protein combinations are used to characterize influenza viruses. There are 18 HA (H1 – H18) and 11 NA (N1 – N11) subtypes, respectively. Of these subtypes, H5 and H7 are of major concern within the scientific community; as they manifest as LPAI in aquatic species, which can evolve into HPAI when introduced to domestic poultry (Canadian Food Inspection Agency, 2015; Centers for Disease Control and Prevention, 2017, 2015; Health canada, 2008; Jensen et al., 2013; Olsen et al., 2006; World Health Organization, 2017b; Zhu et al., 2014). The exact mechanism that causes this evolution not fully understood. However, it has been postulated that evolutionary mutations, only occurring in H5 and H7 proteins, motivate increased pathogenicity (Gamblin and Skehel, 2010; Heider et al., 2015; Monne et al., 2014; Mueller et al., 2010; Xu et al., 2016). One must first understand the roles of HA during infection to understand how these mutations promote an increase in pathogenicity.

2.1.1 Role of Hemagglutinin During Viral Infection

During infection, the HA and NA virion surface proteins both recognize sialic acid. The first step of infection occurs when HA receptors bind to sialic acids, which are located on carbohydrate side chains of cell membrane glycolipids and glycoproteins (Gamblin and Skehel, 2010). During virus replication, NA removes sialic acid from infected cell membranes as well as virus glycoproteins; thereby allowing newly replicated viruses to infect additional cells (Gamblin and Skehel, 2010). HA contains three identical subunits, each containing two
polypeptides that result from the cleavage of a single precursor. The cleavage site in most HA subtypes is a single Arg residue, and cleavage occurs extracellularly (Böttcher et al., 2006; Gamblin and Skehel, 2010). However, H5 and H7 subtypes, have acquired multiple residues at the cleavage site. In this case, a subtilisin-like protein digesting enzyme recognizes these residues; thereby increasing cleavage efficiency, virus infectivity, and pathogenicity (Gamblin and Skehel, 2010; Garten and Klenk, 1999; Klenk and Rott, 1988; Perdue et al., 1997; Steinhauer, 1999; Stieneke-Grober et al., 1992).

2.1.2 Mutations Found in H5 and H7 Subtypes Promoting Increased Pathogenicity

It has been postulated that enhancements in HA sialic acid receptor-binding specificity are associated with the emergence of HPAI virus strains (Heider et al., 2015). Enhancements are linked with a series of amino acid residue substitutions and nonsense mutations near the HA receptor-binding site (RBS), which contains residues 110 – 140. (Monne et al., 2014; Xu et al., 2016). Heider et al. observed that HPAI H5 and H7 viruses possess a 5- and 2-fold enhanced receptor-binding specificity to 3'-Sialyl-N-acetyllactosamine (3'-SLN) and sialic acid in comparison to LPAI viruses (Heider et al., 2015). Additionally, it was determined that this enhancement was linked directly to amino acid substitutions near the receptor-binding site of HA. The H5 and H7 of LPAI possess a Gly residue at position 136 of the RBS; whereas the H5 and H7 of HPAI possess an Asn residue at the same position (Heider et al., 2015). Furthermore, it was found that some amino acid substitutions in HPAI generate additional glycosylation sites on the HA protein (i.e. Ala134Thr generates an additional glycosylation site) (Heider et al., 2015). Heider et al. also determined that substrate-specificity profiles of NA do not correlate with the pathogenicity of the virus (Heider et al., 2015; Russell et al., 2006). The findings presented by Heider et al. are consistent with previously reported data (Daniels et al., 1987). Therefore, HPAI virus strains exhibit enhanced receptor-binding
specificity due to mutations on HA, while the substrate specificity profiles of NA do not correlate with pathogenicity of the virus.

Additionally, Monne et al. observed an emergence of HPAI from a LPAI ancestor based on data obtained from the H7 epidemic of 1999 to 2001 in northern Italy (Monne et al., 2014). It was observed that the evolution from LPAI to HPAI involved more than amino acid residue insertion/substitution at the H7 cleavage site. Across the viral genome, it was found that HPAI isolates of H7 subtype possess 19 amino acid substitutions, as well as a nonsense mutation (Monne et al., 2014). The nonsense mutation results in a C-terminal removal of six residues, which promotes transport of the non-structural influenza protein 1 (NS1) into the nucleoli (Monne et al., 2014). Once in the nucleoli, NS1 may favor viral transcription and translation or may alter the cell cycle for viral needs (Keiner et al., 2010). A total of 69 HPAI H7 viruses were examined in this study. Out of those, 66 were found to possess an Ala143Thr substitution on residue 143 of the H7 gene, which generates an additional glycosylation site adjacent to the RBS (residue 141) (Monne et al., 2014). Interestingly, this mutation was also present in a 2003 H7N7 HPAI isolate from the Netherlands, which caused one human fatality. This strain demonstrated increased viral replication in Madin-Darby canine kidney epithelial cells and altered viral attachment to human lung tissues (de Wit et al., 2010; Monne et al., 2014). The additional glycosylation site caused by the substitution on residue 143 was also found on five LPAI precursors in Italy, which indicates that the HPAI viruses likely evolved from these five progenitors (Monne et al., 2014). The H7 outbreaks in Italy are a prime example of how LPAI can quickly evolve into HPAI in domestic poultry stocks. Due to this trend, there is a genuine and pressing need to detect H5 and H7 LPAI subtypes early, before they evolve into HPAI in poultry stocks. Furthermore, it is important to monitor and detect HPAI viruses as well, as they will constantly be present in the world, post mutation.
2.2 Avian Influenza Infections in a North American Context

Over the past 10 years, there have been approximately 280 recorded AI outbreaks in North America, resulting in over 71,000 fatalities in domestic poultry stocks (OIE, 2017). Additionally, 16.8 million animals were destroyed to prevent propagation of the infection. North American LPAI and HPAI outbreak data can be observed in Tables 1 and 2, respectively.

**Table 1:** Recorded instances of LPAI infection in North America (OIE, 2017). N/A indicates that data was unavailable. All numbers, save number of outbreaks, are reported as number of animals.

<table>
<thead>
<tr>
<th>Month</th>
<th>Year</th>
<th>Country</th>
<th>Province/State</th>
<th>Strain</th>
<th>No. of Outbreaks</th>
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<tr>
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<td>5043</td>
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<tr>
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<td>British</td>
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<td>94</td>
<td>81</td>
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<td>California</td>
<td>H5N8</td>
<td>1</td>
<td>114000</td>
<td>N/A</td>
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<td>N/A</td>
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<td>11805</td>
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<td>South Dakota</td>
<td>H5N2</td>
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<td>Indiana</td>
<td>H5N8</td>
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<td>10</td>
<td>10</td>
<td>67</td>
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<tr>
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<td>Iowa</td>
<td>H5N2</td>
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<td>22</td>
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<td>H5N2</td>
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<td>May</td>
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<td>N/A</td>
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<tr>
<td>May</td>
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<td>Iowa</td>
<td>H5N2</td>
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<td>145236</td>
<td>N/A</td>
<td>N/A</td>
<td>145236</td>
</tr>
</tbody>
</table>

It can be observed from Table 1 that there has been an almost-even distribution of H7 and H5 LPAI occurrences in North America. However, when compared to the data presented in Table 2, it was observed that H5 was responsible for most HPAI occurrences. Generally, LPAI viruses do not cause mortality in domestic poultry stocks, which is supported by the data presented. A detailed comparison of symptoms between LPAI and HPAI infections can be observed in Table 3.
Table 3: Comparison of symptoms between LPAI and HPAI infections in domestic poultry stocks (Canadian Food Inspection Agency, 2015). The main difference is that HPAI causes sudden death, resulting in high mortality rates.

