IMMUNOCHROMATOGRAPHY ASSAYS TO DIAGNOSE TROPICAL VIRAL PATHOGENS USING GOLD NANOPARTICLES

by

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Submitted to the Department of Mechanical Engineering in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Mechanical Engineering at the MASSACHUSETTS INSTITUTE OF TECHNOLOGY
June 2017

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Report

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Submitted to the Department of Mechanical Engineering, on May 22\textsuperscript{nd}, 2017 in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Mechanical Engineering

ABSTRACT

Immunochromatography assays are ideal candidates for diagnosis of disease in remote areas, due to their low cost and rapid readout. Moreover, they can be stored at relatively high temperatures, and do not require electric power, specialized personnel, equipment or reagents. We use those devices to diagnose viral mosquito-borne tropical diseases that have caused major epidemics and hospitalization in the last years. By allowing mobile phone readability of the diagnosis results, we enable real-time epidemiologic data on the spread of the disease.

Immunochromatography assays use capillary flow and the accumulation of ligand-coated nanoparticles to detect the presence of target proteins. We build multiplexed diagnostics that allow the detection of the four serotypes of Dengue and Zika, and validate the performance of these diagnostics by using patient samples from endemic areas from the Americas and India. Moreover, we build a multiplexed diagnostic that can detect Dengue, Zika and Chikungunya by using a low volume of patient sample.

In order to provide a rapid response to epidemics, lateral flow immunoassays need to be rapidly tested and manufactured. However, years of research are necessary to identify, screen and test disease-specific antibody pairs. To provide a faster response to outbreaks, we explore cross-reactive antibodies developed against a related pathogen. To avoid nonspecific signal from the related pathogens, gold nanoparticles of different colors are combined with cross-reactive antibodies of different affinities and used in order to distinguish between the two infections as well as co-infections. In this context, I present an Ebola and Marburg diagnostic and a Dengue and Zika diagnostic.

Limit of detection, as well as sensitivity/specificity are critical issues in the development of rapid diagnostics; these parameters are dependent on the nature of the ligand-target pair and binding thermodynamics when attached on a surface. In this thesis, I explore strategies to increase the sensitivity and specificity of the lateral flow devices. These new, effective, fast, reliable and inexpensive lateral flow devices represent significant improvements to field detection of disease and real-time epidemiology in situations where the lack of specialized personnel, reagents or materials challenge the suitability of the standard diagnosis methods.

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ACKNOWLEDGEMENTS

The work presented in this thesis has been possible with the help of many people. Many faculty, fellow students, friends and family have greatly contributed to my experience. First of all, I would like to thank my advisor, Prof. Lee Gehrke, for his guidance and advice during the years I have spent in his research group at MIT. The time and knowledge he has shared with me has a priceless value, as it has helped me grow both as a researcher and personally. It has been great to learn from a mentor that is welcoming and committed to promoting a good working atmosphere as well as the interests of all the members of the group. I would also like to thank him for the opportunity that he gave me to T.A. in his makerlab class. Teaching alongside him, José and Anna was a very fun and enriching experience. I learnt a lot from both the instructors and the students.

I would also like to thank Prof. Kimberly Hamad-Schifferli, my mentor since I first entered MIT as a visiting student and my M.S. thesis advisor. She has been a catalyst when providing me with knowledge and advice that have been invaluable for shaping this thesis, and that will also accompany me in my future endeavors. Moreover, I would also like to thank her for such kind and balanced character that encouraged me to persevere in research, as well as in achieving my goals.

I am grateful to Prof. Kim Vandiver, not only for serving as the committee chair during my years in the Ph.D., but also for his enthusiasm for the research question, and for always keeping my mind on the next steps to take. Moreover, the opportunity of getting an R.A. with him during my first years in the Ph.D. was also key for me to be able to work on this project. I am also grateful to Prof. Rohit Karnik, for his kindness and his always insightful comments and feedback. I am very glad he was able to join as a committee member in this thesis.

Furthermore, I would like to thank the MechE department of MIT, especially our Academic Administrator, Leslie Regan for always being so knowledgable, friendly and involved when replying to my many questions.

I am very lucky for having shared this years with terrific labmates. Irene Bosch, and her unstoppable character, José Gómez and his capacity to think out of the box, J. Tam for always being a helping hand and support, Diana for her will to work, learn and ask questions, and my lab neighbor, Chunwan. I would also like to thank the two people with
whom I have simultaneously shared the Ph.D. Ann Durbin and John Lian. It has also been
great to interact with Ale, Elena and Jane-Jane in the lab, as well as in our weekly
meetings and many lunch times. I am also thankful for the members of the Hamad-
Schifferli group with who I shared part of this experience: Anna and Paula (“¡felices
domingos de laboratorio!”), as well as Maria and Cristina. I would finally like to thank Marc Carré, for his capacity to transfer his enthusiasm in research.
I would as well like to thank my parents, family and friends, who have greatly contributed
to making this experience even more enjoyable, my extended catalan family in Boston:
Marcel, Jose, Jaume, Biel, Maria, Cris, Fer, Dunjis(x3) and Andreu. My fellow MechE
friends, especially Poorya and Federico. My friends in the Spain@MIT group, especially
Enrique, Maite, Noel and Ferran. And my roomates, especially Sagi, Nuria, Eider, Margot,
Alicia, Gemma, Sara and Ferry. Also, Majo, in particular, for making sure I was eating
while doing this thesis; and finally, Guillermo, for our good times, and for inspiring me to
achieve a bit more every day.
Finally, I would like to acknowledge the financial support from SUTD-MIT, MIT-TATA
center, Broschy fellowship, La Caixa and Rafael del Pino. Being part of each of them has
made this work possible, and it also has allowed me to access a network of fellows –
many of whom are good friends nowadays- with similar interests to mine. In particular, I
would like to thank MIT-TATA center, in particular, Prof. Robert Stoner and Prof. Chintan
Vaishnav, for their guidance and for enabling my trips to India and thus test the devices
presented in this thesis in the field, which has been very valuable for informing the
feasibility of my studies. Also, I thank research collaborators, Rajas Warke, Prof. Michael
Diamond, Prof. Sean Whelan, as well as the Center for Materials Science and
Engineering (CMSE) and the Biophysical Instrumentation Facility (BIF), for the use of
their materials and equipment.
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INTRODUCTION

MOTIVATION

In the currently ongoing outbreak, Zika (ZIKV) is co-circulating with Dengue virus (DENV) and Chikungunya (CHKV), which also share the same vector, the mosquitoes Aedes aegypti and Aedes albopictus. These diseases are major international health concerns because their dissemination has increased greatly in urban areas due to travel and globalization. The WHO estimates that there are 3.2 billion people at risk of at least one of the three diseases, and between 50-150 million and 0.5-6 million annual infections of Dengue and Chikungunya, respectively. According to the Brazilian Ministry of Health, in only four months of 2015, there were in Brazil alone between 0.4-1.3 million Zika cases, and the worldwide burden of Zika is still unknown. The three diseases are difficult to distinguish based on the patient's symptoms (Table 1), causing difficulties in disease diagnosis. Infection by any of the three diseases leads to similar influenza-like symptoms, such as fever and pain in both muscles and joints. Moreover, the incubation periods are similar, and there is a high percentage of asymptomatic patients, which further complicates the prediction of epidemics.

Table 1: Symptoms of Dengue, Zika and Chikungunya

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Zika</th>
<th>Dengue</th>
<th>Chikungunya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fever</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Rash</td>
<td>+++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Conjunctivitis</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Articulargia</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Myalgia</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Headache</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Mouth/nose bleeding</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>~% asymptomatic</td>
<td>80</td>
<td>75</td>
<td>3-28</td>
</tr>
<tr>
<td>Incubation period</td>
<td>2-7 days</td>
<td>5-7 days</td>
<td>3-7 days</td>
</tr>
</tbody>
</table>

However, the outcomes of each of the three diseases are different (Table 2). Dengue can cause hemorrhagic fever and shock that can be life threatening, Chikungunya can cause disabling joint pain and arthritis, and Zika can cause microcephaly and Guillain Barre syndrome. Although there is no treatment and no widely disseminated vaccines for any of the diseases, specific diagnostics are needed in order to identify the pathogen in order
to define an appropriate course of clinical treatment, for prediction and alert public health officials of the threat of epidemics, as well as to assess the efficacy of vaccination campaigns.

Table 2: Potential clinical complications of Dengue, Zika and Chikungunya

<table>
<thead>
<tr>
<th>Clinical complications</th>
<th>Zika</th>
<th>Dengue</th>
<th>Chikungunya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemorrhagic fever</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Shock</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Guillain-Barré</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Microcephaly</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Disabling joint pain</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Arthritis</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

The currently used diagnosis methods for Dengue, Zika and Chikungunya include thermal cycling amplification (PCR) and enzyme-linked immunoabsorbent assays (ELISA). However, these methods require specialized equipment, reagents, and highly trained personnel, have a complex methodology, and a slow turnaround for readout, challenging their suitability in the field. Immunochromatography assays, also known as lateral flow tests, on the other hand, are simple, easy to use, low-cost diagnostic tools that provide fast detection of antibodies or antigens. One issue when developing lateral flow immunoassays for outbreaks is that their time-to-manufacturing can be quite long, often requiring several years of research, due to the needs to validate the diagnostic as well as the requirement to produce disease-specific antibodies. The development delays are compounded by the limitations of disease surveillance for early warning of circulating pathogens, due to the lack of adequate diagnostics, the differences between reporting methods in different countries, as well as poor communication between public health officials and healthcare centers. The end result is a delay in response to public health threats. Better methods to detect and report disease outbreaks are urgently needed, in order to allow for improved detection and early response to prevent or limit epidemics. Therefore, a method to rapidly develop diagnostics that can be readily deployed in the field is needed. Moreover, a device to diagnose disease caused by the four serotypes of Dengue, as well as Zika and Chikungunya in one easy to use, low cost, specific and sensitive diagnostic would have a significant impact on the millions of individuals who are infected by, or at risk for infection by viruses. In addition, real-time mapping of the disease
spread could provide advance warning to diminish the effects of an advancing outbreak, thereby improving the health of many patients.

OBJECTIVES AND STRATEGIES

This work centers on the goal of building machine-readable multiplexed lateral flow immunochromatography devices to detect several tropical disease markers. If readable by a mobile phone, the device could provide real-time epidemiologic data to monitor disease distribution based on diagnostic results (Fig. 1). Immunochromatography devices rely on the specific binding of antibody-conjugated nanoparticles with a disease marker—antigen—, and a second antibody immobilized on a nitrocellulose porous membrane. A design challenge is to ensure that the gold nanoparticle conjugates and the antibodies used to detect the disease biomarkers have sufficient sensitivity to detect the pathogen even at low target protein concentrations, such as in early stages of the disease.

The first objective of the thesis is to build an immunochromatography assay that can identify the four serotypes of Dengue, as well as co-circulating viruses, such as Zika and Chikungunya. This objective has been achieved by two different strategies. First, serotype-specific Dengue antibodies as well as Zika antibodies have been generated and tested in lateral flow devices, which have been further validated with patient serum samples from endemic areas. Moreover, a code to analyze the test has been written in ImageJ to allow objective, automated analysis of the lateral flow tests (chapter 3). Second, a triplex test for Dengue, Zika and Chikungunya has been built and tested (chapter 4).
The second objective of this thesis is to reduce the time to manufacturing of new lateral flow devices, in order to provide a faster response to emerging pathogens. In chapter 5, we explore a methodology that relies on the use of cross-reactive antibodies originally generated against one antigen that can be repurposed in order to detect a closely-related antigen from a different viral pathogen. This is possible through the use of nanoparticles of different colors, together with monoclonal antibodies exhibiting differential affinities for each of the pathogens. In this context, three different tests are presented as examples of how cross-reactive antibodies and multicolored nanoparticles can be exploited in immunoassays: an Ebola/Marburg diagnostic using antibodies raised in response to a Marburg virus antigen; a diagnostic for the four serotypes of Dengue using antibodies raised in response to a Dengue serotype 3 virus antigen immunization, and a Dengue/Zika diagnostic built using antibodies raised in response to Dengue virus antigen. In order to further reduce the response time to emerging threats, I use the same approach to convert a commercially available Dengue diagnostics into a multiplexed, specific test for both Dengue and Zika.

The third objective of the thesis is to improve the detection limit, and in turn, the sensitivity and specificity of the immunochromatography assays. In chapter 6 we explore the use of different nanoparticles and conjugation strategies that achieve lower limits of detection in lateral flow devices. Through these efforts, it is possible to decrease the device cost, by decreasing the quantities of antibodies and nanoparticles required. Achieving a low cost is essential for delivering the rapid tests to individuals who are in great need of diagnostics, but lack resources for expensive tests.

BACKGROUND

MOSQUITO-BORNE VIRUSES: DENGUE ZIKA AND CHIKUNGUNYA

Dengue, Zika and Chikungunya have many similarities in clinical symptoms, epidemiology and transmission routes by the Aedes mosquitoes. Their main transmission vectors, Aedes Aegypti and Aedes albopictus thrive in tropical and subtropical regions, and the viruses are mainly transmitted between humans and the vector. It is believed that the three diseases originally evolved in Africa of Asia from zoonotic transmissions between mosquitoes and nonhuman primates, with humans as
secondary hosts\textsuperscript{18}. The viruses spread to new regions as the mosquitoes were transmitted to new areas through human traffic and globalization. The mosquito probably first adapted to the Americas and the Caribbean during Atlantic slave trade from the 15\textsuperscript{th} to the 19\textsuperscript{th} centuries and spread through Asia and Southern Europe through commercial trade in the 18\textsuperscript{th} and 19\textsuperscript{th} centuries\textsuperscript{20}. During the last 50-60 years the expansion of global trade and globalization have increased the opportunities for the Aedes mosquitoes to expand geographically\textsuperscript{1} (Fig. 2).

![Figure 2: Aedes mosquito spread worldwide](image)

**HISTORICAL ASPECTS AND CURRENT SITUATION**

**Dengue**

Dengue is an old disease. The first reports of major epidemics possibly caused by Dengue date back to 1779-1780, although reports of disease suspected of being Dengue date from much earlier\textsuperscript{2}. After late 1970s, Dengue was characterized by large epidemics in intervals of 10-40 years. The first known epidemic of Dengue hemorrhagic fever dates back to after World War II, in 1953, in Manila, Phillipines, and by 1970 it had spread throughout Southeast Asia\textsuperscript{18}. Nowadays, there are more than 2.5 billion people at risk of Dengue in more than 100 countries suffering from endemic Dengue transmission\textsuperscript{21}. There are 390 million estimated Dengue infections yearly, with hemorrhagic fever as the outcome in 1\% of the infected individuals\textsuperscript{22}. The burden of Dengue in endemic regions is around 1300 disability-adjusted life years (DALYs) per million population\textsuperscript{19}.

Dengue fever is caused by infection by any of the four Dengue Virus serotypes (DENV-1, DENV-2, DENV-3, DENV-4), and a fifth Dengue serotype has been isolated in Borneo\textsuperscript{23}. The four Dengue viruses originated in sylvatic transmission cycles between monkeys and mosquitoes and independently started infecting humans in Africa or Southeast Asia between 100 and 800 years ago\textsuperscript{20}. Dengue remained a geographically
constrained disease until mid 20th century. Currently, travel and globalization contribute to the spread of the disease. The four Dengue serotypes were initially isolated between 1940-1960\(^2\), infection by one of the Dengue serotypes provides life-long immunity against that particular serotype, however, secondary heterologous infections can increase the risk of Dengue hemorrhagic fever and Dengue shock syndrome.

**CHIKUNGUNYA**

Chikungunya, similarly to Dengue, also exists in sylvatic forest cycles between mosquitoes, nonhuman primates and other small mammals in Africa\(^3\)\(^4\). Further spread of the virus beyond Africa probably occurred during the 18\(^{th}\) century. Chikungunya was first isolated around 1952 in an outbreak in Tanzania\(^25\), followed by outbreaks from 1954-1968 in the Philippines. 10 years after, CHIKV had already spread in Southeast Asia and the Indian Ocean Islands through large epidemics and then sporadic activity (probably due to herd immunity of the population). In the 2000s, Chikungunya caused massive outbreaks in Kenya, the Indian Ocean Islands and India, and from there it was transmitted, probably via air travel to dense populations previously not immune to the virus in Europe, the Americas and other parts of Africa.