<table>
<thead>
<tr>
<th>Pathogenicity of Virus</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Symptoms</strong></td>
<td>• watery, greenish diarrhea</td>
<td>• high mortality and sudden death</td>
</tr>
<tr>
<td></td>
<td>• excessive thirst</td>
<td>• watery, greenish diarrhea</td>
</tr>
<tr>
<td></td>
<td>• swollen wattles (chin) and combs (top of head)</td>
<td>• excessive thirst</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• swollen wattles (chin) and combs (top of head)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• reduced egg production</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• coughing, sneezing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• huddling, depression, closed eyes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• decreased food intake</td>
</tr>
</tbody>
</table>

Additionally, a mortality rate of approximately 100% is present in Table 2 (entries for which both number of confirmed cases and animal death data were available). It should be noted that the state of Minnesota has the most recorded outbreaks of AI; 97 outbreaks in 2015 alone, causing economic losses exceeding 309 million US dollars (Ohio State University, 2015; OIE, 2017). The available data, in combination with the mutation mechanisms presented, clearly demonstrate the need to detect LPAI H5 and H7 subtypes early, before they evolve into HPAI in poultry stocks.

2.3 Current Avian Influenza Detection Methods

Current methods of AI detection include conventional one-step RT-PCR, QIAGEN RT-PCR test, HI tests, ELISA-based tests, virus culturing in embryonated eggs, and chicken pathogenicity tests (Jensen et al., 2013; United States Department of Agriculture, 2015; World Organization for Animal Health, 2016). In the United States, AI screening tests are conducted at one of 45 USDA approved laboratories within the National Animal Health Laboratory Network (NAHLN) (United States Department of Agriculture, 2015). These labs determine if
the virus is of H5 or H7 subtype, and then forward any positive samples to the USDA’s National Veterinary Services Laboratory (NVSL) for confirmatory testing (United States Department of Agriculture, 2015).

Rapid tests carried out by the NAHLN are as follows: matrix screening for AI viruses, H5 subtype screening, H7 subtype screening, and N1 subtype screening; all of which are RT-PCR based tests (American Plant Health Inspection Service, 2008; United States Department of Agriculture, 2015). The test samples are usually obtained from fecal or tracheal swabs from live specimens. Typically these tests take 24 hrs to run, require expensive equipment, and require highly trained technicians (American Plant Health Inspection Service, 2008). It should also be noted that these RT-PCR tests can only detect the virus HA subtype, they cannot determine pathogenicity of the virus (American Plant Health Inspection Service, 2008).

In the case that a NAHLN lab detects H5 or H7 serotypes, the samples are forwarded to the NVSL in Ames, Iowa for confirmatory testing (United States Department of Agriculture, 2015). There are three types of confirmatory tests: virus isolation tests (in embryonated eggs), genetic sequencing tests, and chicken pathogenicity tests. The two latter types are used to test for pathogenicity of the virus, while the first type is used to confirm H5 or H7 subtype presence (American Plant Health Inspection Service, 2008). Virus isolation tests are conducted by inoculating embryonated eggs with the viral strain and allowing the virus to “grow”. Simultaneously, RT-PCR tests are used on the culture to identify HA and NA subtypes (American Plant Health Inspection Service, 2008; United States Department of Agriculture, 2015). In the case of genetic sequencing tests, the entire genetic sequence of the virus is compared to a database containing all AI virus sequences. If the detected virus matches a known HPAI genetic sequence, the virus is deemed highly pathogenic (American Plant Health Inspection Service, 2008). Lastly, the chicken pathogenicity involves inoculating ten young disease-free birds with the suspected HPAI virus; the result is considered positive if a mortality
rate of 75% is observed (United States Department of Agriculture, 2015). The turnaround for this test is approximately 10 days (American Plant Health Inspection Service, 2008). It should be noted that similar screening and confirmatory tests are conducted in Canada.

2.4 Nanomaterials used in Biosensor Applications

Although current detection methods show high specificity, reproducibility, and selectivity towards H5 and H7 AI subtypes; they are very resource intensive, suffer from poor diagnostic turnaround, and require highly specialized facilities (Jensen et al., 2013; United States Department of Agriculture, 2015; World Organization for Animal Health, 2016). Due to poor diagnostic turnaround, LPAI H5 and H7 infections in poultry may evolve to HPAI infections before the test results come back. Therefore, there is a need for a more rapid H5 and H7 AI subtype detection method, that can be employed at the infected premises in a point-of-care fashion. This can be accomplished by developing a point-of-care biosensor that leverages nanomaterials to effectively reduce reaction time and reagent cost, while also eliminating the need for specialized personnel and facilities.

One advantage of using nanomaterials to sense biomarkers (i.e. HA antigens) is the high specific surface that they provide; thereby enabling immobilization of an increased number of bioreceptor units (i.e. HA antibodies) (Holzinger et al., 2014). This high surface area is also responsible for a significant reduction in the reaction time, in comparison to conventional testing methods. The important factors in material choice when developing biosensors are biocompatibility, stability, and conductivity (Holzinger et al., 2014; Zhang and Wei, 2016).

2.4.1 Leveraging Nanomaterials for Disease Detection

Recent advancements in nanotechnology have produced novel materials with unique physicochemical properties that are well suited for biosensing applications. Of these, graphitic
materials (e.g. carbon nanotubes and graphene) have received substantial research interest in this domain (Turcheniuk et al., 2015). Aside from their exceptional properties, these materials can be readily produced with ease and in high quantities.

2.4.1.1 Comparison of Graphene and Carbon Nanotubes

The preference for carbon nanotubes usage in nanohybrids has declined over the years due to the emergence of graphene, which is less expensive and possesses some advantageous properties when compared to carbon nanotubes (Ma et al., 2013). Graphene possesses a 2D basal plane structure that allows the loading of microspheres (ca. 200+ nm), which is beneficial in comparison to carbon nanotubes nanoparticle decoration (Zhou et al., 2010). Additionally, graphene possesses a higher surface area, improving the interfacial contact with secondary components (i.e. nanocolloids) in comparison to carbon nanotubes; whereas carbon nanotubes often exhibit aggregation of secondary components. Furthermore, graphene lacks metallic impurities, which is the major downfall of carbon nanotubes use in biosensor development, and therefore can be incorporated into complex sensors through common microfabrication approaches (i.e. layer-by-layer (LBL) electrode deposition) (Jin et al., 2010; Khalil et al., 2016). A list of general material properties exhibited by graphene can be observed in Table 4.

**Table 4:** Material properties of graphene

<table>
<thead>
<tr>
<th>Material Property</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Optical Transmittance</strong></td>
<td>97.7%</td>
<td>(Bonaccorso et al., 2010)</td>
</tr>
<tr>
<td><strong>Density</strong></td>
<td>$0.77 \frac{mg}{m^2}$</td>
<td>(Basu and Bhattacharyya, 2012; Chowdhury and Balasubramaniam, 2014)</td>
</tr>
<tr>
<td><strong>Resistivity</strong></td>
<td>$10^6 \Omega cm$</td>
<td>(Basu and Bhattacharyya, 2012; Chowdhury and Balasubramaniam, 2014)</td>
</tr>
<tr>
<td><strong>Planar Surface Area</strong></td>
<td>$2630 \frac{m^2}{g}$</td>
<td>(Stoller et al., 2008)</td>
</tr>
<tr>
<td><strong>Young’s Modulus</strong></td>
<td>1100 GPa</td>
<td>(Lee et al., 2008)</td>
</tr>
<tr>
<td><strong>Fracture Strength</strong></td>
<td>125 GPa</td>
<td>(Lee et al., 2008)</td>
</tr>
<tr>
<td><strong>Mobility Charge Carrier</strong></td>
<td>$200,000 \frac{cm^2}{Vs}$</td>
<td>(Bolotin et al., 2008; De la Fuente, 2017)</td>
</tr>
</tbody>
</table>
Due to its advantageous properties over carbon nanotubes, graphene was used as a scaffold for nanoparticle decoration.

2.4.1.2 Amplification of the Electrical Properties of Graphene – Nanocomposites

Nanocomposites are multiphase materials: one phase is dispersed in a second phase (matrix or scaffold) in nano-size form; whereby the properties of the individual component materials are combined (Bai and Shen, 2012). Due to their advantageous properties in biosensing applications, graphene-inorganic metal and metal oxide nanocomposites have received substantial research interest (Bai and Shen, 2012). These composites can provide enhanced effective surface area, superior catalytic properties, increased specificity, and limit of detection (LOD) in comparison to using graphene alone (Bai and Shen, 2012). As an example, individual graphene sheets tend to form irreversible clusters due to van der Waals forces and \( \pi-\pi \) stacking, thereby reducing their electrochemical properties (Stankovich et al., 2007). However, the incorporation of a second phase (i.e. metal/metal oxide nanoparticles) provides a nano-spacer, which increases the graphene interlayer distance to minimize clumping. This effectively increases the conductance in two ways: the first being that both sides of graphene sheets are now accessible and the second being the addition of a conductive metal layer (Si and Samulski, 2008; Tien et al., 2010).

In addition to the enhanced properties of graphene-metal nanocomposites, biomolecules (e.g. proteins, antibodies, etc.) can easily be directly immobilized on the surface of the metal nanoparticles (Wu et al., 2012). In contrast, direct immobilization on carbon nanotubes or graphene sheets has proven to be unstable, as the biomolecules can be lost through frequent washing steps during biosensor fabrication (Chen et al., 2003; Mohanty and Berry, 2008). Inevitably, this contributes to poor reliability/repeatability and non-specificity of the biosensor.
Due to pitfalls of graphene with respect to biomolecule immobilization, graphene-metal nanocomposites were used for biosensor development.

2.4.2 Graphene-Gold Nanocomposites in Biosensor Development

Many noble metal nanostructures have been researched; however, gold (Au) nanoparticles are among the most researched nanomaterials. This is due to their superior surface chemical properties, increased chemical stability, exceptional catalytic activity, and biocompatibility (Connor et al., 2005; Han et al., 2009; Zhong et al., 2004). In this sense, biocompatibility refers to low cytotoxicity and the ability to easily immobilize biomolecules on nanoparticle surface. These properties make Au nanoparticles of paramount importance for DNA detection and rapid identification of microorganisms (i.e. viral and bacterial pathogens) (Elghanian et al., 1997; Khan et al., 2013; Li and Rothberg, 2004). For these reasons, it is expected that enhanced signals can be achieved by using Graphene-Au nanocomposites in biosensor development. In this respect, graphene-Au nanocomposites possess approximately 230% better electrocatalytic current density and Raman signals than Au nanoparticles alone (Govindhan et al., 2015; Wang et al., 2013). Additionally, the addition of Au nanoparticles on graphene increases the surface enhanced Raman scattering (SERS) by a factor of 45 in comparison to graphene alone (Sidorov et al., 2012). Graphene-Au nanocomposites will be employed in hope to increase the electrochemical signal of the biosensor.

3. METHODOLOGY

3.1 Materials and Reagents

Gold (III) chloride trihydride (HAuCl₄·3H₂O), L-polylysine, hydroquinone, potassium hexacyanoferrate (K₃[Fe(CN)₆]), potassium hexacyanoferrite (K₄[Fe(CN)₆]), phosphate buffer
saline (PBS), gold cleaning solution, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), CdTe quantum dots and pyrene carboxylic acid were purchased from Sigma-Aldrich (MO, USA). Graphene (4% wt. water dispersion) was obtained from ACS Materials (CA, USA). All the chemicals were of analytical grade and used as received without further purification. Screen-printed gold electrodes were purchased from Dropsens (Spain). Whole chicken blood and influenza virus A (H1N1) surface protein were purchased from Cedarlane Labs (ON, Canada). Anti-influenza A (H5N1) virus hemagglutinin (HA) antibody and influenza virus A (H5) surface protein were purchased from Abcam, Inc., (Cambridge, UK). Anti-H4 (H4N6) polyclonal antibody was purchased from MyBioSource Inc., (San Diego, USA). Milli-Q water (18.2 MΩ, DI water) was used throughout the experiments.

3.2 Avian Influenza A (H4N6) and (H9N2) Virus Cultures

All influenza A virus cultures were generously donated by Dr. Eva Nagy from the department of pathobiology, Ontario Veterinary College, University of Guelph. The following protocol was carried out in her facility by her staff.

Low pathogenic AIV H4N6 (avian influenza A/Duck/Czech/56 (H4N6)) was propagated in 11-day-old embryonated chicken eggs by inoculation into the allantoic cavity (Szretter et al., 2006). Infectious titer in allantoic fluid was determined at 72 h post-inoculation and expressed as a 50% tissue culture infective dose 128 HAU/50 µL.

Inactivated AIV H9N2 (A/Turkey/Ontario/1/66) was propagated in 11-day-old embryonated SPF chicken eggs. The egg-derived virus was inactivated with formalin (final concentration 0.02%) for 72 h at 37 °C. The protein content of the inactivated virus preparation was determined using haemagglutination inhibition (HI) assay and expressed as 50% tissue culture infective dose 128 HAU/50 µL (Singh et al., 2016).
3.3 Fabrication of Non-Spherical Graphene-Gold Nanocomposite Scaffolding

The nanocomposites were synthesized in a one-pot, *in-situ* method resulting in a final working solution volume of 20 mL. First, 18 mL of 40X diluted graphene solution (final solution concentration of 1 mg/mL) was sonicated for 15 minutes to separate the graphene sheets. Next, 1 mL of HAuCl₄ (final solution concentration of 2.5 x 10⁻⁴ M) was added to the graphene solution under constant stirring. The solution was then stirred for 30 minutes. Next, 1 mL of hydroquinone (final solution concentration of 2.5 x 10⁻⁴ M) was added to the graphene gold solution to simultaneously reduce Au³⁺ to Au⁰ and graphene to reduced graphene. The solution was stirred for 1 hour at room temperature to allow complete reduction. The solution was then centrifuged at 15,000 rpm for 5 minutes to remove any unused reactants. The supernatant was removed from each tube, followed by a washing step with DI water. This centrifugation and washing procedure was carried out three times to ensure that any remaining reducing agent had been removed. The solution was then returned to a single 20 mL glass vile after the final washing step. The nanocomposite solution was then stored in a refrigerator at 4°C for future use.