**ZIKA**

Zika Virus was isolated in 1947 from a rhesus monkey in the Zika Forest in Uganda\(^26\). The first evidence of human infection was in 1952, and it was known to be active in Africa and Asia around 1970. However, it was considered a minor pathogen due to the mild symptoms that it caused. It is believed that Zika originated in East Africa around 1920. There is only one strain recognized, and only two main virus lineages, the African and the Asian. However, in 2007 Zika, from the Asian lineage, caused an outbreak in the Yap Island, of the Federated States of Micronesia, and spread to the Pacific Islands\(^27\). In 2013, an outbreak of Zika in French Polynesia caused autoimmune and neurological complications, including Guillain Barré syndrome, and by 2015-2016 Zika caused explosive outbreaks in the Caribbean, Brazil and the Americas, and has been declared a public health emergency by the WHO, due to the reported association with microcephaly in newborns of infected pregnant women in Brazil. By the end of 2015, the possible links between Zika and microcephaly were first reported by Brazilian authorities\(^28\)\(^29\). Between
2010-2015, the yearly average of microcephaly cases had been 163 (5.6 per 100000 live births); in 2015, there were 3530 cases of microcephaly (121.7 per 100000 births), 35% of the suspected Zika-caused microcephaly cases happened in Pernambuco, a Brazilian state that had a high burden of Zika\textsuperscript{28}. Zika RNA has been found in amniotic fluid of pregnant women, and in the placenta\textsuperscript{4}. It can also be sexually transmitted. Currently, Zika is widely spread in Latin America and is starting to spread in South East Asia.

**VIROLOGY**

**DENGUE AND ZIKA**

The four Dengue serotypes (DENV-1-DENV-4) and Zika belong to the genus Flavivirus, family Flaviviridae. There are approximately 70 known viruses in the Flavivirus genus, which include, among others, Dengue, Zika, Yellow Fever, West Nile Virus, Japanese Encephalitis, or Tick Borne Encephalitis. Flaviviruses are spherical enveloped, single stranded, positive sense RNA viruses with a diameter of 40-50nm. Their genome (Fig. 3) is 11000 bases long and encodes for three structural proteins (C, prM and E) that form the virus particle and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5).

![Flavivirus genome organization](image)

**CHIKUNGUNYA**

Chikungunya belongs to the genus Alphavirus, family Togaviridae. There are three known genotypes that cause the disease: Asian, East/Central/South African and West African. Unlike Dengue, previous infection with one genotype provides lifelong immunity against the other two genotypes. Alphaviruses are spherical enveloped positive-sense, single-stranded RNA viruses with a diameter of ~70nm. Their genome (Fig. 4) is 11000-12000 bases long and encodes for three main structural proteins (C, E1 and E2) that form the virus particle and four non-structural proteins (nsP1, nsP2, nsP3 and nsP4).
TRANSMISSION OF DENGUE, ZIKA AND CHIKUNGUNYA

The three diseases are transmitted to humans by the bite of an infective mosquito\textsuperscript{1}. The main vectors of Dengue, Zika and Chikungunya in urban settings are *Aedes Aegypti* and *Aedes albopictus*. *Aedes aegypti* is a highly-domesticated mosquito which prefers to lay eggs in human-made containers typically inside or around homes, such as flower vases, car tires or buckets to collect rainwater. The adult *Aedes aegypti* mosquitoes prefer to stay indoors, and tend to feed on humans rather than animals. They are day biters and have two peaks of biting activity: early in the morning or 2-3 hours before dark. *Aedes Aegypti* is a sneaky biter and may bite the same person or several people more than once during one blood meal, which makes it an efficient epidemic vector\textsuperscript{19,30}. *Aedes Albopictus*, on the other hand, is mostly an outdoor mosquito, and it bites humans as well as many other vertebrates. *Aedes Albopictus* can lay eggs in human-made containers, although it prefers tree-holes and bamboo internodes with water. After the female lays eggs, both *Aedes Aegypti* and *Aedes Albopictus* spend 7-9 days in the immature cycle, and can live around three weeks as adult mosquitoes. Vertical transmission (transovarial transmission from female mosquito to egg) of Dengue\textsuperscript{31} and Zika\textsuperscript{32} occur, and is possible for Chikungunya\textsuperscript{33,34}.

After a person is bitten by an infective mosquito, the virus is incubated during a period of 3-14 days, after which fever and other symptoms may appear. Typically, during this febrile phase, which lasts between 2-10 days, the viruses circulate in the infected patient's blood\textsuperscript{35}, and if an *Aedes Albopictus* or *Aedes Aegypti* bites the infected patient the mosquito may in turn become infected in the following 4-10 days and further transmit the disease to other people during the rest of the mosquito's life span. Nevertheless, in the case of Zika, other routes of transmission have been reported, such as perinatal infection\textsuperscript{36}, sexual transmission\textsuperscript{37}, blood transfusion\textsuperscript{38} and laboratory exposure.

The three diseases are also transmitted in a sylvatic cycle between primates and *Aedes* mosquitoes in rainforests in Africa, Asia and possibly the Americas. Although the sylvatic
versions of Dengue, Zika and Chikungunya can cause disease, and even hemorrhagic fever, in the case of Dengue, in humans\textsuperscript{39}, these viruses are typically confined to the forests\textsuperscript{20}. However, cross-species transmission from sylvatic viruses has led to the four Dengue serotypes, the Zika epidemic and the three Chikungunya genotypes that circulate today in human populations\textsuperscript{20}, and it is not impossible for a new serotype to emerge\textsuperscript{20}.

**CLINICAL DIAGNOSIS AND TREATMENT**

**DENGUE**

Dengue infections can cause a disease spectrum from mild to severe and fatal hemorrhagic disease. Dengue fever typically presents as a temperature rise to 102-105°F and fever during 2-7 days, together with nonspecific symptoms such as rash, retro-orbital pain, rash, nausea and vomiting and joint pains. The acute phase of the illness may last 2-7 days, but the convalescent phase can last for weeks\textsuperscript{2}.

Dengue hemorrhagic fever is characterized by a sudden onset of fever together with nonspecific symptoms, and is difficult to distinguish from Dengue Fever or other illnesses found in tropical areas until the fever remits. Between 24h before or after the temperature drops to normal the patients can show skin hemorrhages such as purpuric lesions, petechiae or ecchymoses. Severe disease can lead to hemorrhage in the gastrointestinal track and shock caused by plasma leakage. Blood tests typically show hemorrhagic manifestations such as low platelet count or hemoconcentration due to vascular leakage. Epidemiological evidence shows that the risk of Dengue Hemorrhagic fever is associated with a secondary infection from a different Dengue serotype than the one causing the initial infections, as was seen in past outbreaks\textsuperscript{19}. Antibody-dependent enhancement has been seen in both animal models and in vitro\textsuperscript{40-42}.

Management of DENV fever is done through symptomatic treatment, which is typically, nonsteroidal anti-inflammatory drugs for pain control. For severe DENV, blood and platelet transfusions may be needed, as well as fluid and electrolyte correction. For patients in shock, hemodialysis, treatment for heart failure or mechanical ventilation might be necessary, in a case by case basis.
CHIKUNGUNYA

Chikungunya presents as abrupt high fevers together with nonspecific symptoms, such as arthralgia, myalgia, chills, rash, vomiting, nausea and headaches, indistinguishable from Dengue. Severe polyarthralgia, which can be disabling are more characteristic of CHIKV than DENV, and has a positive predictive value of 80% for CHIKV viremia, even in Dengue endemic areas. The acute phase of the illness may last 1-12 weeks, most patients improve 1-2 weeks after the onset of symptoms, but a high proportion have arthralgia from several months to years. Chikungunya is not considered a neurotropic virus, however, neurological complications that required hospitalization were reported in the large outbreaks in India and in the Indian Ocean islands. In adults, Guillain Barré Syndrome, enchelopathy, seizures, encephalitis and encephalomyelitis. Infected neonates with mother to child transmission in utero could suffer from central nervous system complications such as white matter lesions or cerebral hemorrhage. Only symptomatic treatment is available for CHIKV. Acute and chronic arthralgia are typically treated with nonsteroidal anti-inflammatory agents.

ZIKA

Zika can be asymptomatic in 80% of patients, and the clinical symptoms may be mild, mainly fever, rash arthralgia, myalgia and conjunctivitis. In 2015, Zika was linked to Guillain Barré syndrome in adults and to microcephaly of newborns. Infections linked to microcephaly would typically occur during the first trimester of pregnancy. Microcephaly can lead to seizures, developmental delays, learning disabilities and impaired motor function. Only supportive care, nonsteroidal anti-inflammatory drugs can be used to treat Zika. For pregnant women, ultrasounds every 3-4 weeks are recommended for early diagnosis of microcephaly as well as other abnormalities, which could be detected after 18-20 weeks of gestation. Neurological monitoring of children should follow during infancy.
Laboratory Diagnosis of Dengue, Zika and Chikungunya

In order to diagnose pathogens, the typical strategy is to either diagnose based on the presence of the pathogen in the patient, or based on the immune response of the patient against the pathogen (Fig. 5).

Figure 5: Scheme of typical methods for disease diagnosis

The three viruses can be isolated from diagnostic serum, blood, plasma or body tissues, by inoculation into mosquitos, cell culture in mosquito cell lines (C6/36) or mammalian cell lines (vero), or by intra-cerebral inoculation of mice. Serotype-specific monoclonal antibodies can be used for virus identification in immunofluorescent assays. The RNA of the viruses can be detected by nucleic acid amplification tests from patients during the acute phase of the disease. When performing PCR, primers against serotype-specific regions of the Dengue viruses can be targeted in order to distinguish the different serotypes.

Viral proteins can be detected in the bloodstream of infected patients during the acute phase of the disease. In the case of Dengue, the nonstructural protein 1, NS1, has raised much interest as it is synthesized by all flaviviruses and is secreted from infected mammalian cells. It can be found in high concentrations in during both primary and secondary infections, and can be detected from the first day of fever up to 9 days after the onset of symptoms. NS1 antigen detection can be done in an ELISA format, in immunohistochemistry of tissue sections or lateral-flow rapid tests. In the case of CHIKV, antigen capture ELISA has been used to detect the virus after 2 days of illness, and rapid tests are under development.

The patient’s humoral response after infection can also be used as a diagnostic tool, however, because these methods rely on the patient’s immune response, the diagnostic can only be done some days after the acute phase of the disease, after the patient has
started developing an immune response against the virus. The acquired immune response against infection from any of the three viruses leads to the production of antibodies (IgG, IgA an IgM), mainly against the envelope E protein of the virus. However, the immune responses against each virus might be specific for each disease.

In the case of Dengue, the relative IgG/IgM responses vary between the primary and secondary infections. Typically, a patient will have higher IgM titre during the primary infection\(^\text{11}\). It is difficult to identify the Dengue serotypes through IgG/IgM assays, due to the cross-reactive nature of the humoral response, that can be nonspecific for the four Dengue serotypes, Zika\(^5^4, 5^5\), and also other flaviviruses\(^5^6\). Dengue IgM can be detected in 50% of hospitalized patients after 3-5 days after the onset of fever and in 93% of patients after 6-10 days. IgM can circulate during up roughly 3 months\(^2\). IgG, on the other hand, is indicative of a past Dengue infection. To diagnose a current infection, paired sera needs to be tested to prove seroconversion (changes in antibody titres between acute and convalescent samples). Although IgG and IgM can be used for Dengue diagnosis, they can be cross-reactive with Zika or other flaviviruses and may not be indicative of a Dengue-specific response.

In the case of Zika, IgG and IgM serological methods are difficult to interpret due to the cross-reactivity with other flaviviruses\(^7\). In order to attempt identifying the primary flavivirus infection, Plaque-reduction neutralization tests can be performed to measure virus-specific neutralizing antibodies, however, the technique is time consuming, laborious, and the results might be difficult to interpret.

In the case of Chikungunya, ELISA and rapid tests can be used to detect CHIKV-specific IgG or IgM antibodies. ELISA and lateral flow tests can be used to detect IgM antibodies against CHIKV can be detected 2-7 days after the onset of symptoms, and can persist in serum for up to 3-4 months. IgG antibodies, on the other hand, can be detected 5-7 days after the onset of symptoms and can last many years.

**Prevention and control**

Prevention of Dengue, Zika and Chikungunya can be achieved by both effective vector control and vaccination. Mosquito control would provide the largest benefit against the three diseases. Currently, mosquito control combines population behavioral education to reduce mosquito bites-such as the use of protective clothing, screened doors and
windows and the use of insect repellent with DEET-,

together with methods to manage

water containers in order to reduce the number of breeding sites near homes -such as emptying containers or the use of larvicides in collected ponds of water- or campaigns for

mosquito spraying in neighborhoods. These methods are effective in the short term; however, they are difficult to adhere over an indefinite period of time in communities, especially without a feedback on the efficacy of the methods. Other novel vector control measures include the release of genetically modified male mosquitoes that sterilize the circulating female population to reduce the egg output, or the introduction of Wolbachia, a nonpathogenic bacteria for humans, that can partially give mosquitoes resistance to

DENV, ZIKV and CHIKV infection.

**Dengue Vaccines**

Research to find vaccine candidates started shortly after Dengue was first isolated. Recently, a vaccine, Dengvaxia, by Sanofi Pasteur, against the four serotypes of Dengue has been licensed for individuals between 9-45 living in endemic areas. The vaccine is a 3-dose life recombinant tetravalent (against the 4 serotypes) administered on a 0/6/12 schedule. The Dengue scientific community is split between those in favor and against the vaccine. On one hand, data shows that the vaccine was efficacious after a 3-year assessment, and showed to reduce Dengue fever due to all four serotypes in one third of the participants. However, there was an unexplained increase of hospitalization in children younger than 9 years old. Many countries such as Mexico, El Salvador, Brazil of Philippines will introduce the WHO recommended Dengue vaccine, Dengvaxia, during 2015-2017. Other countries, such as India are unlikely to introduce the vaccine in the short term, claiming that there is limited data or clinical trials to assess the efficacy and safety of the vaccine.

There are more than 23 other vaccine candidates against Dengue in different stages of development and clinical trials based on a range of platforms (inactivated, live attenuated, live vectored, chimeric, virus-like particle, subunit protein, DNA) on phase 1 or 2 clinical trials.
ZIKA VACCINES
Research to find a ZIKV vaccine candidate is ongoing in preclinical trials with rhesus monkeys and mice with different platforms, such as purified inactivated virus or a DNA vaccine expressing a prM-Envelope immunogen.

CHIKUNGUNYA VACCINES
Although the is currently no commercial vaccine against CHIKV, more than 15 vaccine candidates based on a range of platforms (inactivated, live attenuated, live vectored, chimeric, virus-like particle, subunit protein, DNA) are on phase 1 or 2 clinical trials. The first Chikungunya vaccine candidate was developed by the U.S. Army and tested in 2003. It was a life attenuated Chikungunya virus strain isolated from a patient in Thailand via serial passage in MRC-5 cells. This vaccine was able to show seroconversion of 98% after 28 days, and neutralizing antibodies persisted in 85% of the patients after 1 year, however, the project was discontinued as research efforts were prioritized to counter bioterrorism.

Moreover, working with CHIKV poses greater difficulties than Dengue or Zika as it is a BSL-3 pathogen, and also because it causes sporadic, unexpected outbreaks, and thus it is difficult to plan phase 3 clinical trials.

FILOVIRUSES: EBOLA AND MARBURG
Ebola and Marburg are highly contagious and deadly viruses that persist in natural reservoir hosts, probably bats, in Africa. Initial transmission of the diseases may be through contact with infected animal's secretions or consumption of meat from intermediate hosts, such as nonhuman primates. After, human to human transmission is the main driver for the outbreak, from direct contact to infected patient’s fluids or body contact. Survivors of the diseases can also transmit the virus weeks or months after recovery.

HISTORICAL ASPECTS AND CURRENT SITUATION
Marburg was discovered in 1967, in Marburg, Germany when laboratory workers became infected when dissecting African green monkeys. The first reports of naturally acquired Marburg in Africa date between 1975-1985. After, large outbreaks appeared in Democratic Republic of Congo (1998-2000, with 83% fatality rate) and Angola (2004-2005, with 90% fatality rate).
Ebola was discovered in 1976 after causing a large outbreak in Democratic Republic of Congo and Sudan\textsuperscript{79}. From then, small outbreaks in remote villages in Central and West Africa occurred until the recent epidemic in Guinea, Liberia and Sierra Leone\textsuperscript{80}.

**Virology**

Marburg and Ebola belong to the Filoviridae family. They have a tubular shape of 80nm diameter and a length of 800-1000nm. They are single-stranded, negative sense enveloped RNA viruses. Their genome is \(~19000\) bases long and encodes for seven structural proteins (NP, VP30, VP35, L, VP24, VP40 and GP) that form the virus particle and one non-structural protein, in the case of Ebola (sGP).