3.4 Nanocomposite Scaffolding Characterization

The graphene-Au nanocomposites were characterized using UV-Visible spectroscopy (Cary 100, Agilent Technologies), transmission electron microscopy (TEM, FEI Tecnai G2 F20 microscope), scanning electron microscopy (SEM), energy dispersive x-ray (EDX) analysis, and electrochemical analysis (cyclic voltammetry).
3.5 Nanocomposite Deposition on Electrodes

All electrodes were first cleaned by dropping 10 µL of gold cleaning solution onto the working electrode. After 10 seconds the electrodes were washed thoroughly with DI water. Next, 5 µL of L-polylysine was dropped onto the working electrode and was spread to cover the entire working electrode area. The electrodes were covered in a petri dish, dried for 2 hours at room temperature, followed by a DI water rinse to remove any unbound L-polylysine. The same process was carried out for depositing the graphene-Au nanocomposite solution. These alternating layers formed L-polylysine/nanocomposite bilayers on the substrate. The above protocol was repeated for each additional bilayer. Two bilayers were used (meaning 4 layers in total) for the electrochemical immunosensing.

3.6 Antibody Conjugation of Electrodes and Quantum Dots

Electrodes were first functionalized by using 1 mM pyrene carboxylic acid (adding a -COOH group to the graphene sheets). 10 µL of pyrene carboxylic acid was deposited onto each working electrode and the electrodes were set to dry for 1 hour. Next, 5 µL of 4 mM EDC was deposited onto the working electrodes followed by 5 µL of 10 mM NHS. The electrodes were left for 10 minutes to allow reaction between EDC and NHS, which was followed by a light DI water wash step to remove any o-acylisourea by-product. Next, 5 µL of the respective primary antibody, anti-Hx (1 µg/mL in 1X PBS buffer (pH 7.0)), was deposited onto the working electrode. The electrodes were incubated overnight at 4°C in a moisture chamber.

CdTe quantum dots were conjugated with anti-N1 antibodies (1 µg/mL) using the same EDC/NHS carbodiimide crosslinking. The quantum dots were also incubated overnight at 4°C. The same procedures were followed to immobilize anti-H4 and anti-H9 antibodies onto the CdTe quantum dots for the H4N6 and H9N2 sensing experiments, respectively.
3.7 Optimization of AIV Immunosensor

The concentration of primary antibodies was tuned by conducting cyclic voltammetry (CV) studies on electrodes with varying antibody concentrations (0.5 µg/mL to 2.5 µg/mL). A 1:1 mixture of 5 mM of K₄[Fe(CN)₆] and K₃[Fe(CN)₆] in 1X PBS (pH 7.4) was used as the electrolyte solution. During testing 100 µL of the electrolyte solution was dropped onto the electrode. The antibody concentration that resulted in the lowest current corresponding to highest surface coverage was chosen for the design. A commercial potentiostat (DRP-STAT200, Dropsens, Spain) was used to measure electrochemical redox current at a scan rate of 0.01 V/s.

Similarly, a study was conducted using various antibody-antigen interaction times (0 min, 5 min, 10 min, 20 min, and 60 min). For this test 5 µL of target antigen (H4N6) spiked blood dilution was dropped onto each respective electrode, immediately followed by 5 µL of CdTe anti-H4 bioconjugate. Incubation time was considered to begin after the CdTe bioconjugates were added. After each incubation time-period, the electrodes were gently washed with DI water. Each electrode was tested by micropipeting 100 µL of PBS buffer onto the antibody-antigen-antibody superstructure. The electrochemical redox current of the CdTe quantum dot reporters was measured to obtained applicable incubation time.

3.8 Electrochemical Immunosensing of AIV

The schematic of electrochemical immunosensing of AIV is illustrated in Fig. 1. Screen-printed gold electrodes were used for these tests. A total of 8 serial dilutions of H5 surface protein were used (1 µg/mL to 1 fg/mL), each concentration was tested in triplicates. Similarly, a total of 8 serial dilutions of H4N6 were used (128 HAU, 1.28 HAU, 1.28 x 10⁻² HAU, 1.28 x 10⁻³ HAU, 1.28 x 10⁻⁴ HAU, 1.28 x 10⁻⁵ HAU, 1.28 x 10⁻⁶ HAU, and 1.28 x 10⁻⁷ HAU). H4N6 dilutions were spiked in three-parts diluted whole chicken blood (PBS, pH 7.4). 5 µL of
each viral dilution was dropped onto respective anti-H₅ modified working electrode. Next, 5 µL of the anti-N1 (for H5N1) or anti-H4 (for H4N6) conjugated CdTe quantum dots was micropipetted onto each working electrode. In the case of H5N1, the electrodes were incubated in a moisture chamber for 1 hr at 4°C to allow for antibody-antigen interaction, which was then followed by a DI water wash to remove any unbound quantum dot reporters. The H4N6 and H9N2 viruses were incubated for an optimized duration of 10 minutes. The electrochemical redox current of the CdTe quantum dot reporters was measured with varying antigen concentrations. Each electrode was tested by micropipeting 100 µL of PBS buffer onto the antibody-antigen-antibody superstructure. All tests were conducted using a scan range of 0.1V to -1 V with a scan rate of 0.01 V/s and a sampling rate of 0.002 V/s.

3.9 Specificity and Cross Reactivity Studies

The designed immunosensor was tested for specificity to H5N1, H4N6, and H9N2. This was done by using H1N1 recombinant protein, H9N2 virus, and 1 mg/mL peptidoglycan

Figure 1: Electrochemical immunosensing mechanism for direct detection of avian influenza A viruses in a whole chicken blood sample matrix.
(dispersion in 1X PBS) as negative controls (in the case for H4N6 as the target). H5 recombinant protein was also used as a negative control for H4N6 virus. The control concentrations used for recombinant proteins and viruses were 1 µg/mL and 128 HAU, respectively. For cross reactivity testing of H4N6 in diluted whole blood, the sample was spiked with 1 mg/mL peptidoglycan and 128 HAU of H4N6. Blanks were also run for both H5N1 and H4N6 as negative controls. These tests were conducted in triplicates. A similar protocol was used for testing selectivity and cross-reactivity of the immunosensor to an H9N2 target antigen. In this protocol, H4N6 was used as a negative control in addition to the other negative controls.

3.10 Validation Studies with Commercial ELISA Kit

A comparison study was performed with a commercial avian influenza A H4N6 (Cat. No: NS-E10156, Novatein Biosciences, Woburn, MA, USA) ELISA Kit to validate the designed immunosensor. Various virus titers were prepared using sample diluent provided in the ELISA kit box and by strictly following the manufacturer's protocol in the performance of the bioassay.

4. RESULTS AND DISCUSSION

4.1 Nanocomposite Characterization

In this study, graphene sheets were decorated with non-spherical nanoparticles in a one-pot \textit{in-situ} simultaneous reduction of graphene and a gold salt using hydroquinone as a reducing agent. The resulting nanocomposites were examined using SEM and EDX to determine their elemental composition (Fig. 2). Through elemental analysis, it was found that C, Au, and O were present in the sample, indicating that the graphene sheets were successfully decorated with gold nanoparticles.
Figure 2: SEM and EDX characterization of the graphene gold nanocomposites. Si was used as the substrate, hence its appearance in spectrums 9 and 10.

The presence of gold in the nanocomposite was also confirmed via UV-visible spectroscopic studies (Fig. 3). From Fig. 3, the nanocomposite exhibited the characteristic $\pi - \pi$ bond of the polyaromatic C – C at 230 nm for graphene, as well as a second broad peak that was associated
with the gold nanoparticles.

**Figure 3**: UV – Vis characterization of graphene-gold nanocomposites and graphene.

TEM images of the fabricated gold-graphene nanocomposites were also obtained (Fig. 4). From Fig. 4, more particularly panels C and D, it is evident that spikey/star shaped Au nanoparticles were formed and deposited onto graphene sheets. The reason non-spherical gold nanoparticles were desired was because they would act as excellent spacers between the graphene sheets, thus reducing $\pi - \pi$ stacking, promoting inter-layer linkage, and increasing the conductivity of graphene.
Due to one-pot in-situ nanocomposite synthesis, the Au nanoparticles bind to graphene electrostatically, thus reducing the overall synthesis time. In addition to the benefit of decreased synthesis time, these composites can provide enhanced effective surface area, superior catalytic properties, increased specificity, and limit of detection (LOD) in comparison to using graphene alone (Bai and Shen, 2012).

As an example, individual graphene sheets tend to form irreversible clusters due to van der Waals forces and π-π stacking, thereby reducing their electrochemical properties (Stankovich 4: TEM images of non-spherical graphene-gold nanocomposites. (A) 200 nm scale demonstrates that graphene has been decorated with numerous nanoparticles; (B) 100 nm scale demonstrates non-spherical shape; (C) 20 nm scale depicts a small group of non-spherical nanoparticles as well as detailed graphene sheets; and (D) 10 nm scale depicts a single non-spherical nanoparticle.
et al., 2007). However, the incorporation of a second phase (i.e. non-spherical gold nanoparticles) provides a nano-spacer, which increases the graphene interlayer distance to minimize clumping. This effectively increases the conductance in two ways: the first being that both sides of graphene sheets are now accessible and the second being the addition of a conductive metal layer (Si and Samulski, 2008; Tien et al., 2010). The non-spherical confirmation allows increased surface area contacts between the nanoparticles and graphene sheet layers. Furthermore, nanospacing allows both sides of the graphene sheets to be conductive by reducing π – π stacking phenomena. The significance of the presented TEM images is that non-spherical Au nanoparticles were formed, and that the graphene sheets were well-decorated with nanoparticles; thus, forming non-spherical graphene – gold nanocomposite scaffolding.

4.2 Electrode Surface Modification with Nanocomposite Scaffolding

Cyclic voltammetry (CV) studies were conducted to determine the signal amplification due to electrode surface modifications with the developed nanocomposite scaffolding (Fig. 5). From Fig. 5, it can be observed that each successive bilayer (L-polylysine and nanocomposite scaffolding) caused an associated increase in the signal. This is because with each successive layer, more gold is being deposited onto the electrode surface, which in turn causes the conductivity to increase. It should be noted that L-polylysine was used as a stabilizing polymer to anchor the nanocomposite scaffolding to the electrode surface.
Figure 5: CV studies of electrode surface modification with L-polylysine/nanocomposite bilayers. Each consecutive bilayer increased the signal by approximately 50 µA. A 1:1 mixture of 5 mM of $K_4[Fe(CN)_6]$ and $K_3[Fe(CN)_6]$ in 1X PBS (pH 7.4) was used as the electrolyte solution.

It was observed that a single bilayer would sometimes wash away after testing, therefore additional bilayers were needed. The second and third bilayers provided more stable surface modifications while also increasing the measured signal. It should also be noted that although signal increases were still observed after two bilayers, each additional bilayer would reduce the overall diffusivity of the surface modification, and thus decrease sensitivity of the sensor. Due to this, a two-bilayer modification was chosen for further experimentation, as it reduced electrode fabrication time, provided a stable modification, and increased the measured signal.
4.3 Antibody concentration optimization

Five different antibody concentrations were used to determine an optimum coverage of the working electrode by the primary antibody. In this case anti-H5 antibody was used. In theory, the antibody concentration with the lowest peak current would indicate the most coverage of the working electrode surface. The concentration studies can be observed in Fig. 6.

![Graph](image)

**Figure 6:** Anti H5 antibody concentration vs. Peak current, which was obtained from cyclic voltammetry testing. The lowest peak current was found to be 150 µA, which corresponded to 1 µg/mL of anti H5 antibody. Due to this, 1µg/mL of anti H5 antibody was chosen as the optimum concentration for further experiments. The same concentration of anti-H4 was used for H4N6 sensing. Buffer: 5 mM K$_3$[Fe(CN)$_6$] - K$_4$[Fe(CN)$_6$] in 1X PBS.

The primary antibody concentration with the lowest current value was found to be 1 µg/mL. However, as concentration was increased beyond 1 µg/mL, the associated peak current began to increase as well. This contradicts electrochemical theory because as antibody concentration is increased, impedance of the electrode should also increase, thus reducing the peak current.
These results may have been affected by steric hindrance as antibody concentration was increased. It may also be possible that the antibodies agglomerated together at higher concentrations, forming a conductive layer. Further studies are required to confirm whether these phenomena are occurring. For this work an antibody concentration of 1 µg/mL was used on the working electrode. The same concentration of secondary antibody was used to form CdTe quantum dot bioconjugates.

The proposed immunosensor must provide sensing results more rapidly than current conventional methods. In attempt to reduce turnaround time, antigen – antibody interaction time studies were conducted (Fig. 7).

![Graph](image)

**Figure 7:** Peak current vs. antigen – antibody incubation time. The incubation period with the second highest response was chosen for all further testing, corresponding to a 10-minute incubation period. Error bars represent standard deviation, n = 3.
It was found that the highest response is received at an incubation period of 60 minutes. This is on-par with current conventional techniques, however, a sensor that could be used rapidly in a point-of-care fashion was desired. Therefore, an incubation time of 10 minutes was chosen, as it occurred on the plateau of the curve and possessed lower noise than a 20-minute incubation period. As a 10-minute incubation period exists on the flat portion of the curve, this shows that exact timing is less critical in the 10-minute zone. This could be of use during field deployment, as the user may take a bit longer to set up the reagents or instrumentation. Furthermore, an incubation time of 10 minutes is much more feasible than 60 minutes when it comes to point-of-care device employment. Therefore, an incubation time of 10 minutes was used for the H5N1, H4N6, and H9N2 studies.

4.4 H5 protein detection in 1X PBS

Detection of H5 recombinant protein was used as a proof of concept to determine if the immunosensor mechanism would work. The mechanism was later adapted to detect H4N6 and H9N2 viruses. The CV profiles of the various H5 protein concentrations are shown in Fig. 8(A). Upon conducting H5N1 sensing experiments, two negative characteristic peaks were found in the CV profiles, one at -0.35V and the second at -0.75V, corresponding to the CdTe bioconjugate reporters. It was found that the characteristic peaks at -0.75V were more prominent, and thus were used to obtain the current – antigen concentration data shown in Fig. 8(B). Fig. 8(B) shows a near sigmoidal relationship between peak current and recombinant protein concentration. It was found that the biosensor could distinctly detect spiked concentrations of H5 protein in 1X PBS (pH 7.4) from 1 µg/mL to 10 fg/mL. A linear range exists between 10 ng/mL and 10 pg/mL of recombinant protein (Fig. 8(B) inset). The coefficient of determination and slope values for this relationship were found to be 0.987 and 0.9242 µA*mL*µg⁻¹, respectively. A reduced linear region was chosen as opposed to a full
non-linear sigmoidal relationship because a linear calibration curve was more desirable to work with than a non-linear curve.