**Clinical diagnosis, treatment and vaccines**

The clinical initial symptoms of MARV and EBOV are nonspecific and can be confused with other diseases, such as malaria, Dengue, Chikungunya, influenza or bacterial sepsis, which are also endemic to Central and West Africa\textsuperscript{81}. The incubation period of the diseases can range 2-20 days. First, the initial nonspecific phase of flu-like illness can last 1-4 days and can include symptoms such as high fever, myalgia, arthralgia, weakness, headache, nausea, vomiting and rash. Bleeding tendencies can occur during the peak of the illness, which can include petechiae, mucosal bleeding, bloody diarrhea, hematemesis and ecchymoses\textsuperscript{82}. Organ dysfunction is also common at this stage. The third phase, late organ/convalescent phase, typically starts at day 12-13 after the onset of fever and may result in death or a prolonged recovery period\textsuperscript{83}. There is no proven treatment other than symptomatic and supportive care through blood and platelet transfusions as needed, as well as fluid and electrolyte correction. Experimental therapies during the EBOV outbreak were convalescent blood or plasma transfusions from recovered patients\textsuperscript{84}, monoclonal antibodies (ZMapp and ZMab)\textsuperscript{85, 86}, lipid-bound siRNA (TKM)\textsuperscript{87} or antivirals (favipiravir)\textsuperscript{88, 89}, among others. Vaccine candidates for EBOV and MARV include glycoprotein-bearing vesicular stomatitis viruses (rVSV-ZEBOV)\textsuperscript{90}, however the efficacy of the vaccines is difficult to assess, as EBOV and MARV cause sporadic, unexpected outbreaks.
LABORATORY DIAGNOSIS
Diagnosis of Ebola and Marburg can be done by virus isolation, though it is complicated as it needs to be done in BL4 facilities. During outbreaks, mobile laboratories were used equipped with PCR, qRT-PCR or IgG, IgM testing by ELISA for screening. Moreover, rapid diagnostic tests can be used to detect Ebola or Marburg VP40 or GP in blood or body fluids.

LATERAL FLOW IMMUNOASSAYS
Lateral flow immunoassays were originally derived from latex agglutination assays and plate based immunoassays (radio-immunoassays and enzyme immunoassays). Lateral flow technology was first introduced in the market in 1980s, in the form of the human pregnancy test to detect hCG. Currently, lateral flow is a widespread technology with more than 200 companies developing diagnostics for diverse industries. Lateral flow is an ideal method for detecting disease in remote areas because the need for refrigerated storage can be obviated; moreover, specialized chemicals and expertise are not needed. Lateral flow assays can be used in both developed and developing countries in order to provide a fast diagnostic for pathogens where the delay of molecular diagnosis tools leads to incorrect treatment against a different pathogen than the one causing the disease, increasing drug-resistance, as well as costs. Moreover, rapid diagnostics have been used in developed countries as a first screening method, followed by a confirmatory test, and have shown to decrease hospital stays and medical costs.

Lateral flow tests use capillary action to move sample conjugates and reagents through a porous nitrocellulose membrane (Fig. 6). The conjugate pad contains the detection conjugate, which can be any species that gives rise to a color. Typically, this is spherical gold nanoparticles bound to an antibody or biomolecule that can specifically capture the test protein. Once the test solution is placed on the sample pad, it flows toward the conjugate pad and mixes with the detection conjugate. It then flows to the absorbent pad, passing through the nitrocellulose membrane, where the detection antibodies have been printed an antibody against the agent to be detected in the test line, a positive control and negative control. A visible line can be observed when gold nanoparticles accumulate in a specific region due to ligand-receptor binding. Therefore, if the test protein is present in the test solution, the gold nanoparticles will accumulate at both the positive control and
test lines. Lateral flow strips are widely used in point of care (POC) devices, such as pregnancy tests, disease diagnostics, etc. because they can be operated by non-experts, they are cheap, portable, and do not require electric power to be operated\textsuperscript{13}.

**Figure 6:** Scheme of a lateral flow immunoassay

**DIPSTICK IMMUNOASSAYS**

Immunoassays can be designed in a dipstick format, or vertical flow (Fig. 7), where the patient fluid is first mixed with the gold conjugate in a tube. After, the nitrocellulose is dipped into the container, and the mixture of the patient sample and conjugate flows in the nitrocellulose by capillarity, similarly to lateral flow assays. This method is useful in laboratory settings, as it allows for rapidly preparing and testing immunoassays. However, this methodology cannot be used with whole blood, as red blood cells could end up masking the signal of the gold nanoparticles. Therefore, the method requires the use of a centrifuge to separate the patient's blood into serum (for diagnosis) and red blood cells.

**Figure 7:** Scheme of a dipstick immunoassay
The central element of lateral flow devices and dipstick immunoassays is the gold nanoparticles that give rise to the colorimetric readout. The strong absorption of light by gold nanoparticles is due to the electron oscillations on their surface, which depends on the size and shape of the nanoparticles. Therefore, it is possible to design gold nanoparticles to have high absorption even at low concentrations.

**Gold nanoparticles**

In the Middle Ages artisans started using gold nanoparticles to give color to stained glasses in the cathedrals. This fact was not recognized until 1857, in M. Faraday's Bakerian Lecture on the “Experimental Relations of Gold (and other Metals) to Light,” when he observed that the red color of colloidal gold comes from the presence of aggregates of gold atoms. Nevertheless, colloidal chemistry did not receive a huge growth until the 20th century, due to the works of Oswald, Mie, Svedberg and Zsigmondy.

One important fact that was already noticed more than a 100 years ago is the increase in the percentage of surface atoms with decreasing particle size. For example, a cube of 1 cm edge, has a percentage of surface atoms of around 10^{-5}%. On the other hand, a cube of 10 nm edge, would have a 10% surface atoms; and in a 1 nm edge cube, each atom would be a surface atom. This is one of the reasons for exploiting the surfaces of colloids as sensors, and why one can expect very different physical and chemical properties in a nanoparticle than those expected in bulk materials.

Nanotechnology comprises the methods and techniques to study, design and fabricate devices that work in the nanometer scale (1-100 nm). Its interest has increased in the last decades, where more than 35 countries have developed programs in nanotechnology since 2000, and the number of published articles regarding nanotechnology has rapidly increased during the last years. One of the interests on nanotechnology is based on the fact that all biological systems have their first level of organization at the nanoscale; therefore, nanotechnology can lead to important improvements in interacting with biology, biotechnology, medicine and healthcare. Hence, a significant amount of research has been devoted to studying the interactions of many types of nanoparticles, including quantum dots, polymeric nanoparticles, carbon nanotubes or gold nanoparticles, with biological systems.
Gold nanoparticles are especially interesting for interacting with biological systems due to their surface chemistry, optical properties, and biocompatibility. They can be synthesized with different sizes and shapes, such as spherical, stars, shells, bones or rods—among others—resulting in diverse physical and chemical properties.

**THESIS OUTLINE**

This thesis consists of seven chapters. The first chapter, Introduction, gives a general overview of the problem that this thesis tries to assess, as well as gives some background on the diseases that have been targeted in this study and on lateral flow assays. Chapter 2 contains the methods used during the thesis, which include the synthesis and surface modification of gold nanoparticles as well as assembly of lateral flow tests and quantification of the results. Chapter 3 contains a diagnostic capable of distinguishing the four serotypes of Dengue as well as Zika that was developed in the context of this thesis. This diagnostic used 5 parallel membranes—one PAN and the others one for each serotype of Dengue—, this test was validated with patient samples. In chapter 4, a multiplexed device for Dengue, Zika and Chikungunya with low cross-reactivity is presented. In the next chapter, chapter 5, a method to use cross-reactive antibodies with different affinities for several biomarkers is introduced. This method exploits cross-reactive antibodies and shows four examples in which cross-reactive antibodies can be used in diagnostics: a Marburg/Ebola diagnostic, a diagnostic for the four serotypes of Dengue, a Zika and Dengue diagnostic and enhancing a commercially available Dengue diagnostic to detect Zika. Chapter 6 introduces key factors to optimize lateral flow immunoassays for diagnostics, and some key points on gold nanoparticles that can be used in the immunoassays. Finally, the conclusions chapter provides some general thoughts on the thesis, as well as some potential future directions.
MATERIALS AND METHODS

REAGENTS

Au chloride trihydrate was purchased from Sigma-Aldrich (CAS: 16961-25-4). Bis(sulphatophenyl)phenyl-phosphine dehydrate (BPS), was purchased from Aldrich (CAS:308103-66-4). N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid) (HEPES) was purchased from United States Biochemical Company (CAT: 16926), sodium periodate (CAS: 7790-28-5) was purchased from Sigma, dithiolalkanearomatic PEG6-NHNH₂ (CAS: 963115-54-7) was purchased from Sensopath Technologies. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was purchased from Sigma. Gold standard for ICP was purchased from Fluka (38168-100 ml). Sodium citrate tribasic was from Sigma Aldrich, tannic acid was purchased from Alfa Aesar and sodium carbonate was from Mallinckrodt. 5kD mPEG was from nanocs. Phosphate Buffer Saline (1x PBS, pH 7.4) was from Gibco (CAT: 10010-049). Innova gold nanoparticles, were purchased from Innova Biosciences. The surfactants cetyltrimethylammonium bromide and benzyldimethylhexadecylammonium chloride were from sigma Aldrich.

Nitrocellulose HiFlow Plus membranes, HF75 (HF07502), HF90 (HF09002), HF120 (HF12002), HF135 (HF13502), HF180 (HF18002) and HF240 (HF24002, currently discontinued) was from Millipore.

Fluorescent Goat anti-Mouse IgG (H+L) Secondary Antibody, DyLight 650 conjugate was purchased from Pierce. Fluorescent ssDNA thrombin binding aptamer (TBA) with the sequence 5’/5ThioMC6-D/-(T₁₅)-GGTTGAGTTAGTTGG/-36TAMSp/ 3’ was purchased from IDT Technologies. Goat anti-mouse IgG, Fc was purchased from Millipore (AQ127).

Zika NS1, as well as the four serotypes of Dengue NS1 native proteins were from Native Antigen. Bovine serum albumin (BSA) was from Sigma (CAT: A9418), and diluted in 1x PBS to prepare a 10mg/ml solution. Filtered human serum was obtained by filtering 1ml of human serum from Sigma Aldrich (H4522) through a 0.2 μm cellulose acetate syringe filter (Pall, Acrodisc 25 mm Syringe Filter, with 0.2 μm HT Tuffryn Membrane).

In the tests involving filtered human serum at different cut-off molecular weights, the human serum was filtered through Vivaspin 4 ml centrifuge filters: 1,000,000 MWCO, PES Membrane, 300,000 MWCO, PES Membrane, 50,000 MWCO, PES Membrane and
10,000 MWCO, PES Membrane for the 1 MDa, 300 kDa, 30 kDa and 10 kDa filtered human serums, respectively. After filtration, the nanodrop was used to measure the final concentration of protein in the serum. The final protein concentration was 38.6, 7.7, 3.2, 0.7 and 0.65 for the 0.2 µm, 1 MDa, 300 kDa, 50 kDa and 10 kDa, respectively.

GOLD NANOPARTICLES

SYNTHESIS

NANOSTAR SYNTHESIS

Synthesis of gold nanostars (NStar): NStars with different extinction spectra were synthesized by tuning the Au/HEPES ratio in solution\textsuperscript{113}. We tuned the concentration of HEPES from 28-140 mM, while keeping the Au concentration in solution constant. We mixed 200, 350, 500, 750 or 1000 µl of 140 mM HEPES with 800, 650, 500, 250 or 0 µl of 18 MΩ deionized water, followed by the addition of 16 µl of 25 mM HAuCl\textsubscript{4}·3H\textsubscript{2}O and further vortexing for the synthesis of NStar200, NStar350, NStar500, NStar750 and NStar1000, respectively. After vortexing, solutions sat undisturbed for 1 h, during which the NStars crystallized. Afterwards, ~ 0.5mg BPS was added for NStar stabilization, and the solution was vortexed and left undisturbed for 1 h. After this time, the NStars were ready to use in experiments. The NStars were separated from excess reagents by centrifugation at 10000 rcf for 15 min. The resulting NStar pellet was resuspended in 1 ml of 18 MΩ water.

NANOROD SYNTHESIS

One-step synthesis of gold nanorods (NR23nm and NR30nm): Gold nanorods (NRs) were synthesized following literature methods\textsuperscript{122}. In a typical synthesis, 2.4mL of 50mM gold chloride trihydrate (HAuCl\textsubscript{4}) were added to 189.6ml of 150mM cetyltrimethylammonium bromide (CTAB) solution mixed with 3ml of 100mM sodium chloride (NaCl), and the solution turned orange; for 2.4ml of 10mM silver nitrate (AgNO\textsubscript{3}) were added to the solution, followed by gentle mixing. Ascorbic acid was tuned to change the size of the NR: for the longer NR30nm, 2.4 ml of 100mM ascorbic acid (AA) were added; and for the shorter NR23nm, 4.2 ml of 750mM, ascorbic acid addition was followed by inversion until the solution turned colorless. Finally, 0.128 ml of 3.125mM sodium borohydride (NaBH\textsubscript{4}) was added. The solution sat on the bench undisturbed
overnight, during which time it turned reddish brown (NR30nm), or blue (NR23nm), indicating the presence of NRs.

**Seeded synthesis of longer gold nanorods (NR45nm and NB57nm):** Long calsupes (NR45nm) and gold nanobones (NB57nm) were synthesized by a binary-surfactant seed-mediated growth method. The co-surfactants were CTAB and benyldimethylhexadecylammonium chloride (BDAC). This synthesis method is typically divided in two steps: preparation of the gold seeds and the growth of the NC. The seed preparation step consisted in a rapid reduction of the gold ion in the presence of CTAB. In a typical protocol, 7.5ml of a 0.2M CTAB solution were mixed with 0.25ml of 0.001M H\textsubscript{2}AuCl\textsubscript{4} in a beaker. While the solution was vigorously stirred, 0.6ml of ice-cold 0.01M NaBH\textsubscript{4} was added and the solution turned brownish yellow. Vigorous stirring continued for 2 minutes. For the growth step of nanorods, in a typical protocol, 25ml of 0.001M H\textsubscript{2}AuCl\textsubscript{4} were added into a mixture of 10ml of 0.2M CTAB and 15ml of 0.3M BDAC in a glass bottle, and the solution turned orange. 1ml of 0.004M AgNO\textsubscript{3} were added to the solution, followed by gentle mixing until the solution turned colorless. 0.05ml of the seed solution was added to the growth solution. The solution sat undisturbed overnight and turned reddish-purple, and NR45nm were formed. In order to turn these long nanocapsules into nanobones, we added 1ml of 100mM AA to 54ml of synthesized NR45nm solution, and left the solution undisturbed overnight to obtain NB57nm.

**Nanosphere synthesis**

**Synthesis of Au NS:** NS were synthesized following the citrate reduction Turkevich protocol. In a typical protocol, NS8nm were synthesized by mixing 1ml of 25.4mM gold (III) chloride trihydrate with 79ml of milliQ water. The solution was heated and stirred until it reached 60°C. Then, 20ml of a mixture containing 6.8mM sodium citrate, 0.03mM tannic acid and 25mM sodium carbonate were added to the solution. Heating and stirring were continued for 15min, and the temperature was kept between 60°C-70°C. NS18nm and NS32nm were synthesized by adding 1 or 0.5ml, respectively of a 6.8mM sodium citrate solution to 50ml of 0.25mM gold (III) chloride, while the gold chloride solution was boiling. Samples were stirred and heated for 15min during which the gold crystals were formed. All samples were left to cool down to room temperature while stirring was continued. Once
they reached room temperature, ~0.5mg of BPS (bis(p-sulphatophenyl) phenylphosphine) was added to the particles, and mixing continued overnight.

**NANOPARTICLE CHARACTERIZATION**

**Characterization of the nanoparticles:** Optical characterization of the nanoparticles (NP) was performed with either Cary 100 UV Vis, or a Cary500i UV-Vis-NIR Dual-Beam Spectrophotometer from Agilent Technologies. Morphology of the nanoparticles was characterized with a FEI Tecnai G2 TEM at 120 kV, equipped with a single-tilt support that was used to tilt the samples ± 30° in order to observe their three-dimensional structure. ImageJ was used to process the images and measure the dimensions of the nanoparticles. In addition, a Zetasizer Nano ZS from Malvern Instruments was used to measure the hydrodynamic diameter ($D_h$) and the zeta potential ($\zeta$) of the NP. Agarose gel electrophoresis was used to confirm the antibody and mPEG binding on the NPs, in short, 1% agarose gels were prepared and NPs were loaded by mixing 8 µl of concentrated NPs with 4 µl of 50 % glycerol in MilliQ water.