Figure 8: (A) Cyclic voltammetry profiles for H5 surface protein concentrations spiked in 1X PBS (pH 7.4) ranging from 0 µg/mL to 1 fg/mL. (B) Calibration curve of H5 surface protein immunosensing derived from the CV. Inset: Linear detection range from 10 ng/mL to 10 pg/mL; $R^2 = 0.987$. Error bars represent standard deviation, $n = 3$. 
The results obtained from the cyclic voltammetry studies with respect to recombinant protein concentration (Fig. 8(A)) exhibit baseline shifts, which make some higher concentration curves appear to have weaker peaks than some lower concentrations. This could be due to electrode to electrode variation resulting from non-uniform drying rates of the nanocomposite scaffolding. The resultant peak current values were used to develop a strong current–protein concentration relationship. The negative peaks at -0.75 V (Fig. 8(A)) agree with previous work on electrochemical reporting properties of CdTe (Amelia et al., 2012).

The immunosensor possessed a lower LOD of 10 fg/mL and an upper LOD of detection of 1 µg/mL in PBS (pH 7.4) based on the obtained sigmoidal data (Fig. 8(B)). These results are agreeable with previously developed ELISA and nanoenzyme techniques (Ahmed et al., 2017). It is quite possible that the biosensor can detect concentrations greater than 1 µg/mL; however, higher concentrations were not tested in this thesis work. It can be said that the linear concentration range (Fig. 8(B) inset) shows promise of ultra-sensitive detection of target antigens in tandem with reduced turnaround time compared to conventional detection methods.

4.5 H4N6 virus detection in blood

The goal of this study was to develop an immunosensor with the capability to detect avian influenza A (H4N6) virus in whole chicken blood. Upon conducting H4N6 sensing experiments in three-parts diluted whole chicken blood, it was found that the sensor can detect the virus with a LOD of $1.28 \times 10^{-7}$ HAU (Fig. 9).
Figure 9: Avian Influenza A (H4N6) sensing results in three-parts diluted whole chicken blood. The sensor exhibits a detection range from 128 HAU to $1.28 \times 10^{-7}$ HAU in blood. Error bars represent standard deviation, n = 3. It can be observed that a linear response exists on the semi-log plot for concentrations between 128 HAU and $1.28 \times 10^{-7}$ HAU, with a sensitivity of 0.718 µA/HAU and a coefficient of determination of 0.975. Additionally, selectivity and cross-sensitivity tests were conducted for H4N6 (Fig. 10).
Figure 10: Selectivity and cross-sensitivity results for avian influenza A (H4N6) virus immunosensing mechanism. The blank, H1N1 protein, H5 protein, peptidoglycan (PG), and H9N2 virus signals are statistically indistinguishable, thus, highlighting selectivity to the target H4N6 antigen. Error bars represent standard deviation, n = 3.

Cross-sensitivity was not observed between PG and H4N6 virus, because even in the presence of PG, H4N6 could be distinctly detected and measured (Fig. 10).

These results highlight the selectivity of the immunosensor, as non-target viruses and viral proteins were statistically indistinguishable from a blank sample. More importantly, the immunosensor is selective to H4N6 even when exposed to H9N2 avian influenza virus. It can also be seen that there is no cross-sensitivity with peptidoglycan, meaning that H4N6 can still be detected in the presence of peptidoglycan. Peptidoglycan is a component of the bacterial cell wall, and thus was used to simulate possible bacterial contamination present in the sample. These results suggest that the developed immunosensor is highly specific to H4N6 in whole
chicken blood and that the sensor is ultra-sensitive even in the presence of bacterial components.

The immunosensor demonstrated ultra-sensitive detection of avian influenza A (H4N6) in three-parts diluted whole blood, with an upper and lower LOD of 128 HAU and \(1.28 \times 10^{-7}\) HAU, respectively (Fig. 9). The designed immunosensor also exhibited excellent selectivity towards H4N6 inactivated virus (Fig. 10). Even in the presence of highly concentrated peptidoglycan (1000X greater than the antigen), H4N6 could be selectively detected. These results are highly significant because some samples (e.g. blood, faeces, mucosa, and sputum) can contain both viral and bacterial contaminants – it is important to be able to detect the target virus in such complex media. Due to the 10-minute incubation time, the turnaround time has been significantly reduced, when compared to conventional ELISA.

4.6 Validation of H4N6 Detection with Commercial ELISA kit

The sensitivity of the H4N6 immunosensing mechanism was compared to that of a commercially available ELISA kit. The goal of this study was to determine if the immunosensor possessed superior sensitivity than conventional detection methods. The ELISA results for H4N6 virus demonstrate a sensitivity of 0.128 HAU (Fig. 11).
Figure 11: H4N6 commercial ELISA test results depicting a lower limit of detection of 0.128 HAU. Error bars represent standard deviation, n = 3.

Concentrations lower than 0.0128 HAU are statistically indistinguishable by the ELISA kit. In comparison, the immunosensor (LOD = 1.28 x 10^-7 HAU) was found to be 10^5 times more sensitive than the commercial ELISA kit. There is a possibility that the ELISA kit had reduced sensitivity due to the complexity of the media used, which is another limitation of using ELISA in place of a biosensor. It is also noteworthy that the ELISA protocol takes several hours and multiple steps, whereas the immunosensor turnaround time is on the order of minutes. Thus, the designed immunosensor exhibits ultra-sensitivity towards H4N6 inactivated virus and a quicker turnaround time when compared to commercial ELISA.
4.7 Validation of H4N6 Virus Binding Using SEM Imaging

To demonstrate that the target antigen was, in fact, binding to the working electrode as hypothesized, SEM studies were conducted (Fig. 12).

Figure 12: SEM images of H4N6 virus on the working electrode: (A) 10um scale to indicate coverage of the electrode, (B) 2um scale to indicate coverage of the electrode, (C) 300nm scale to indicate virus particle size, (D) 100 nm scale to illustrate viral coronae.

From panels A and B, it was observed that the modified electrode surface did possess many particles, which could be viral in nature. Upon further analysis and increased magnification (panels C and D) it was evident that the particles were roughly the size of influenza virus particles (~180 nm in diameter). Moreover, the particles present in panels C and D also exhibit a hazy or blurred outline. This could be the coronae of the virus, which would indicate that virus particles have successfully bound to the immunosensor. However, due to limitations of resolution, even with a thin 20 nm gold plating, it was not possible to obtain clearer images with the SEM. For these reasons, these images in tandem with previously measured
electrochemical signals, were used to determine that the virus was successfully binding to the immunosensor.

4.8 H9N2 virus detection in blood

The goal of this study was to showcase the versatility of the immunosensing mechanism. By changing the target antibodies of the mechanism (i.e. from H4N6 to H9N2), the sensor can detect additional target antigens. To demonstrate this, influenza A (H9N2) virus was spiked in whole chicken blood for these tests. Upon conducting H9N2 sensing experiments in three-parts diluted whole chicken blood, it was found that the sensor can detect the virus with a LOD of $1.28 \times 10^{-7}$ HAU (Fig. 13).

![Graph showing the relationship between H9N2 concentration and sensor signal intensity. The equation $y = 0.1468 \log(x) + 1.78143$ with $R^2 = 0.945$ represents the fit. Error bars indicate standard deviation, $n = 3$.]