Au ion concentrations were measured with the Activa-S ICP-AES from Horiba Jobin Yvon. 2 ml of the NStars were separated from unreacted gold in the solution by centrifugation at 10000 rcf, and resuspended in 1 ml of 18 MΩ deionized water. 150 µl of the purified Au nanoparticles were dissolved in 0.5 ml of aqua regia overnight, after which time they were diluted to 5 ml to produce a final concentration of 2% nitric acid. Au standards of 0, 2, 5, 10, and 20 ppm were prepared by diluting the 1 mg/ml Au standard from Fluka with 2 % nitric acid.

Ferguson Analysis and light scattering were used to calculate the hydrodynamic diameter and zeta potential ($\zeta$) of gold NStars which has been previously described by others.\textsuperscript{123, 124} For the Ferguson analysis, gels of 0.5, 1, 1.5, 2 and 4 % agarose were run at 12 Vcm\textsuperscript{-1}, and the mobility of the nanoparticles was measured with ImageJ. NPs were loaded by mixing 8 µl of concentrated NStars with 4 µl of 50 % glycerol in 18 MΩ water. Spherical gold NPs synthesized by citrate reduction were used as standards to calculate the hydrodynamic diameter of the gold NStars and their $\zeta$.

**Theoretical Methods:** DDA method with the DDSCAT package,\textsuperscript{125} freely available in NanoHub.org- by Draine and Flatau was used to simulate the NStar optical response. In short, TEM images of NStars were analyzed with ImageJ to obtain average dimensions.
of every NP synthesis. These values were used to create 3D models of the NStars in AutoCAD (Autodesk), and area and volume of the particles were calculated. The 3D models were meshed using Blender and exported as .obj files (Fig. 8). After, DDSCAT Convert, from Draine and Flatau was used to convert the .obj file into a collection of dipoles, which were used as input by DDSCAT to simulate the optical properties of the NStars. The medium surrounding the NStars was considered to be water with a refractive index of 1.33. Au dielectric values were obtained from Johnson and Christy\textsuperscript{126}.

![Figure 8: NStar 3D model in AutoCAD (left), meshed in Blender (middle), dipole collection in DDSCAT convert (right)]

**NANOPARTICLE BIOCONJUGATION**

**Bioconjugation of the NStars:** For covalent, directional conjugation, a heterobifunctional linker consisting of a hydrazide on one end and a dithiol on the other, linked by a short polyethylene glycol (PEG) chain (hydrazide dithiolalkanearomatic PEG6-NHNH\textsubscript{2}) was attached to the antibodies, this conjugation step of the linker to the antibody was not done for passive electrostatic conjugation. First, 300\textmu{l} of a 1mg/ml solution of antibody in 40 mM HEPES (pH 7.4) was mixed with to 30\textmu{l} of 100mM sodium periodate (NaIO\textsubscript{4}, Sigma) and allowed to react while agitating for 45 min at room temperature in the dark, thus oxidizing the carbohydrate moieties on the Fc region of the antibody to aldehydes. Then, 1.5ml of PBS and an excess of linker (2 \textmu{l} of 33.3 mg/ml in ethanol) were added to the oxidized antibodies and agitated for 30 min, during which time the aldehyde group of the antibodies bound to the hydrazide on the linker. To remove unreacted linker, antibodies were buffer-exchanged into PBS using a 10 kDa centrifugal filter column (Amicon) and resuspended to a 1mg/ml solution in PBS. Prior to antibody conjugation, the NPs were separated from excess reagents by centrifugation at 12000 rcf for 12 min. The resulting NP pellet was resuspended in 100 \textmu{l} of 40mM HEPES at pH 7.7 and 300 \textmu{l} of MilliQ water, followed by the addition of 10 \textmu{l} of
1 mg/ml of the modified antibody, vortexed, and further agitated overnight, to enable antibody binding to the NP. In order to avoid nonspecific binding on the NP, 10 μl of 0.1 mM mPEG was added, the solution was vortexed and further agitated for 20 min, to enable mPEG to passivate any bare gold surfaces. Finally, NPs were centrifuged for 12 min at 10000 rcf twice to separate excess reagents, and then used in the immunoassays. Fluorescence spectroscopy was used to quantify the coverage of antibodies bound per nanoparticle, by a supernatant-loss method\textsuperscript{113}.

Single-stranded DNA (ssDNA) aptamers were covalently bound to the NStars by Au-thiol covalent conjugation. In short, dithiol bonds in the terminal 5′ dithiol modifier of the ssDNA were reduced by mixing 5 μl of 100 mM TCEP at 4 °C with 5 μl of 100 μM ssDNA. The solution sat undisturbed for 1 h. In the meantime, a 20 μl pellet of NStars was prepared by centrifuging 1 ml of as prepared BPS-NStars at 10000 rcf for 15 min. The 20 μl NStar pellet was resuspended in 100 μl of 0.5x TBE and 4 μl of reduced ssDNA, vortexed and then concentrated by water evaporation in a Savant SpeedVac at room temperature for 4 h, when 20-30 μl of solution were remaining in the tubes. After concentration, samples were sonicated at room temperature in a water bath and left undisturbed overnight. To separate bound and unbound DNA, 5 μl of the NStar pellets were diluted in 195 μl of water and centrifuged at 14000 rcf for 30 min. The supernatant, which contained the unbound DNA, was collected and fluorescence spectroscopy was used to quantify the bound ssDNA on the Au NStars.

**Corona formation around nanoparticles:** A protein corona was formed around the antibody-conjugated Au NPs by mixing 20 μl of the NP pellet with 500 μl of filtered human serum (HS) vortexing and incubating the mixture during 1 h. After, the NP mixture was centrifuged for 12 min at 10000 rcf to separate excess serum proteins, the supernatant was discarded and the pellet was further washed three times by resuspending the NPs in 0.5 ml of PBS and centrifuging at 10000 rcf for 12 min, in order to minimize the presence of non-corona proteins in the NP pellet.

**Commercially available (Innova) NPs:** They were used in chapters 3, 4 and part of 5. 40 nm gold nanoparticles were purchased from Innova Bioscience. The serotype-specific DENV nanoparticle-antibody conjugates and the ZIKV nanoparticle antibody-conjugates were prepared according to manufacturer's instructions. For single antibody
conjugations, the antibody was first diluted to 0.1 mg/ml in the supplied dilution buffer. Next, 12 ul of diluted antibody was mixed with 42 ul reaction buffer. 45 ul of the mix was then used to resuspend the lyophilized gold nanoparticles (20 OD particles). The antibody-nanoparticle mix was incubated for 10 minutes at room temperature, followed by the addition of 5 ul of proprietary quencher solution to stop the coupling reaction.

**Antibodies**

**Production of Monoclonal Antibodies**

DENV and ZIKV anti-NS1 monoclonal antibodies were produced in mice under contract (Covance, Inc; Denver, PA), following an approved animal care protocol. BALB-c mice were immunized with purified recombinant DENV or ZIKV NS1 proteins that were expressed in mammalian cells (Native Antigen, UK) to facilitate protein folding and post-translational modifications. From each of the immunized groups, one seroconverted animal with high titers of antibodies recognizing a pool of the NS1 DENV antigens was used for cell fusion to generate hybridomas. Supernatants from cloned hybridomas were tested by ELISA to generate “fold over background” values that gave us an approximate idea of serotype specificity or DENV/ZIKV specificity (see Table S4). From the ~200 DENV hybridomas and 100 ZIKV hybridomas identified in the initial screening (Table S1), supernatants from 30 DENV clones and 16 ZIKV clones were used to stain virus-infected Vero cells, followed by analysis in flow cytometry. This step was included to ensure that the selected hybridomas produced antibodies that recognized native NS1 protein expressed by virus-infected cells. Approximately 15 DENV anti-NS1 hybridomas and 16 anti-ZIKV NS1 hybridomas were expanded in low IgG serum (Invitrogen) containing hybridoma cloning supplement (Roche). The expressed monoclonal antibodies were isotyped and purified by affinity chromatography on a protein L matrix (GE Healthcare). Purified antibodies were concentrated and buffer exchanged into PBS. Eleven purified DENV antibodies were then tested pairwise in immunochromatography tests, as were 10 ZIKV antibodies to identify pairs that exhibited high differential NS1 binding and low non-specific background interactions.

Anti-ZIKV monoclonal antibodies were generated using a modified immunization and B cell harvest protocol. Both a rapid lymph node approach (B cell isolation from lymph
nodes and cell fusion at 23 days after immunization) and the more traditional spleen B cell hybridoma method (B cell isolation from B cells and cell fusion at 105 days post-immunization) were used. To select antibodies with minimal potential for cross reactive DENV NS1 recognition, an enhanced screening approach was also applied. Approximately 100 anti-ZIKV NS1 hybridoma supernatants were screened by indirect ELISA against not only ZIKV NS1 protein, but also DENV serotypes 1-4 NS1 proteins, as well as a panel of flavivirus NS1 proteins including West Nile Virus, Usutu Virus, Tick Borne Encephalitis Virus, and Japanese Encephalitis virus. From the starting pool of about 100 anti-ZIKV mAbs, 16 anti-ZIKV were selected for flow cytometry analysis.

**Flow cytometry analysis:** To ensure that the antibodies expressed by hybridomas recognized native NS1 proteins, hybridoma supernatants were used as a source of anti-NS1 antibodies for immunostaining Vero cells that were infected by DENV or ZIKV. Vero cells (90% confluency) were infected with DENV or ZIKV at multiplicity of infection (MOI) of 1 and incubated for 24 hours. The cells (50,000 cells per well of a 96-well plate) were prepared for immunostaining by using the BD cytofix/cytoperm reagent, following the protocol provided by the manufacturer (BD Biosciences). Washed and fixed cells were incubated with 100 ul of primary mouse hybridoma media for 1 hour, washed and incubated for 1 hour with phycoerythrin (PE)-labeled anti-mouse secondary antibody. Immuno-reacted cells were analyzed using a Guava flow cytometer (Millipore). Live cells were gated and fluorescence was quantified using a positive control antibody anti-E flavivirus detecting Dengue 1-4 or Zika infected cells to assess infection levels.

**Preparation of antibodies for lateral flow assays:** After hybridoma cell cultures had produced the antibodies, they were harvested, centrifuged to separate any cells in solution, and concentrated using Millipore centrifugal units (30k Da MW). Protein L columns (GE Healthcare) were used to purify the kappa light chain mouse antibodies. After purification, the antibodies were buffer-exchanged into PBS, concentrated and stored at 4 °C. A NanoDrop 2000 UV-Vis Spectrophotometer at 280 nm was used to calculate the concentration of the purified antibody, and a TapeStation with a P200 ScreenTape from Agilent Technologies was used to confirm the purity of the monoclonal antibodies.
LATERAL FLOW ASSAYS AND HALF STRIPS

**Preparation of the nitrocellulose strips:** Nitrocellulose membrane (EDM Millipore HF18002XSS, chapter 3 and 4, or HF13502XSS chapter 5 and 6, or others as indicated) was cut into strips using a laser cutter (Universal Laser Systems; model VLS2.30; 30 watt) at 30% power and 90% speed. The strip pattern was designed in Adobe Illustrator. Strips were attached to a wick (GB003 Gel Blot Paper) with adhesive paper (MIBA-010 Backing Card, 0.020" thickness; DCN Diagnostics, Carlsbad CA). For the positive control area, 0.33 ul of anti-mouse Fc antibody (EDM Millipore AQ127) at 1 mg/ml was spotted on the control line. The anti-NS1 capture line on the nitrocellulose was generated by pipetting 0.33 ul of test band antibody (2 mg/ml, or 4mg/ml chapter 3) at the NS1 test area. Strips were air-dried and stored in a dessicator at room temperature before use.

**Running the assays:** Each immunochromatography strip was run in a separate microcentrifuge tube, and groups of tubes/strips were run together. In a typical experiment, the nitrocellulose was placed inside a test solution containing 4 μl of 50% w/v sucrose in water and 8μl of 1% v/v Tween 80 in PBS, either 1 μl of the homemade Ab-NP conjugates (chapter 6), 4μl of Innova NPs and additional 5μl of the Innova proprietary quencher (chapters 3 and 4) or a mixture of 1μl of homemade Ab-NP conjugates and 3μl of Innova NPs (chapter 5), and the analyte, which would typically be a patient sample, vero cell supernatant or purified NS1 (Dengue or Zika) or E2 (Chikungunya) proteins in filtered human serum (total volume of 30 μl), and the tests were run by letting the solution migrate through the strip via capillary action until the samples dried. The run time varied with the sample and humidity; it was always less than one hour and sometimes as short as 15 minutes. The strips were left to dry and then imaged for quantitative signal analysis. Strips were typically run in filtered human serum, although in chapter 6 some strips are run in phosphate buffered saline (PBS), or bovine serum albumin at 10 mg/ml in PBS, as indicated.

**Forming a protein corona around the nitrocellulose** (chapter 6 only): In order to form a protein corona around the nitrocellulose, filtered human serum was allowed to migrate through the nitrocellulose by capillary action followed by the migration of PBS through the
nitrocellulose, in order to wash the pores from unbound human serum proteins. After that, the tests were placed inside the test solutions containing NPs, sucrose, tween and the analyte solution, and allowed to run through capillary action until the samples dried.

PATIENT SERUM SAMPLES

Each of the collaborating laboratories contributed a panel sample size of 6-20 de-identified retrospective serum samples. MIT IRB and local IRBs approved the protocols for purposes of pathogen identification. Lanciotti PCR primers and probes were utilized to define DENV serotypes. The serum panels used for the retrospective rapid test analysis of DENV and ZIKV acute sera were stored at each of the collaborating laboratories at -80°C until use. To use the serum samples in immunochromatography, the serum was first passed through a Spin-X Centrifuge tube filter with 0.2 um Nylon filter (Costar, catalog #8169) to remove any cryo-aggregates. For ZIKV detection, 150 ul of cleared filtered serum was then 5X concentrated using a centrifugal filter (Amicon ultra 0.5 ml 30K, Catalog # UFC503096) that was centrifuged for 10 minutes at 13000 rpm using in an Eppendorf tube centrifuge. Primers and probe sequences for ZIKV were provided by CDC, Puerto Rico; 1086f ZIKV 5’CCG CTG CCC AAC ACA AG 3’; 1162 ZIKV 5’ CCA CTA ACG TTC TTT TGC AGA CAT 3’; 1107-FAM 5’ FAM AGC CTA CCT TGA CAA GCA GTC AGA CAC TCA A. If sufficient volume of the serum sample was available, it was analyzed using the laboratory ELISA as described above. A sample size of 30 ul of DENV serum, or 30 ul of 5X concentrated (from 150 microliters) ZIKV sample serum or urine, was used for the rapid tests.

IMAGE ANALYSIS OF THE STRIPS

Automated image analysis of the nitrocellulose strips (chapter 3): Rapid test results were analyzed using image processing software to machine-read and quantify test results. After completion of the test run, the strips were taped to a sheet of paper with a printed red box outline that was slightly larger than the strip length. Within the red box, short black vertical lines served as fiduciary markers for image processing, separating the strips and identifying top/bottom. The image of the strip inside the red box was captured with a mobile phone camera and analyzed using an ImageJ macro to quantify test results.
The ImageJ macro provided instructions to localize and scan the positive control area and test area in each test. Briefly, a Workflow (DeskConnect, Inc.) script was written to run on the mobile phone, with instructions to GPS-localize where the image was captured, followed by uploading to Dropbox. A Python script running on a desk computer was activated upon Dropbox sync, calling ImageJ to perform the image analysis on the uploaded file. An ImageJ macro identified the red square and black line fiduciary markers, followed by drawing a rectangle in between the black markers, on the strips. ImageJ quantified the signal at the test area and blank/background area, and generated a normalized intensity, calculated by dividing the maximum intensity value at the test band by the average value of a blank area in each test strip. Test data were stored and used later for sensitivity/specificity and ROC curve analysis.

**Statistical analysis** (Chapter 3): Origin (OriginLab Corporation) was used to calculate and graph the box and whiskers plots shown in chapter 3, where the black X represent the maximum and minimum measured normalized intensity values, the black □ represents the mean value and the box represented the 25-75% range of the data. Individual colored points represented individual patient samples measured. Unpaired, two-tailed T-tests were performed to test for statistical significance. For the serotype-specific dipsticks, we tested for statistical significance between the “expected positive” serotype-specific dipstick and each of “expected negatives” serotype-specific dipsticks. No statistically significant difference was observed between the “expected negatives” serotype-specific dipsticks. For the PAN DENV and ZIKV tests, we tested for statistical significance between the PCR confirmed positive tests and the PCR confirmed negative tests.

**Limits of detection** (chapter 3): Limits of detection for each of the antibody pairs were measured using recombinant NS1 proteins antigens. Antigen solutions were diluted serially and chromatographed on DENV 1-4 dipsticks, the pan-Dengue dipstick (P), or the ZIKV dipstick (Z). The signal intensities were quantified (ImageJ), normalized by the intensity at the highest concentration, and plotted against antigen concentration and fit to a sigmoidal curve. The limit of detection was calculated from the sigmoidal curve fit as the NS1 concentration found at the intersection with a line representing the value of 5-times the standard deviation of background signal intensity. Background was determined
by analyzing triplicate strips in nitrocellulose areas outside of the test and positive control regions.

**Receiver Operator Curve (ROC) Analysis** (ROC curves, chapter 3): The individual rapid tests (DENV 1-4; pan-DENV; ZIKV) were validated using PCR- and/or ELISA- confirmed serum samples containing variable NS1 protein levels, resulting in normalized test signal intensities (test signal intensity/background signal intensity) ranging from 0.5 to 5. Based on these data, MATLAB was used to plot and calculate ROC curves, as well as to find the optimal cutoff values for each rapid test type (DENV 1-4; pan-DENV; ZIKV). In practical use, rapid tests with normalized intensity values greater than the cutoff are defined as positive, while those with normalized intensity values less than the cutoff are negative. To generate the optimal cutoff values and ROC curves, ImageJ was used to scan each of the test strips, yielding a signal intensity for each test area and also for background in an area of the strip outside of the test and control areas. These normalized intensity values were inputs to a MATLAB script. The script used theoretical cutoffs between 0.5 to 5, and calculated both the sensitivity (number of true positives divided by total confirmed positive values) and specificity (number of true negatives divided by the total confirmed negatives) at each of the theoretical cutoffs. After calculating the sensitivity and specificity at each theoretical cutoff, the optimal cutoff value was defined at the highest sum of sensitivity and specificity. In this way, the optimal cutoff value reflected the optimal sensitivity and specificity performance of the test. To calculate the confidence intervals, the ImageJ intensity measurements were fitted with a generalized linear regression model with binomial distribution and a link logit function, using MATLAB’s fitglm function. MATLAB’s perfcurve function with a bootstrap of 1000 was used to evaluate the linear regression model and calculate the 95% confidence intervals of the areas under the curve, sensitivity and specificity. The optimum cutoffs obtained for patient samples were 1.14, 1.18, 1.2, 1.37, 1.19 and 1.08 for lanes 1, 2, 3, 4, DENV PAN and ZIKV, respectively.

**Image analysis of the nitrocellulose strips** (chapters 4, 5 and 6): Once the tests had been dried, images of the finished tests were scanned and quantified with ImageJ\textsuperscript{128}. For the limit of detection analysis, gray values of the detection areas were obtained by subtracting the grayscale values from the detection areas from the grayscale values of
the background, and normalized following: $gray_n = \frac{gray-gray_0}{gray_{max}-gray_0}$. Where $gray_0$ is the measured gray value of the blank, $gray_{max}$ is the gray value of the highest concentration point (at saturation), and $gray$ is the gray value at each concentration. After normalization, gray values were plotted and fitted in a Langmuir equation of the form: $gray_n = \frac{[\text{antigen}]}{K_{D}^{eff} + [\text{antigen}]}$. Where $[\text{antigen}]$ is the concentration of antigen present in the 30 µl of sample in the solution, and $K_{D}^{eff}$ represents the effective binding constant in a Langmuir-like system. The limit of detection was measured as the concentration of antigen capable of showing a signal at 5-times the value of the standard deviation of the blank.

**Image analysis of the nitrocellulose strips** (chapter 5): RGB information was extracted during ImageJ analysis by splitting the images of the strips into their red, green and blue components. Matlab was used to train a Linear Dimensional Analysis (LDA) with six predictor variables (red, blue and green intensities of two test bands in each assay) to distinguish the clusters (for example, the Dengue and Zika test had three clusters: Dengue, Zika and the mixtures of Dengue and Zika at different ratios of NS1). Confusion matrices were plotted by comparing the expected results from LDA (True class), versus the LDA results (Predicted class). The resulting confusion matrix gave an idea of the accuracy of the predictor. Values in the diagonal represented correctly-classified tests, while values out of diagonal represented incorrectly-classified results. The classifier accuracy was always over 80%, typically around 95%. Principal component analysis was done in Matlab and used to plot the results from six to two dimensions to help visualizing the separate clusters.
RAPID DIAGNOSTICS TO DETECT AND DISTINGUISH DENGUE VIRUS AND ZIKA VIRUS INFECTIONS

ABSTRACT

The recent Zika virus (ZIKV) outbreak demonstrates that cost-effective clinical diagnostics are urgently needed to detect and distinguish viral infections to improve patient care. Unlike Dengue virus (DENV), ZIKV infections during pregnancy correlate with severe defects, including microcephaly and neurological disorders. Because ZIKV and DENV are related flaviviruses, their homologous proteins and nucleic acids can cause cross reactions and false results in molecular, antigenic, and serologic diagnostics. Here, monoclonal antibody pairs are characterized and further translated into rapid immunochromatography tests to specifically detect the viral nonstructural 1 (NS1) protein antigen and distinguish the four DENV serotypes and ZIKV without cross reaction. To complement visual test analysis and remove user subjectivity in reading test results, image processing and data analysis were used for data capture and test result quantification, generating standardized objective data. Using a 30µl patient serum sample, the sensitivity and specificity values of the DENV serotypes 1-4 tests and the pan DENV test (detects all four Dengue serotypes) ranged from 0.76 to 1.00. Similarly, sensitivity/specificity for the ZIKV rapid test was 0.81/0.86 using a 150µl serum input. These new approaches and reagents have immediate application in differential clinical diagnosis of acute ZIKV and DENV cases; moreover, the approach platform can be applied toward developing rapid antigen diagnostics for emerging viruses.

INTRODUCTION

Dengue (DENV) and Zika (ZIKV) are transmitted by genus *Aedes* mosquitoes; they are related flaviviruses and important global pathogens that bear significant burden on public health systems. Dengue has a wide distribution, with more than a quarter of the world’s population at risk, and hundreds of millions of new infections annually. There are four immunologically distinct types of Dengue viruses (DENV serotypes), and infection with one of the four DENV serotypes fails to provide long-lasting immunity against the remaining three viral serotypes. Some reports suggest that disease severity varies
among DENV serotypes. ZIKV gained global attention in 2015 when thousands of clinical cases appeared suddenly in northern Brazil, with accompanying reports of devastating congenital defects, including microcephaly and Guillain-Barre syndrome. The World Health Organization subsequently declared ZIKV a global public health emergency. Cost-effective diagnostics are urgently needed to detect and distinguish DENV and ZIKV, as well as other pathogenic viruses. The flavivirus NS1 protein is a useful infection marker because of its release from infected cells into the bloodstream and its accumulation in Dengue patients at concentrations up to 50 µg/ml.

A number of commercial DENV NS1 rapid tests have been reported and compared. However, these studies were performed prior to the ZIKV epidemic, and a recent publication confirms DENV-ZIKV NS1 cross-reactivity using a commercial rapid test. In addition, none of the commercial rapid tests distinguishes the DENV serotypes. Combining NS1 and IgM/IgG in a dual test improves sensitivity and specificity for DENV compared to NS1 alone. However, the primary antigen for IgG and IgM during flavivirus infections is the viral envelope (E) protein, and the similarity of the flavivirus envelope proteins fuels additional concerns about cross reactivity and false positive test results. There are currently no approved or widely used vaccines to prevent ZIKV or DENV infections, nor low-cost rapid antigen-based diagnostics demonstrated to identify ZIKV infections without cross reactive interference of related Dengue viruses. In this chapter, I describe viral NS1 antigen-based rapid tests that are founded on the identification and characterization of monoclonal antibody pairs that detect and distinguish the four DENV serotypes, as well as ZIKV, without cross reactivity.

**BASIC PRINCIPLE OF THE ASSAY**

Immunochromatography assays (IA) rely on ligand-target binding to provide a fast detection of antigens. In a typical IA, nanoparticle-conjugates mixed with a patient sample are put in contact with a porous nitrocellulose membrane which has antibodies specific for the biomarker immobilized on the test area. If the antigen of interest is in the sample, it binds to the antibodies on the NPs while the NP-antibody/antigen complex migrates though the nitrocellulose through capillary flow. The complex accumulates at the test area via binding of the antigen to the immobilized antibodies, forming a sandwich.
immunoassay (Fig. 9). This results in a visible dot or band at the test area. The positive control area on a test strip has antibodies that can bind to the crystallizable fragment (Fc) of the antibodies on the NP, indicating that sample and nanoparticle flow through the nitrocellulose was successful and that the data are therefore interpretable. If the antigen is present in the test solution, the captured gold NPs will accumulate in both the positive control and the test areas, indicating a positive result (Fig. 9 right, lane 2). On the other hand, if the antigen is not present in the test solution, the gold nanoparticles will accumulate only in the positive control, and no signal would appear in the test band (Fig. 9 right, lane 1).

![Diagram of lateral flow assay](image)

*Figure 9: Scheme of a lateral flow assay. Negative (1) and positive (2) test*

**SEROTYPE-SPECIFIC DENGUE DIAGNOSTICS**

Because of the strong similarity between the four serotypes of Dengue (Table 3), strategic screening was necessary to identify monoclonal antibodies that would be able to detect and distinguish the four serotypes of the disease. On a first attempt, we purchased commercially available antibodies from different vendors; moreover, we tested antibodies generated under contract to the monoclonal antibody core at the Dana-Farber Cancer Institute (DFCI; Boston, MA). The DFCI antibodies were generated using a recombinant Dengue NS1 protein antigen that was expressed in bacteria. Proteins expressed in bacteria often form inclusion bodies that must be solubilized with urea or other agents; therefore, and the solubilized protein may not fold properly to recapitulate the native protein conformation. The analysis of the DFCI antibodies (data not shown) revealed that there was no detectable recognition of NS1 protein that was released by virus-infected mammalian cells. The second approach was to use anti-NS1 antibodies that were
generated by infecting mice with Dengue virus. These antibodies were generated in the lab of Dr. Michael Diamond, from Washington University School of Medicine in St. Louis, after intraperitoneal infection of alpha/beta interferon receptor-deficient C57BL/6 mice with $10^5$ PFU of a mixture of two DENV-2 South East Asia strains (16681 and NGC)\textsuperscript{140}. One additional antibody, (9NS1), also from Prof. Michael Diamond lab, was generated by injecting mice with purified insect-cell generated, recombinant West Nile Virus NS1 (5μg injection). This antibody (9NS1) was cross-reactive with NS1 of all the flaviviruses that were tested in the lab\textsuperscript{141,142}. Taken together, the Diamond lab antibodies were useful in detecting NS1 from infected cells, as well as NS1 in patient samples. However, there was high cross-reactivity among the four serotypes, a high limit of detection, and only some strains of the viruses isolated from the americas could be detected with the antibody pairs. Therefore, we moved towards a third attempt of developing Dengue serotype-specific antibodies by injecting mice with the native form of the NS1 protein (native antigen). This third attempt resulted in the generation of highly specific Dengue antibodies that were able to detect Dengue with high sensitivity and specificity in both infected vero cell supernatants and patient samples. In the following paragraphs results from each of the three attempts to obtain serotype-specific antibodies will be discussed.

\textit{Table 3: Amino acid identity and homology between the four serotypes of Dengue and Zika}

<table>
<thead>
<tr>
<th>AMINO ACID IDENTITY</th>
<th>DENV1</th>
<th>DENV2</th>
<th>DENV3</th>
<th>DENV4</th>
<th>ZIKV</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV1</td>
<td>74</td>
<td>80</td>
<td>69</td>
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<td>85</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>ZIKV</td>
<td>73</td>
<td>72</td>
<td>73</td>
<td>72</td>
<td></td>
</tr>
</tbody>
</table>

DANA-FARBER ANTIBODIES AND COMMERCIAL ANTIBODIES
A total of nine NS1 antibodies was purchased from ViroStat (5162, 5163, 5164 and 5165), USBiological, Novabiotech, Abcam (ab41623 and ab41616) and Genetex (GTX103346). Although data from the companies showed that they were able to bind to denatured Dengue NS1 protein in western blots, it was not possible to obtain any binding in the immunochromatography assays developed in the lab by using supernatants from Dengue-
infected vero cells. These results suggested that that the antibodies would probably not bind to the native secreted form of NS1 that is found in patient samples.

A similar trend was observed with the antibodies generated at the DFCI Monoclonal Antibody facility. These antibodies were generated by injection of mice with DENV-1 NS1 expressed in bacteria (the resulting antibodies were 8H7G10, 7C5, 7C5E1, 8H12H11), or DENV-4 NS1 expressed in bacteria (the resulting antibody was F4-24). They were able to detect Dengue NS1 in Western Blots, FACS as well as recombinant Dengue NS1 that had been expressed in bacteria (Fig. 10 lane 3, rDENV-2). In particular, the pair of antibodies F4-24 and 8H7G10 was able to detect bacteria-expressed NS1 with high sensitivity, however, it could not detect the native NS1 protein from Dengue-infected Vero cell supernatants (Fig. 10, lane 1, sDENV-2). Interestingly, we observed that the antibodies produced with DFCI were only able to detect the native Dengue proteins when those had been previously denatured under acidic conditions, (Fig. 10, lane 2, sDENV-2a), indicating that the NS1 binding site of the antibodies was a peptide that was not available unless the NS1 protein was denatured. Because it was not practical to selectively denature only the NS1 proteins without denaturing the antibodies on the nanoparticles and nitrocellulose in the context of a rapid test, we moved towards testing antibodies that were specific for the eukaryotic cell produced, non-denatured form of the NS1 protein.

![Diagram](image)

*Figure 10: DFCI antibodies were able to detect the denatured NS1, but not the native protein.*

DIAMOND DENGUE ANTIBODIES

The commercially available antibodies, as well as the antibodies generated in DFCI were not good candidates for our serotype-specific diagnostic to distinguish native NS1
proteins, as we were interested in identifying pairs of antibodies that would be able to detect the native NS1 protein as found in patient serum. In order to overcome the obstacles presented by the antibodies that detected only the bacteria-expressed NS1, or the denatured form of the protein, but not the native secreted protein that would be found in patient samples, we tested a group of antibodies that had been generated through two protocols in Prof. Michael Diamond’s laboratory. In the first case, antibodies were generated through Dengue serotype-2 infection of alpha/beta interferon receptor-deficient C57BL/6 mice, instead of challenging the mice with only the purified protein. These infections resulted in the generation of several antibodies that primarily recognized the viral envelope protein, although a few antibodies that recognized nonstructural proteins were also obtained (of those, we tested DV2-1, DV2-2, DV2-4, DV2-5, DV2-9, DV2-15, DV2-19, DV2-23)\textsuperscript{140}. In the second approach another antibody, namely 9NS1 was tested. 9NS1 had been produced by challenging BALB/c mice with purified insect-cell generated, recombinant West Nile Virus NS1\textsuperscript{141}.

In order to determine if the antibodies were able to bind to NS1 released by infected vero cells into the cell culture supernatants, we tested them in pairs, by binding one antibody on the gold nanoparticles, spotting the other antibody on the nitrocellulose and testing each pair against vero cell supernatants that had been infected with one of the four Dengue serotypes (Scheme in Fig. 11, left). Testing throughput was increased by using the half-strip dipstick format (12), where dipsticks are run in in rapid format (approximately 20 minutes, depending on humidity conditions) by placing them in microcentrifuge tubes containing small volume suspensions of conjugated nanoparticles and sample without need for sample paper pads and conjugate paper pads that are characteristic of lateral flow chromatography. In order to define the serotype-specificity of the antibodies tested, we plotted their binding results in a matrix format (Table 4) in which strong binding to vero supernatants of either Dengue 1,2,3 or 4 infections or uninfected supernatants were tested. A dark green color represented a strong visual signal in the rapid test, a faint green color represented a weak signal, and gray indicated that there was no binding observed.
Out of the 10 antibodies that were tested initially, only 7 (namely, DV2-1, DV2-4, DV2-5, DV2-15, DV2-19, DV2-23 and 9NS1) were able to detect NS1 in pairs. Only 9NS1 was able to detect the four Dengue serotypes, while the others mainly detected DENV-1 and DENV-2 NS1 protein. In order to detect DENV-3, we used NPs from a commercial Dengue diagnostic (Dengue Duo, Standard Diagnostics, Alere). However, we observed that the SD nanoparticles had a strong cross-reactivity with NS1 of many flaviviruses. We assembled a serotype-specific Dengue diagnostic by using four different antibody pairs (Fig. 11, right). In lane 1, we conjugated antibody DV2-4 on the nanoparticles and spotted the antibody DV2-1 on the nitrocellulose, demonstrating that this pair was able to detect both NS1 from DENV-1 and DENV-2. In lane 2, we conjugated DV2-19 on the nanoparticles, and spotted DV2-1 on the nitrocellulose, which detected DENV-2 only. In
we spotted 9NS1 on the nitrocellulose and used the SD Kit NPs as the conjugate, representing a pair that recognized the four Dengue serotypes. Finally, in lane 4 we paired 9NS1 with itself, which detected DENV-2 and DENV-4. Therefore, a Dengue 1 sample yielded a positive signal in both lane 1 and 3, while a Dengue 2 sample generated positive signals in all four lanes. The Dengue 3 sample yielded a positive signal in the third lane, and finally, a Dengue 4 sample gave rise to positive signals in lanes 3 and 4 (Fig. 11, right). These results are evidence that building a serotype-specific Dengue diagnostic was possible.