**Figure 13:** Avian Influenza A (H9N2) sensing results in three-parts diluted whole chicken blood. The sensor exhibits a detection range from 128 HAU to $1.28 \times 10^{-7}$ HAU in blood. Error bars represent standard deviation, $n = 3$. 
It can be observed that a linear response exists on the semi-log plot for concentrations between 128 HAU and $1.28 \times 10^{-7}$ HAU, with a sensitivity of 0.1468 $\mu$A/HAU and a coefficient of determination of 0.945.

The sensitivity of the H9N2 sensor was much less when compared to the H4N6 sensor. This could be due to, in part, to the differences between virus structures. Each hemagglutinin (HA) has a different binding site, meaning that H9 is quite different from H4 due to the sialic acid linkage site. Moreover, hemagglutinin and neuraminidase proteins are slightly different for each influenza virus strain, which could give rise to varying detection sensitivities to each antigen.

Another cause for decreased sensitivity could be antigen-antibody pairing. Sometimes, it is not possible to obtain a commercial product that is specific to only one H9N2 virus strain. For example, the strain used in this study was H9N2 (A/Turkey/Ontario/1/66), and it was not possible to find commercial antibodies raised against this specific strain – rather a generic, less specific, H9N2 antibody was used, which could detect all H9N2 viruses. Due to these factors, a reduced detection sensitivity was expected.

Cross-sensitivity and selectivity trials were also conducted for the H9N2 immunosensing experiments. The results are highlighted in Fig. 14.
Figure 14: Selectivity and cross-sensitivity results for avian influenza A (H9N2) virus immunosensing mechanism. The blank, H1 protein, H5 protein, peptidoglycan (PG), and H4N6 virus signals are statistically indistinguishable, thus, highlighting selectivity to the target H9N2 antigen. Error bars represent standard deviation, n = 3.

Cross-sensitivity was not observed between PG and H9N2 virus, because even in the presence of PG, H9N2 could be distinctly detected and measured (Fig. 14).

These results highlight the selectivity of the immunosensor, as non-target viruses and viral proteins were statistically indistinguishable from a blank sample. More importantly, the immunosensor is selective to H9N2 even when exposed to H4N6 avian influenza virus. It can also be seen that no cross-sensitivity exists with peptidoglycan, meaning that H9N2 can still be detected in the presence of peptidoglycan. These results suggest that the developed immunosensor is highly specific to H9N2 in whole chicken blood and that the sensor is ultra-sensitive even in the presence of bacterial components.
The immunosensor demonstrated ultra-sensitive detection of avian influenza A (H9N2) in three-parts diluted whole blood, with an upper and lower LOD of 128 HAU and $1.28 \times 10^{-7}$ HAU, respectively (Fig. 13). The designed immunosensor also exhibited excellent selectivity towards H9N2 inactivated virus (Fig. 14). Even in the presence of highly concentrated peptidoglycan (1000X greater than the antigen), H9N2 could be selectively detected. It is also important to note that this study highlights the versatility of the immunosensor – the bioreceptor element can be modified to target additional viral antigens. The H9N2 immunosensor was not validated with a commercial ELISA kit as these experiments were sufficient to showcase the versatility of the sensing mechanism.

To demonstrate the novelty of this versatile immunosensor, it was compared with previous related works (Table 5).
**Table 5:** Comparison of this thesis work to recent electrochemical-based influenza biosensor studies.

<table>
<thead>
<tr>
<th>Sensor (method)</th>
<th>Mechanism</th>
<th>Antigen(s)</th>
<th>Sample matrix</th>
<th>LOD</th>
<th>Detection range</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrochemical Imunosensor (CV, EIS, CA)</td>
<td>SPGE/RGO/CA/Ab/Ag on microfluidic chip</td>
<td>H1N1 virus</td>
<td>PBS</td>
<td>0.5 PFU</td>
<td>1 – 10⁶ PFU</td>
<td>(Singh et al., 2017)</td>
</tr>
<tr>
<td>Electrochemical Imunosensor (N/A)</td>
<td>SPCE/Ab/BSA/Ag</td>
<td>H1N1 virus</td>
<td>chick embryo allantoic fluid</td>
<td>0.43 HAU</td>
<td>4 – 64 HAU</td>
<td>(Zhang et al., 2017)</td>
</tr>
<tr>
<td>Aptamer Sensor (CV, DPV)</td>
<td>SPCE/Au NP/DNA-aptamer/Ag/Ab-ALP</td>
<td>H5 protein</td>
<td>PBS</td>
<td>100 fM</td>
<td>100 fM – 10 pM</td>
<td>(Diba et al., 2015)</td>
</tr>
<tr>
<td>Electrochemical Imunosensor (DPV)</td>
<td>GCE/Au NP/Ab/BSA/Ag/Ab/Pt-pZnO-hemin</td>
<td>Not mentioned</td>
<td>PBS; 10 parts diluted human sera</td>
<td>0.76 pg/mL</td>
<td>0.001 – 60 ng/mL</td>
<td>(Yang et al., 2016)</td>
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<tr>
<td>Electrochemical Immunosensor (EIS, SWV)</td>
<td>SPGE/Au NP/scFV/BSA/Ag-His</td>
<td>H5 protein</td>
<td>PBS</td>
<td>0.6 pg/mL</td>
<td>4 – 20 pg/mL</td>
<td>(Góra-sochacka et al., 2016)</td>
</tr>
<tr>
<td>Electrochemical Immunosensor (CA)</td>
<td>Au electrode/ZnO NR/Ab/BSA/Ag/Ab₂ on microfluidic chip</td>
<td>H1 protein; H5 protein; H7 protein</td>
<td>PBS</td>
<td>1 pg/mL</td>
<td>1 pg/mL – 10 ng/mL</td>
<td>(Han et al., 2016)</td>
</tr>
<tr>
<td>Electrochemical Immunosensor (CV, LSV)</td>
<td>Au electrode/ G-Au NP/MAb/Ag/PAb-Ag NP-G</td>
<td>H7 protein</td>
<td>PBS</td>
<td>1.6 pg/mL</td>
<td>1.6 pg/mL – 16 ng/mL</td>
<td>(Huang et al., 2016)</td>
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<tr>
<td>Electrochemical Immunosensor (CV)</td>
<td>SPGE/PL/G-Au NP-Ab/Ag/Ab-CdTe; SPCE/PL/G-Au NP-Ab/Ag/Ab-CdTe; SPCE/PL/G-Au NP-Ab/Ag/Ab-CdTe;</td>
<td>H5 protein; H4N6 virus, H9N2 virus</td>
<td>PBS; 3 parts diluted chicken whole blood</td>
<td>1 fg/mL; 1.28 x 10⁻⁷ HAU</td>
<td>1 fg/mL - 1 ug/mL; 1.28 x 10⁻⁷ – 128 HAU</td>
<td>This work</td>
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</table>

This thesis work is the first to detect real virus culture in a whole chicken blood sample matrix, to the best of my knowledge. Most of the previously reported work first test the target analyte in buffer followed by spiking the target in biological fluid. This leads to inconsistency in results between buffered targets and spiked targets. Moreover, this work has demonstrated lower limits of detection than previously conducted studies. The goal of this design was to develop a sensing mechanism that is more sensitive than conventional ELISA and to reduce the time-to-results – both of which have been successfully accomplished in this thesis work.