This serotype-specific diagnostic was tested with a few sera from patients infected with Dengue, and we obtained high sensitivity and specificity. Sensitivity is a measure of the test's performance in identifying individuals who have disease (true positive), while specificity is a measure of the test's performance in identifying individuals who do not have disease (true negatives). For Dengue 1, the sensitivity was measured to be 75% and a 100% specificity, for Dengue 2 we obtained an 82% sensitivity and 100% specificity, for Dengue 3, an 83% sensitivity and 100% specificity and for Dengue 4 a 100% sensitivity and specificity. Although the values obtained for sensitivity and specificity seemed acceptable, the diagnostic suffered from major issues that led us to ultimately decide for changing the antibody pairs. (1) First, it was not possible to detect double infections, which was a major issue, as the four serotypes of Dengue currently co-circulate in many countries, and double and triple infections have been reported. (2) In the second place, some of the antibody pairs were strain-specific, which meant that a pair of antibodies that could detect DENV-1 from Venezuela or Colombia was not able to detect DENV-1 from
Brazil (Table 5). For example, the pair of antibodies 9NS1 on the nitrocellulose with DV2-1 on the nanoparticles detected only DENV-2 from Brazil, but was able to detect the four serotypes of Dengue from both Venezuela (Ven) and Colombia (Co), of different outbreaks. Moreover, the test was using two pairs of antibodies (SD-NPs with 9NS1, and the pair 9NS1 on both the NP and nitrocellulose) that were potentially cross-reactive with many flaviviruses. Finally, the limits of detection of the diagnostics, were high (150ng/ml, as measured with recombinant NS1 produced in mammalian cells from Native Antigen), and therefore only acute samples with high viremia were detectable. These results suggested that the group of antibodies tested did not have characteristics favorable for a high sensitivity, serotype-specific diagnostic device.

Table 5: Different virus strains could yield to different results with some antibody pairs

<table>
<thead>
<tr>
<th>TESTING ISOLATES OF ALL SEROTYPES FROM DIFFERENT LOCATIONS</th>
<th>9NS1 Isolate location</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOCK D1V D2V D3V D4V</td>
<td>Brz Ven Co</td>
</tr>
<tr>
<td>DV2-1</td>
<td></td>
</tr>
</tbody>
</table>

**GEHRKE LAB ANTIBODIES**

In order to overcome the aforementioned obstacles of a high limit of detection, the cross-reactivity among the serotypes and other flaviviruses and the differences between the native and the bacteria-expressed recombinant NS1 proteins, we generated anti-NS1 antibodies. We purchased recombinant NS1 protein of the four Dengue serotypes produced in mammalian cells from Native Antigen Company (UK). Because the protein is expressed in eukaryotic cells, it has secondary modifications, including glycosylations, and is secreted in soluble form from the cells in a form that mimic the native NS1 present in patient samples. Groups of mice were injected separately with DENV serotypes 1-4 recombinant NS1. B cells from the spleen were fused with mouse myeloma cells to generate hybridomas. We developed a step-wise strategy to screen the hybridomas: (1) Hybridoma screening (from a starting pool of 209 DENV hybridomas) was performed by the ELISA method, using individual recombinant Dengue NS1 proteins as capture antigens. An unrelated 6His-tagged protein was included as a negative control because the recombinant NS1 antigen from Native Antigen contained a His Tag for purification.
Further controls included a mixture of NS1 proteins from flaviviruses (Zika, Yellow Fever, Japanese Encephalitis, West Nile Virus and Tick Borne Encephalitis). By screening the hybridoma supernatants against individual DENV serotypes 1-4 NS1 proteins, the relative ELISA values provided an initial evaluation of differential binding properties for each antibody. Because of the similarities among the DENV NS1 proteins (Table 3), each group of mice immunized with a single purified recombinant NS1 protein yielded a pool of both serotype-specific and cross-reactive antibodies. In the second step, hybridoma supernatants were used to stain permeabilized Vero cells that had been infected with known DENV viral serotypes, followed by flow cytometry. Flow cytometry was used to demonstrate that the monoclonal antibodies recognized native NS1 protein expressed by virus-infected cells, and also to provide a quantitative analysis of cross reactive binding when the antibodies were used to stain different Dengue serotypes. From these experiments, 11 DENV mAb recognized NS1 protein present in the virus-infected cells without significant cross reaction; therefore, the hybridomas were expanded in cell culture, and the supernatants containing secreted monoclonal antibodies were collected. The mAb isotype was defined in preparation for affinity chromatography purification using Protein A or Protein L columns (GE Healthcare Life Sciences).

Finally, the purified antibodies were tested in immunochromatography pairs, with one antibody conjugated to gold nanoparticles, and one antibody adsorbed to nitrocellulose membrane. The 11 DENV hybridomas were tested in a matrix for interactions with DENV serotype 1-4 NS1, ZIKV NS1, or without added antigen as negative controls. The number of DENV combinations tested was therefore \((11 \times 11 \times 6 = 726)\).
Table 6: Binding antibody pair matrix (Gehrke Lab Dengue antibodies)

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<thead>
<tr>
<th>NANOPARTICLES</th>
<th>323</th>
<th>136</th>
<th>271</th>
<th>243</th>
<th>912</th>
<th>29</th>
<th>900</th>
<th>626</th>
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The immunochromatography results defined eight DENV mAb that were ultimately incorporated into the rapid tests used to analyze patient samples. The complete serotype-specific immunoassay consisted of five lanes (Fig. 12). The first lane (Fig. 12, 1) detected only DENV-1 and had nanoparticles conjugated to the antibody 912, paired with the antibody 271 spotted on the nitrocellulose. The second lane (Fig. 12, 2) detected only DENV-2, it contained nanoparticles conjugated with 243, with the antibody 1 on the nitrocellulose. The third lane (Fig. 12, 3) detected DENV-3 only, and it contained nanoparticles conjugated to the antibody 411, and the antibody 55 on the nitrocellulose. The fourth lane (Fig. 12, 4), specific for DENV-4, used nanoparticles conjugated to the antibody 626, and with the antibody 55 on the nitrocellulose. Finally, the fifth lane (Fig. 12, P) was a Dengue PAN lane that was able to detect the four Dengue serotypes. This one contained the antibody 323 on the nitrocellulose, paired with a mixture of the antibodies 243, 626, 271 and 411 on the nanoparticles. In addition, we observed that that the 912 and 243 antibodies displayed excellent single serotype specificity for the DENV-1 and DENV-2 NS1 proteins (Table 6). These antibodies also detected DENV-1 and DENV-2 as 912/912 and 243/243 homo-pairs, respectively. However, experimental results showed that the limits of NS1 detection could be improved by using hetero-pairs without sacrificing specificity; therefore, 912 was paired with 271, and 243 with 1. The 411, 55, and 626 antibodies did not display the single serotype specificity observed with 912 and 243 (Table 6); however, when used as pairs in the rapid tests, 411/55 (DENV-3) and 626/55 (DENV-4) showed excellent specificity. The 323 mAb recognized DENV serotypes 1-4; however, we found that when used as a 323/323 homo-pair, the detection of Dengue serotype 4 NS1 in the pan-Dengue test was not optimal. Therefore, by conjugating a mixture of mAb on the pan antibody nanoparticles (271, 243, 411, 626), we achieved improved limit of detection results in the pan-DENV dipstick test.
LIMITS OF DETECTION:

The limit of detection is a critical benchmark for a rapid diagnostic test, for it defines the lowest antigen concentration that can be detected, and is related to defining the earliest diagnosis time in a viral infection. We validated the rapid tests by chromatographing solutions of 500ng/ml of purified NS1 from the four Dengue serotypes, as well as other flaviviruses, such as Zika, West Nile Virus, Japanese Encephalitis, Tick Borne Encephalitis and Yellow Fever. No false positives were observed with any of the negative controls (data not shown). The limits of detection for the pan-Dengue, serotype-specific DENV, and ZIKV NS1 tests were defined by chromatographing dilutions of recombinant NS1 proteins, followed by quantifying the signal intensities, normalizing them to the plateau maximum (Fig. 13) and plotting sigmoidal data fits. The limits of detection were calculated as the NS1 concentrations intersecting a line representing a signal intensity point 5-fold greater than the standard deviation of the background signal. The results showed that the limits of detection for purified NS1 proteins in the serotype-specific lanes (Fig. 13, left) were 4ng/ml, 21ng/ml, 20ng/ml and 4ng/ml for the serotypes DENV-1,
DENV-2, DENV-3 and DENV-4, respectively, while for the PAN Dengue tests (Fig. 13, right) the LoD were 1ng/ml, 8ng/ml, 2ng/ml and 11ng/ml for NS1 of serotypes DENV-1, DENV-2, DENV-3 and DENV-4 respectively. These limits of DENV NS1 detection are far below the reported concentrations of DENV NS1 protein in serum (up to 50µg/ml)\textsuperscript{130}. Because the concentration of NS1 in the serum of patients changes during the course of the disease, having a low limit of detection ultimately means that the disease can be detected during more days of the infection.

**Figure 13:** Titration curves of the antibodies pairs with each Dengue serotype. Left, serotype-specific tests. Right, PAN tests

**VALIDATING THE IMMUNOASSAY WITH VERO CELL SUPERNATANTS**

As a validation step preceding patient sample analysis, the dipstick strips were tested in the laboratory by chromatographing native NS1 protein released by virus-infected Vero cells into cell culture supernatants (Fig. 14). Although the recombinant NS1 proteins used in Figs. 12-13 were expressed by eukaryotic cells to optimize antigen protein folding with secondary modifications such as glycosylations, testing the binding of native NS1 proteins released by virus-infected cells is a more robust proxy for analyzing clinical serum samples. Vero cells were infected individually with DENV serotypes 1-4, or with ZIKV (Asian/American strains as a negative control). The supernatants were chromatographed on the dipsticks. The data in Fig. 14, show the recognition of test dipsticks 1-4, as well as the pan-Dengue strip, with each of the DENV 1-4 Vero cell supernatants. These results are evidence that native NS1 protein expressed by DENV infected Vero cells yielded positive signals on the corresponding DENV 1-4 and pan-Dengue tests, and were free of detectable cross reactive binding (arrows indicate the positive tests). In summary, despite the high level of homology and identity among the flavivirus NS1 proteins (Table 3), our
screening strategy identified antibody pairs that detected and distinguished the four DENV serotype NS1 proteins, without detectable cross-reactive interactions.

![Image of immunoassays with vero cell supernatants]

**Figure 14: Validation of the immunoassays with vero cell supernatants**

In order to gain further insight on the sensitivity and specificity of our diagnostic when working with virus isolates in cell supernatants, we next tested Dengue-infected cell supernatants from cells infected with virus found in serum of infected patients (defined as “first pass patient samples”) or cells infected by Dengue viruses of the four serotypes using stocks amplified in the laboratory, to form a total of eight DENV-1, four DENV-2, three DENV-3 and four DENV-4 samples. Representative serotype-specific DENV detection, using Dengue virus isolates and first pass patient samples, is presented in Fig. 14. Each sample was tested using a panel consisting of five strips, with strips 1-4 recognizing DENV serotypes 1-4, respectively, while strip P is the pan-Dengue strip that recognizes all four serotype NS1 proteins. In each panel, strip P (pan-Dengue) confirms a DENV infection, while the signal in strips 1-4 defines the viral serotype. Visually, the specific serotype signals are clear, providing evidence that the serotype-specific tests detected and distinguished each of the DENV serotype NS1 proteins from the cell supernatants. However, to remove any subjectivity in reading and interpreting the results, I captured images of the test strips using a mobile phone camera, and performed automated image recognition and processing using ImageJ, a Java-based public domain program.
Figure 15: Representative DENV-4 picture that was analyzed with the automated ImageJ approach

As shown in Fig. 15, a red box and vertical black lines served as fiducial markers to create standard orthogonal targets for the image processing algorithm to bound the analysis area. Using these references, the software scanned each strip, detecting the control and NS1 test areas, as well as background levels, and quantified the signals. The DENV serotype specific test signals (Fig. 16) were quantified and data plotted for analysis. The data demonstrate that that each of the specific serotype signal signals was distinct from the other serotype signals.

Figure 16: Box and whiskers plots of the validation of the assay using supernatants from infected vero cells

Receiver Operator Characteristic (ROC) curves could be used to illustrate the performance of the rapid tests as a function of the discrimination threshold, and were plotted as (sensitivity vs 1-specificity). Sensitivity is a measure of the test's performance in identifying individuals who have disease (true positive), while specificity is a measure of the test's performance in identifying individuals who do not have disease (true negatives). Quantification by ImageJ allowed us to evaluate sensitivity and specificity as a function of an intensity cutoff value, above which a test was scored positive, and below which a test was considered negative. In practical terms, using a higher cutoff value decreases the sensitivity of the test, while increasing the test specificity. I plotted ROC curves as a function of the discrimination threshold as (sensitivity vs 1-specificity) in Fig.
17, while the numerical values for the tests are shown in table 7. The areas under the ROC curves are a proxy of test performance, where 1 represents a perfect test and 0.5 a random predictor. We measured areas of 0.98, 0.98, 0.95, 1 and 1 for DENV1, DENV2, DENV3, DENV4 and Pan DENV, respectively. Matlab was used to define the cutoff value for each test that maximized sensitivity and specificity.

![ROC curves showing the performance of the immunoassays with infected vero supernatants.](image)

The data presented in table 7 summarize the results of the analysis at the optimal cut off. 95% confidence intervals of the Area Under the curve (AUC), sensitivity and specificity were calculated using a bootstrap technique with 1000 iterations. The calculated optimal cutoff values were 1.16, 1.18, 1.13, 2.1 and 1.19 for DENV1, DENV2, DENV3, DENV4 and pan-DENV, respectively. Sensitivity is defined as the number of measured positives divided by the total confirmed positives, and specificity is defined as the measured negatives divided by the total of confirmed negatives. Using the optimal cutoff value, the test sensitivity and specificity numbers are 1 (8/8) and 0.95 (35/37) for the DENV1 test; 1 (4/4) and 0.98 (44/45) for the DENV2 test; 1 (3/3) and 0.85 (33/39) for the DENV3 test; 1 (4/4) and 1 (44/46) for the DENV4 test; 1(12/12) and 1 (11/11) for the pan-DENV test.

**Table 7: Summary of results of the immunoassay's performance with vero cell infected supernatants**

<table>
<thead>
<tr>
<th></th>
<th>D1V</th>
<th>D2V</th>
<th>D3V</th>
<th>D4V</th>
<th>PAN</th>
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<tr>
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<td>0.98</td>
<td>0.95</td>
<td>1</td>
<td>1</td>
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<tr>
<td><strong>95% CONF. INT.</strong></td>
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<td>[0.91-1]</td>
<td>[0.73-1]</td>
<td>[1-1]</td>
<td>[1-1]</td>
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<tr>
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<td>1.18</td>
<td>1.13</td>
<td>2.1</td>
<td>1.19</td>
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<tr>
<td><strong># NEGATIVE</strong></td>
<td>37</td>
<td>45</td>
<td>39</td>
<td>46</td>
<td>11</td>
</tr>
</tbody>
</table>
VALIDATING THE IMMUNOASSAYS WITH CLINICAL SERUM SAMPLES

We next turned to detecting DENV NS1 in a retrospective study of clinical serum samples. Serum samples were tested in Brazil, Mexico, Colombia, Panama, Guatemala, and India (Fig. 18), following approved human subjects use protocols. The sera were banked frozen samples representing blood that was drawn from febrile patients during routine care.

Figure 18: Map showing the endemic areas where the tests were deployed to analyze human serum samples

Representative serotype-specific DENV detection, using clinical serum samples, is presented in Fig. 19. Each PCR-confirmed serum sample was tested using the defined antibody panel consisting of five strips, with strips 1-4 recognizing DENV serotypes 1-4, respectively, while strip P was the pan-Dengue strip that recognized all four serotype NS1 proteins. After running each patient sample, pictures were taken and the intensity of the strips were analyzed by ImageJ, using the same methodology as for the Vero supernatants that were used to validate the tests. The analysis demonstrates that that each of the specific serotype signal signals was statistically distinct from the other serotype signals. The statistical significance at p<0.05, p<0.01, and p<.001 are indicated as *, **, and *** respectively;
The data obtained from the analysis of patient serums were analyzed further to define sensitivity and specificity values. We used a ROC curve analysis (Fig. 20, top) to evaluate the sensitivity and specificity as a function of an intensity cutoff value, above which a test was scored positive, and below which a test was considered negative. The areas under the ROC curves were measured at 0.88, 0.95, 1, 0.98 and 0.95 for DENV1, DENV2, DENV3, DENV4 and Pan DENV, respectively. Matlab was used to define the cutoff that maximized sensitivity and specificity. The data presented in Fig. 20 (table, bottom) summarizes the results of the analysis at the optimal cut off. 95% confidence intervals of the Area Under the curve (AUC), sensitivity and specificity were calculated using a bootstrap technique with 1000 iterations. The calculated optimal cutoff values were 1.14, 1.18, 1.12, 1.37 and 1.9 for DENV1, DENV2, DENV3, DENV4 and pan-DENV, respectively. Sensitivity is defined as the number of measured positives divided by the total confirmed positives, and specificity is defined as the measured negatives divided by the total of confirmed negatives. Using the optimal cutoff value, the test sensitivity and specificity numbers are 0.76 (13/17) and 0.89 (33/37) for the DENV1 test; 0.89 (8/9) and 0.98 (44/45) for the DENV2 test; 1 (16/16) and 1 (39/39) for the DENV3 test; 1 (6/6) and 0.96 (44/46) for the DENV4 test; 0.88 (51/58) and 1 (11/11) for the pan-DENV test.
ZIKA-SPECIFIC DIAGNOSIS

In order to develop a Zika-specific diagnostic, we used, again, a strategic approach to generate, screen and identify monoclonal antibodies that would be able to detect Zika and distinguish it from other flaviviruses, and the four Dengue serotypes. Groups of mice were injected separately with ZIKV recombinant NS1. B cells from the spleen or lymph nodes were fused with mouse myeloma cells to generate hybridomas. Initial hybridoma screening from 104 ZIKV hybridomas was performed by ELISA using recombinant Zika NS1 protein as the antigen, and as negative control an unrelated 6His-tagged protein, and a mixture of NS1 from the four Dengue serotypes, as well as similar flaviviruses, such as Yellow Fever, Japanese Encephalitis, West Nile Virus and Tick Borne Encephalitis. We obtained both flavivirus cross-reactive antibodies, as well as ZIKV-specific antibodies. Using the ELISA results, 8 anti-ZIKV antibodies were selected and tested in flow cytometry, in order to confirm that the antibodies recognized native NS1 protein from infection of ZIKV on vero cells, and not merely to the recombinant protein. The 8 ZIKV hybridomas recognized the native form of NS1 were therefore expanded in cell culture. The monoclonal antibodies were purified from cell supernatants using protein A or protein L columns (GE healthcare) depending on the isotype of the monoclonal antibodies. The monoclonal ZIKV antibodies, as well as one Dengue antibody that cross-reacted with Zika NS1 (136, Table 8, red) were tested in a matrix format in pairs, by spotting one antibody
on the nitrocellulose and conjugating the other antibody on the gold nanoparticles (Table 8). Purified ZIKV NS1 was used as the antigen, and we ran negative controls with both a mixture of NS1 from the four Dengue serotypes, as well as uninfected human serum.

Table 8: Binding antibody pair matrix (Gehrke Lab Zika antibodies)

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The matrix-based screening showed that several of the ZIKV antibody pairs were cross-reactive with at least one of the DENV serotypes. We further tested the pairs (NP/nitrocellulose): 45/337, 45/110, 130/110, 130/45, 130/337, 130/437, 136/136 and 45/855. Of these, 136/136 had a high limit of detection (217ng/ml), which was expected, as 136 was a mAb originally developed against DENV-1, which was cross-reactive with ZIKV, and therefore was not used further in this chapter of the thesis. Interestingly, the antibody 855 was able to bind only to the Uganda strain of ZIKV (both the purified NS1 protein as well as infected vero supernatants infected with the Uganda Zika strain, but not to the current outbreak, and was therefore not used with patient samples. The other pairs showed a similar behavior in detecting NS1 from vero cell infected supernatants, therefore the pair that was chosen for the lateral flow immunoassay was 130 conjugated on the gold nanoparticles paired with 110 spotted on the nitrocellulose, as ELISA results...
showed that these two antibodies i) had lowest cross-reactivity with the other flaviviruses, ii) their signal in immunoassays was high and iii) they detected ZIKV from both contemporary ZIKV strains as well as and the Uganda ZIKV strain, which has been passaged more than 100 times by injection into mouse brains.

**Limits of detection:**

![Scheme of the ZIKV-specific diagnostic](image)

*Figure 21: Scheme of the ZIKV-specific diagnostic (left). The ZIKV diagnostic does not cross-react with other flaviviruses (middle), titration curve analysis of the ZIKV specific diagnostic (right)*

We tested the ZIKV antibody pair, 110 immobilized on the nitrocellulose membrane and 130 conjugated to the gold nanoparticles (Fig. 21, left) by running recombinant purified NS1 proteins at 500ng/ml (Fig. 21, middle). We observed that there was no signal was observed when the dipsticks were chromatographed with human serum containing NS1 protein from Yellow Fever virus (rYFV), West Nile Virus (rWNV), Japanese Encephalitis (rJEV), tick-borne encephalitis (rTBEV) or any of the Dengue serotypes (rDENV1, rDENV2, rDENV3, rDENV4). The only NS1 protein antigen that gave a signal was NS1 from Zika spiked into human serum (rZIKV, arrow), showing that the pair of antibodies was able to specifically detect ZIKV NS1, but not any of the potentially cross-reactive flavivirus species. We then tested dilutions of ZIKV NS1 spiked into human serum in order to calculate the limit of detection of the mAb pair, by quantifying the intensity of the signals in the test bands, normalizing to the plateau value and fitting a sigmoidal curve (Fig. 21, right). The limit of detection at 5-times higher than the standard deviation of the blank was 18ng/ml.
VALIDATING THE IMMUNOASSAY WITH VERO CELL SUPERNATANTS

Prior to testing the ZIKV assay with patient samples, the diagnostic was first tested in the laboratory by using supernatants Vero cells infected with Zika virus (Fig. 22, left). The supernatants contain native NS1 protein that is released from the virus-infected cells, thereby testing the potential for the device to detect the native form of the NS1 protein as expressed by infected eukaryotic cells. The first tests showed that the diagnostic was capable of detecting Zika from isolates both Uganda Vs ZIKV-U and Brazil Vs ZIKV-B, and did not exhibit any cross-reactivity with supernatants collected from Vero cells infected with any of the Dengue serotypes (Vs DENV1-4) or uninfected supernatants (Mock). We used imageJ to quantify the signals of the vero sups, and plotted them in a box-and-whiskers plot, where it was possible to observe that the signal from infected supernatants was greater than the signal from supernatants collected from uninfected Vero cells (Fig. 22, right).

Figure 22: Validation of the ZIKV diagnostic with vero cell infected supernatants or blank. Representative tests (left) and box and whiskers plot of the results (right)

Virus preparations that are amplified a single time by infecting cells grown in culture are referred to as “first passage viruses”. We tested first passage patient samples that were expanded by infecting Vero cells or C6/36 cells (Fig. 23, left contains, Zika samples as well as one Dengue 2 first pass sample). Alternatively, a “virus isolate” refers to a virus that has been amplified multiple times in the laboratory. Both first pass samples and virus isolates are used for the statistical analysis. We used a Receiver Operator Characteristic (ROC) curve to illustrate the performance of the rapid tests as a function of the discrimination threshold, and are plotted as (sensitivity vs 1-specificity) in Fig. 23, middle, while the numerical values for the tests are shown in Fig. 23, table, on the right. The areas under the ROC curves are a proxy of test performance, where 1 represents a perfect test
and 0.5 a random predictor. We measured an area of 0.96, with a 95% confidence interval of 0.82-1, as measured by a bootstrapping technique with 1000 iterations. The calculated optimal cutoff value was 1.34, where the sensitivity calculated was 0.92 (13/14) and the specificity was 0.89 (8/9).

![ ROC curve analysis](image)

**Figure 23:** ZIKV test was validated with vero cell infected supernatants from different outbreaks. Representative samples (left), ROC curve analysis (middle), table with summary of results from ROC curve analysis (right)

**Validating the Lateral Flow Immunoassays with Clinical Serum Samples**

We used clinical samples of febrile patients to assess the performance of the ZIKV diagnostic. Serum samples were collected from different countries and locations to ensure that the results would be representative (Fig. 18, map). The patient serum samples were first confirmed by either PCR or ELISA and then tested in the lateral flow immunoassays. Prior to running the patient samples in the immunochromatograpy assay, the samples were concentrated 5-fold by centrifugation, starting with a 150µl input and concentrating the serum until 30µl. After the tests ran, a picture was taken with a mobile phone camera and analyzed automatically in ImageJ with a script that scanned the strips and quantified both the signal from the test band and the background. The intensities obtained were plotted in a box and whiskers plot. It was possible to observe a significant difference between infected and uninfected samples, with a p value of 0.003 (Fig. 24, left).
Figure 24: ZIKV NS1 test was validated with human serum from endemic areas. Box and whiskers plot (left), ROC curve analysis (middle) and summary of results from ROC curve analysis (right)

The quantification by ImageJ allowed us to evaluate sensitivity and specificity as a function of an intensity cutoff value, above which a test was scored positive, and below which a test was considered negative. In practical terms, using a higher cutoff value decreases the sensitivity of the test, while increasing the test specificity. The generated ROC curve is presented in Fig. 24, center, while the numerical results from the ROC curve for the ZIKV test in patient samples are shown in Fig. 24, right. We measured an AUC of 0.82. Matlab was used to define the cutoff value for each test that maximized sensitivity and specificity. The calculated optimal cutoff value of 1.08, where the sensitivity was 0.81 (25/31) and the specificity was 0.86 (6/7).

The results presented here demonstrate that we have successfully screened, identified, and characterized monoclonal antibodies that detect and distinguish DENV NS1 antigen for serotypes 1-4, pan-Dengue, as well as ZIKV NS1, without detectable cross reactivity. We have also demonstrated that the DENV and ZIKV NS1 tests do not cross react with NS1 proteins from a number of other related flaviviruses (WNV, USV, TBE, YFV, JEV). The limits of detection of each of the antibody pairs have been defined using recombinant NS1 proteins, demonstrating that DENV NS1 can be detected using serotype-specific tests and pan-Dengue tests in the 1 ng-20 ng/ml range, while ZIKV NS1 can be detected at about 20 ng/ml. The strips have been validated using recombinant NS1 protein, NS1 present in the supernatants of virus-infected Vero cells or C6/36 cells, and NS1 protein present in clinical serum samples from several geographic areas in the Americas and India. ZIKV infections are characterized by low viremia\textsuperscript{143}, and detection of serum NS1 in ZIKV-infected patients required 5X concentration. The use of a mobile phone camera...
linked to ImageJ analysis of the rapid tests permits objective analysis of the test data, and increases test accuracy. Taken together, these results suggest that we have characterized NS1 antigen tests that have the important and novel characteristics of distinguishing the DENV serotypes and detecting ZIKV and DENV infections without cross reactivity, in anticipation of broader clinical applications.

**DISCUSSION**

Generally, within one hour or less from sample collection, rapid diagnostics provide critical information that informs patient care and assesses patient risks. Despite urgent needs, efficacious and inexpensive rapid tests are not available for many infectious diseases, including arboviruses that are the subject of this paper. Viral antigen-based tests provide significant patient benefits in geographic areas where molecular tests are not available, have very slow turnaround times, or are prohibitively expensive. Accurate pathogen identification is essential, a goal that is complicated when distinguishing closely related co-circulating viruses such as DENV and ZIKV, which have significantly different risk profiles. The similarity of clinical symptoms is a compounding problem in many cases, and also a justification for improved diagnostics that provide accurate data on which to base clinical decisions. DENV, ZIKV, and Chikungunya (CHIKV, an alphavirus) infections exhibit common symptoms including varying degrees of fever, headache, myalgia, arthralgia, nausea, and rash, with hemorrhagic fevers as outcomes of severe infections in about 1% of infected individuals\textsuperscript{22}. However, the ZIKV outbreak stunned the virology and medical communities with a distinct set of risks and severe outcomes not previously associated with flavivirus infections, including fetal microcephaly and Guillain-Barre syndrome. Because DENV and ZIKV have major impact on economically challenged countries in the tropics, our approach has been guided by the World Health Organization acronym “ASSURED” to describe the ideal characteristics of a diagnostic test that can be used at all levels of the healthcare system: affordable, sensitive, specific, user-friendly, rapid, equipment-free, and delivered to those who need them\textsuperscript{11, 144}.

This chapter describes DENV and ZIKV rapid tests that specifically detect the viral NS1 protein to identify and distinguish the four DENV serotypes and ZIKV without observed cross-reaction with ZIKV. These results are significant for several reasons. First, we have
identified antibody pairs that distinguish closely related NS1 proteins, and applied them to create rapid tests. Success in detecting/distinguishing the individual serotypes was dependent on a) immunizing groups of mice with the native dimer/hexamer forms of recombinant NS1 that was expressed by eukaryotic cells, b) initiating hybridoma screening against multiple NS1 proteins in parallel, enabling us to begin sorting and selecting from the most diverse pool, using ELISA as well as flow cytometry methods, and c) performing systematic unbiased pairwise screening of the selected clones to identify specific antibody pairs to recognize specific serotypes (Table 6, DENV and Table 8, ZIKV). Similar approaches may be used to identify and distinguish other closely related proteins. Epidemiological surveillance is a second area of significance for serotype-specific detection. Epidemiological surveillance is a critical component of both patient care and public health preparedness\textsuperscript{143, 145, 146}. The serotype specific tests described here could be used in inexpensive routine patient screening during routine medical care to identify not only incoming serotypes, but also patients in endemic areas who may have asymptomatic infections. The introduction of an additional DENV serotype into a region may cause outbreaks with severe hemorrhagic fever presentation because the individual serotype infections do not provide long-lasting cross-protection and heterologous infections increase the hemorrhagic fever outcome of Dengue. Therefore, we propose that rapid testing may diminish the impact of emerging epidemics by enabling early detection of outbreaks. Third, serotype specific detection has significance for DENV vaccine development because current vaccine candidates may not show equal protection across the serotypes\textsuperscript{64}, and knowledge of circulating serotypes can inform trials and outcomes. Finally, the approaches for rapid tests described here represent a platform that can be applied toward detecting the future emergence of new pathogens. Kuno et al.\textsuperscript{147} report approximately seventy viruses in the genus Flaviviridae alone, and the methods described described here can be applied toward detecting and distinguishing other potentially emergent flaviviruses while minimizing cross reactivity.

In addition to detecting and distinguishing DENV serotypes, we report here a rapid test for ZIKV NS1. Laboratory testing demonstrated that the ZIKV NS1 test does not cross react with DENV NS1 (Figs. 22 and 23) using either spiked purified protein in human serum, nor by using supernatants of infected vero cells. Detection without cross reaction
was also confirmed using patient samples (Fig. 24). Detecting ZIKV NS1 in patient samples was more challenging than detecting DENV NS1 because ZIKV NS1 concentrations were about 10-fold lower than DENV patient NS1. A straightforward solution to address testing with the low ZIKV NS1 levels was to concentrate the sera using a centrifugal filter (a 5-minute centrifugation at 10,000g). As shown in Fig. 24, ZIKV NS1 was detectable in samples that were concentrated 5-fold; that is, to 30 microliters from a starting volume of 150 microliters.