5.0 CONCLUSIONS

Due to the high virulence and zoonotic potential associated with H5 and H7 avian influenza pathotypes, it is of utmost importance to control outbreaks by reducing diagnostic turnaround. Current methods of detection exhibit many limitations with respect to sample handling/transport, expensive equipment and reagents, poor diagnostic turnaround and the need for specialized facilities. This thesis work presented aims to provide a rapid electrochemical immunosensor that has potential to be used in a point-of-care fashion on-site. The proposed immunosensor could be employed on farms in the form of a portable hand-held device. With such technology, farmers themselves could monitor the health of their flocks by simply taking a droplet of blood from their chickens, placing it onto a pre-coated electrode, and inserting the electrode into a reader.

The various characterization experiments demonstrated that the nanocomposite scaffolding contained both graphene and non-spherical gold nanoparticles. The immunosensor was then fabricated by using optimized parameters (number of bilayers, antibody concentration, and antibody-antigen incubation time), which in turn, paved the way to a proof of concept immunosensor for H5 protein detection in 1X PBS. The results of the proof of concept sensor were
quite promising and demonstrated that the developed mechanism could be used to detect antigens using immunochemistry.

With respect to avian influenza A H5 recombinant protein, the imunosensor exhibited a lower LOD of 10 pg/mL and an upper LOD of 10 ng/mL in the linear range; however, a sigmoidal detection range from 10 pg/mL to 1 μg/mL was also established. With respect to avian influenza A H4N6 virus, the immunosensor exhibited selectivity to H4N6, a lower LOD of $1.28 \times 10^{-7}$ HAU, and an upper LOD of 128 HAU. In the case of H4N6 detection in blood, the immunosensor was found to be $10^5$ times more sensitive than its commercial ELISA counterpart. Through SEM imaging, it was also shown that the H4N6 virus did bind to the free-standing antibodies present on the working electrode surface, thus indicating that the mechanism works as described.

To showcase the versatility of the developed sensor, it was also adapted to detect H9N2 in whole chicken blood. It was determined that the immunosensor possessed a lower LOD of $1.28 \times 10^{-7}$ HAU, and an upper LOD of 128 HAU. For these tests ELISA validation was not conducted, as this run of tests was just to prove that other antigens could be detected using the developed sensing mechanism.

With respect to recent electrochemical immunosensor studies, this work is the first to perform total analysis (optimization and detection) of viruses in a whole chicken blood matrix, which is a very complex medium. The detection limits were much lower for this study in comparison to recent works. Thus, the designed immunosensor exhibited ultra-sensitivity in comparison to conventional ELISA methods and recent studies.

In conclusion, this biosensor design is moving in the direction of rapid point-of-care detection in blood, but this study is just the groundwork for a bigger and brighter future of avian influenza virus
detection. For this tool to be most effective, future work should be done in partnership with the National Animal Health Laboratory on H5 and H7 virus subtypes as these pathotypes are what we need to detect early for outbreak control.

6.0 RECOMMENDATIONS FOR FUTURE WORK

There are two tangents that branch from the thesis work presented: 1) altering the antibodies to enable detection of H5 and H7 avian influenza subtypes using active virus species; and 2) development of a hand-held electrode reader which could be used by farmers and/or medical personnel to detect avian influenza. The first area of future research focuses on further refinement and versatility of the immunosensing mechanism, whereas the last tangent focuses on moving from lab-based technology to a realizable point-of-care device. Each line of future work is discussed in detail in the following subsections.

6.1 H5 and H7 Future Research

It must be noted that the work discussed in this section would need to be conducted in partnership with the National Animal Health Laboratory in Winnipeg, Manitoba, as H5 and H7 influenza pathotypes are regulated both provincially and federally.

The designed immunosensor can be modified to detect H5 and H7 influenza pathotypes by changing the biorecognition element of the sensor. In the case of HPAI H5 viruses, an anti-H5 antibody would be used as the biorecognition element, which would enable the detection of H5 viruses, in theory. Moreover, work using H5 virion surface proteins was completed in this thesis, thus indicating that detection of H5 viruses is possible for the future. In the case of HPAI H7 viruses, an anti-H7 antibody would be used as the biorecognition element, which would
theoretically enable the detection of H7 viruses. As H7 virion proteins were not studied in this thesis, it is expected that work involving H7 detection will require some optimization of the sensing mechanism. The scientific importance of this future line of research is invaluable when it comes to disease detection and outbreak control.

The work outlined above would be carried out in a Level 3 biohazard lab (National Animal Health Laboratory) and would follow the immunosensor design, optimization, and testing protocol described within this thesis, save changing the target antibodies.

6.2 Point-of-Care Device Development

In this thesis, calibration curves were developed for H5 protein, H4N6 virus, and H9N2 virus using a lab-based immunosensor. The calibration curves could be used to develop a hand-held virus detection kit, which in turn, could be used by farmers or medical personnel to detect avian influenza on-site. This would involve three main components: bio-printed electrodes that house immobilized target antibodies (depending on the target virus); a reagent kit containing 1X PBS buffer and the bioconjugated reporter molecule (CdTe in this case); and a palm-sized potentiostat electrode reader.

6.2.1 Bioprinting of Antibodies

The principle of bioprinting is printing biological molecules or cells (the ink) onto a substrate or 3D matrix in a relatively rapid fashion. In this case, the substrate would be a screen-printed carbon electrode that has been pre-coated with a graphene gold nanocomposite scaffold. Bioprinting will reduce the electrode manufacturing time while also providing a more consistent layer of immobilized antibodies. By using a series of bioprinters, it will be easier to manufacture
different lines (i.e. target antibodies) simultaneously in a fraction of the time that it would take to manually drop-cast onto electrodes. Bioprinting of electrodes can be conducted by a commercial supplier, which is suggested, as they provide less electrode-to-electrode variation.

6.2.2 Reagent and Reporter Kit

The point-of-care detection kit would come with all reagents necessary to conduct testing. This includes 1X PBS buffer, bioconjugated reporter molecules (CdTe – Ab), a push-button safety lancet (for obtaining a blood sample), a 10 µL pipette, and a 100 µL pipette. The kit will come with a detailed protocol that will be adapted from the testing protocol outlined in this thesis.

6.2.3 Hand-Held Potentiostat – Electrode Reader

A potentiostat electrode reader can be developed using the calibration curves presented in this thesis work. The Bionano laboratory has a business contact at DropSens, Spain, which can commercially manufacture a calibrated potentiostat using the calibration data for each virus. Each virus would require its own calibration card, all of which could be swapped in and out of the main potentiostat reader. The potentiostat would operate in a fashion like that of a blood glucometer, save the peroxidase mimic detection mechanism. A theoretical schematic the field-deployable device is shown in Fig. 15.
Figure 15: Schematic of how a point-of-care electrochemical Immunosensing device would look and function. Blood is taken from a chicken using a lancet and is deposited onto the bio-printed electrode, which contains virus-specific antibodies. The reporter molecules are then added. After 10 minutes of incubation, the blood is shaken off and 1X PBS is applied to the working electrode surface. Finally, the electrode is inserted into the potentiostat reader. The result is displayed to the user via a LCD screen output. In this schematic, the subject in question is infected with the target avian influenza virus.
7.0 REFERENCES


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8.0 SCIENTIFIC CONTRIBUTIONS AND DISTINCTIONS

8.1 Conferences


8.2 Publications


8.3 Professional Workshops

Microfluidics Professional Workshop, University of Toronto (2016)

8.4 Awards and Distinctions Obtained

CEPS Dean’s Scholarship (2018) for academic standing

Best Oral Presentation Award (2017) QC – ON Biotechnology Annual Meeting, Université du Québec à Rimouski.