Mobile phone camera images analyzed using image-processing methods were used to quantify test results. The use of image processing and computer software to analyze rapid test has been described previously in a non-clinical analysis. The justifications for using images and image processing are 1) ensuring objective and quantified data under varied use conditions and signal intensities, and 2) generating standardized data that can be shared and compared on a global basis. Test results (Fig. 19, DENV and Fig. 24, ZIKV) showed both very high intensity signals, especially with DENV detection, and also low intensity signals, often with Zika virus NS1. However, low signal intensity is not restricted to ZIKV because DENV NS1 signals can also be very low immediately after the onset of disease symptoms (days 0-1) and also approximately 6-7 days after onset of symptoms, when the virus is being cleared. Although the human eye is extremely sensitive, machine vision offers improved performance for quantitative measurement. Andries et al. reported wide differences in sensitivity and specificity when the same diagnostic was evaluated by different people at different sites, suggesting that training and quality assurance are required in the use of rapid tests. We propose that image processing and data quantification, as described here, provide the quality assurance. The approach is neutral regarding the computation capacity of the phone because the computation is performed “in the cloud”. Immediate internet connectivity is not required in field applications of the tests because coded de-identified image data can be stored on the phone and uploaded or transferred at later times for analysis. Mobile phones have wide use throughout the world, and the global analysis of disease burden would benefit from sharing standardized data.

The DENV and ZIKV tests described here compare very well with rapid tests described previously, with the added crucial benefit of avoiding detectable cross
reactivity between ZIKV and DENV and among ZIKV/DENV and a number of other flavivirus NS1 proteins. Nonetheless, the current DENV and ZIKV tests described here have some limitations, and opportunities for improvement remain. Using whole blood rather than serum would simplify use as a point of care diagnostic in the clinic. The tests reported here were performed using serum because the clinical samples available for retrospective analysis in the endemic areas were frozen serum samples that were validated for virus infection by RNA extraction and nucleic acid amplification and/or by ELISA for the NS1 protein. For whole blood analysis, we have developed and successfully tested lateral flow chromatography devices that incorporate our antibodies and use a specialized sample pad paper that removes red blood cells; therefore, we do not anticipate that using blood is a significant obstacle for future versions of the rapid tests. The sensitivity and specificity of the ZIKV tests ranged from 0.7-1.0. The sample availability for Zika testing was very limited, due to the fact that ZIKV is a new epidemic that was not recognized immediately. Further ZIKV testing and device optimization will be possible as the availability of patient samples improves. ZIKV test performance was improved by concentrating serum samples. Although centrifugation often requires instrumentation and power, in contradiction with the “ASSURED” paradigm, unpowered “whirlygig” centrifugation devices have been described recently, which could concentrate samples or separate serum while meeting the “ASSURED” criterion of “equipment free”. The concentration step was not needed to detect serum ZIKV NS1 using the lab-made ELISA with 150 microliters of serum. A rapid test that could accommodate a 150 microliter sample volume (currently limited by the wick absorption) may permit greater sensitivity, although it would likely be necessary to increase the number of conjugated nanoparticles used in the test.

Device cost and detection during secondary infections are important issues. The current cost for each strip is nearly $5.00, which is due primarily to using commercial gold nanoparticles (~4.50$/test), coupled with small-scale antibody production (<0.50$/test). The cost of the nanoparticles can be decreased by 1000-fold by using lab-made nanoparticles that have excellent performance (Chapter 6); moreover, antibody production scale-up will further decrease costs. We considered the possibility that the efficacy of our rapid tests for detecting NS1 might be compromised in secondary Dengue
infections, where circulating anti-NS1 antibodies could bind NS1 to form immunocomplexes that would shield NS1 from recognition by the rapid test antibodies. Using our DENV diagnostic, we tested a number of secondary Dengue samples that were confirmed by serological rapid tests of IgG/IgM levels, and by hemagglutination tests for IgG/IgM levels. The results revealed that we successfully detected DENV NS1 protein in secondary infections (data not shown); moreover, recent reports confirm success in detecting NS1 in secondary infections using rapid tests. We speculate that detecting NS1 in secondary Dengue infections might be more feasible than detecting envelope (E) protein because the interfering levels of anti-E antibodies are present in larger quantities in the serum and in higher amounts compared with the anti-NS1 antibodies. Therefore, a test based on E detection may be blocked via competition between serum polyclonal antibodies and the monoclonal antibodies used in the nanoparticle.

The rapid NS1 antigen test described here is effective only when analyzing samples collected during the acute phase of the virus infection, when flavivirus RNA and NS1 levels are detectable, prior to virus clearing by the immune system. Serological tests for anti-envelope protein IgG/IgM protein or anti-NS1 protein IgG/IgM are useful for evaluating patients post-acute phase. Several companies are marketing DENV and ZIKV IgG/IgM tests, and although in many cases their cross-reactivity has not been evaluated. A new approach, reported recently, shows high sensitivity and specificity for ZIKV immunoglobulins. We propose that optimal patient care will be provided through use of several diagnostic approaches that are applicable for a range of clinical needs. Rapid immunochromatography tests are well suited for fast turnaround times without need for specialized reagents, equipment, or trained personnel. They are generally low-cost, and can often be transported without refrigeration, and used in austere environments. Nucleic acid amplification methods are highly specific with low limits of detection, as compared to immunochromatography strips; however, disadvantages include requirements for equipment powered by electricity/batteries, specialized reagents, and a cold chain for maintaining enzyme activity. Synthetic biology approaches may offer a hybrid approach wherein the simplicity of paper diagnostics can be combined with isothermal nucleic acid amplification. There is great need for all of these technologies in order to improve the time-to-diagnosis in patients infected by pathogens worldwide.
CONTRIBUTIONS

Work by researchers from the Gehrke lab other than myself has been necessary for the compilation of this chapter. First, Dr. Irene Bosch has grown the hybridoma cells that were used to obtain the antibodies. Purification of the antibodies from Michael Diamond's lab was done by Dr. Justina Tam, Dr. Chunwan Yen and myself. Although I did most of the pairwise detection and limits of detection of the antibodies, some were also tested by Dr. Justina Tam, Dr. Chunwan Yen and Megan Hiley. Patient sample testing was done by Dr. Irene Bosch, Marc Carré and myself, mainly by travelling to endemic areas due to the difficulty of shipping patient samples abroad. PCR confirmation of infected patient samples was done by the groups that donated the samples, as well as Dr. Irene Bosch. Dr. Irene Bosch tested the Zika positive patient samples and developed the protocol of serum concentration to allow diagnosis at low concentrations of NS1 for Zika patients. Most of the contents of this chapter have been submitted for publication. Prof. Lee Gehrke drafted the discussion section of the manuscript and I, as well as other authors, contributed to revisions and editing.
MULTIPLEXED DETECTION OF ZIKA, DENGUE, AND CHIKUNGUNYA

ABSTRACT
Zika virus (ZIKV) shares the same vector, the mosquitoes *Aedes aegypti* and *Aedes albopictus*, with Dengue (DENV) and Chikungunya (CHIKV), and all three disease co-circulate throughout tropical and subtropical regions. Unambiguous clinical diagnosis, based solely on symptoms of the patients is challenging because infection with any of these three viruses leads to similar flu-like symptoms, complicating proper disease management. Point of care (POC) diagnostics that are specific for these diseases and which can identify double or triple infections are critically needed for rapid response in patient treatment and resource allocation. Immunochromatography assays (IA) are attractive because they are simple, low-cost diagnostic tools that can detect biomarkers within minutes. To address this unmet need, we describe a multiplexed IA that can identify Dengue, Zika and Chikungunya virus infections by using antibodies specific for each disease. We are able to provide a specific diagnostic for the three co-circulating diseases in a dipstick format, using 30 µl of human serum a volume that could be obtained by a fingerprick and therefore could allow this diagnostic to be performed at home. Even though this multiplexed diagnostic has not yet been validated with clinical patient samples, we show here that the test can detect the diseases by using purified antigens for each of the viruses spiked into human serum, as well as with supernatants from Dengue, Zika and Chikungunya-infected vero cells. Our approach allows for distinguishing single, double, and triple infections simultaneously, which are unlikely, but not impossible to occur.\(^{157}\)

INTRODUCTION
The current Zika virus outbreak has rapidly evolved into a global public health emergency\(^{27}\), with an estimated 1.5 million cases in Brazil alone.\(^{158}\). Since then, Zika has spread to most countries in the Americas. The emergency situation has been further exacerbated by simultaneous outbreaks of Dengue and Chikungunya. Currently, there are more than 3.2 billion people living in areas endemic to at least one of these diseases. Unfortunately, specific diagnosis of Zika, Dengue, and Chikungunya remains a major
challenge, and the WHO has recommended the rapid deployment of Zika-specific diagnostics\textsuperscript{159}. Initial symptoms of all of these diseases—fever, rash, joint pain—are non-specific, making it difficult to distinguish among them. However, clinical disease management and outcomes of each of the diseases are dramatically different. Zika is now tied to microcephaly in infants,\textsuperscript{160} and is possibly to Guillain-Barré syndrome,\textsuperscript{161} the incidences of which have spiked since the outbreak. Infection with Dengue, if there is a history of prior infection with a different serotype, can result in Dengue hemorrhagic fever or Dengue shock syndrome, and can be fatal.\textsuperscript{16} Infection with Chikungunya can result in joint pain that can be crippling and disabling.\textsuperscript{162} While PCR can identify these infections with high sensitivity and specificity, areas endemic to these diseases often have limited access to laboratories, with turnaround times for lab tests of almost a week or more. Consequently, point-of-care (POC) diagnostics have gained considerable attention for disease management and reporting, especially in emergency outbreaks.

Serological assays for antigens specific to the disease lend themselves well for rapid POC diagnostics because they require no special reagents or training to operate. However, in order to ultimately empower patients and allow them to use the device without the need for venipuncture, POC diagnostics need to run with very small volumes of sample, ideally, under 100\mu l\textsuperscript{163}. Because it is possible that a patient is infected with multiple viruses, it is necessary to be able to detect not just single but also double or triple infections\textsuperscript{157}. Thus, a multiplexed assay for Zika, Dengue, and Chikungunya would be valuable as it could provide a differential diagnosis, for these co-circulating diseases that have similar symptoms. Moreover, making the test multiplexed allows for diagnosing more diseases with a lower volume of sample. However, because Zika and Dengue are both flaviviruses and are highly similar, differential diagnosis is challenging because antibodies targeting their antigens are likely to exhibit cross-reactivity.

Here, we demonstrate a single strip multiplexed assay that can detect and distinguish among DENV, CHIKV, and ZIKV antigens simultaneously. The test is a dipstick immunoassay that can detect non-structural protein 1 (NS1) of Zika and Dengue and E2 of CHIKV in human serum with calculated LODs of 2 ng/ml, 12 ng/ml, and 5 \mu g/ml, respectively. The assay can detect the purified antigen spiked in human serum and also in the supernatant of infected vero cells. Crossover reactivity between the different
antigens is not observed, nor with a panel of related flaviviruses (Japanese Encephalitis, Tick Borne Encephalitis, West Nile Virus, and Yellow Fever Virus).

RESULTS

BASIC PRINCIPLE OF THE ASSAY

As already mentioned in the previous chapters, lateral flow immunoassays (IA) rely on ligand-target binding to provide a fast detection of antigens. In short, NP-conjugates mixed with a patient sample are put in contact with a porous nitrocellulose membrane which has antibodies specific for the biomarker immobilized on the test area. If the antigen of interest is in the sample, it binds to the antibodies on the NPs while the NP-antibody/antigen complex migrates through the nitrocellulose. The complex accumulates at the test area via binding of the antigen to the immobilized antibodies, forming a sandwich immunoassay (Fig. 7, Introduction). This results in a visible dot or band at the test area. The positive control area on a test strip has antibodies that can bind to the crystallizable fragment (Fc) of the antibodies on the NP, and indicates a proper migration of the sample. Therefore, if the antigen is present in the test solution, the gold NPs will accumulate in both the positive control and the test areas, indicating a positive result. In this chapter, we explore the possibility of using three different test bands in only one diagnostic, in such a way that by running only 30 µl of the patient sample we would be capable of detecting and distinguishing three diseases as well as double or triple infections. In this setup, the location where the signal appears on the nitrocellulose indicates the disease that is running in the test. In order to do that, we use disease-specific antibodies immobilized on the nitrocellulose. Strategic selection of the antibodies on the nitrocellulose and on the nanoparticles, was necessary in order to avoid nonspecific binding between the nanoparticles that detected each of the diseases and the antibodies on the membrane.

The biomarkers for the flaviviruses, Zika and Dengue were chosen to be their nonstructural protein (NS1), which is secreted from infected cells. The concentration of NS1 in infected Dengue patients is known to be around 15 µg/ml 4 days after infection\textsuperscript{130, 164-166}, making it an ideal target for lateral flow immunoassays. For ZIKV little is known about the NS1 concentration in patient’s serum. Because these diseases are closely related, Zika NS1 may have a similar behavior to Dengue\textsuperscript{167, 168}. From ELISA studies with
patient samples, we estimate that the concentration may be around 10x lower than for Dengue patients, which correlates well with the low viremia reported for Zika. By performing BLAST analysis on the NS1 of the four Dengue serotypes and Zika, we measured high similarities between the NS1 of the two viruses. The full NS1 sequence identities were an average of 54% comparing Zika and the four Dengue serotypes, and an average of 73% among the four Dengue serotypes (See Table 3, chapter 3). For Chikungunya, the antigen chosen was the viral envelope protein E2 (Fig. 4) which has very low sequence similarity with either Dengue or Zika NS1.

CHARACTERIZATION OF ANTIBODIES AGAINST DENGUE, ZIKA AND CHIKUNGUNYA
Monoclonal antibodies against Zika and Dengue were produced. In short, mice were immunized with purified mammalian cell expressed Zika and Dengue NS1 proteins (Native Antigen) to produce monoclonal antibodies specific for the two diseases. The antibodies were characterized by ELISA and by flow cytometry (see chapter 3) to obtain the best binding pairs. Some monoclonal antibodies showed serotype-specificity for Dengue or cross-reactivity with Dengue and Zika (Table 6, DENV and Table 8, ZIKV). This was accounted for in the design of the lateral flow immunoassay, which was strategically designed by immobilizing the disease-specific antibodies on the nitrocellulose, in such a way that cross-reactivity of Zika and Dengue in the immunoassay would not be observed, and that all the serotypes of Dengue could be measured in the same location in the lateral flow test. Chikungunya antibodies were from Prof. Michael Diamond’s lab, and were produced by immunizing C57BL/6 mice deficient for interferon regulatory factor 7 (Irf7 -/-) with 10^4 PFU of La Reunion 2006 OPY-1 strain of CHIKV. These antibodies were originally developed as therapeautic antibodies and were able to neutralize CHIKV.

In order to assemble the triplex diagnostic three capture antibodies were immobilized on three tests bands of the assay, and three nanoparticle conjugates were prepared: for detection of DENV NS1, one anti-DENV antibody was immobilized onto the nitrocellulose on the test area. On the NP, three different anti-NS1 antibodies were conjugated to the NP to ensure recognition of DENV NS1 from all the serotypes. To detect ZIKV NS1, an anti-ZIKV antibody for NS1 was immobilized on the test area. On the NP, two different antibodies for ZIKV were conjugated to the NPs. We used two anti-ZIKV antibodies in
order to be able to ensure recognition of the NS1 from multiple Zika strains. To detect CHIKV E2, an anti-CHIKV antibody was immobilized on the test area, and another anti-CHIKV antibody was conjugated to the NPs.

Prior to assembling the multiplexed diagnostic we tested the limits of detection as well as cross-reactivity of each of the three antibody pairs.

**Testing DENV NS1 Detection for the Multiplexed Rapid Test**

We tested the anti-DENV antibody pairs to recognize DENV NS1 where anti-DENV was immobilized on the test area (T). On the control area (C), an anti-Fc antibody that could bind to the NP-Ab complex was immobilized. The strip was run with NP-anti-DENV. We titrated DENV NS1 spiked into human serum at concentrations 3846, 1613, 385, 161, 38.5, 16.1 and 0 ng/ml (Fig. 25 right, lanes 1-7 respectively). For DENV NS1 at 3846 ng/ml (Fig. 25, right, strip 1), a spot appeared at the test area, indicating the DENV NS1 could successfully bind to the immobilized Ab and also the NP-anti-DENV, resulting in NPs accumulated at the test line (scheme in Fig. 25, left). A spot also appeared at the control area, indicating that the immobilized anti-Fc could bind to the NP-Ab. Thus, the two spots indicated a positive test. As the DENV NS1 concentration decreased, the intensity of the spot at the test line decreased, and no spot appeared for no DENV NS1 present (Fig. 25, right, strip 7), suggesting that there is no non-specific adsorption of the NP-Ab on the test line.

**Testing ZIKV NS1 Detection for the Multiplexed Rapid Test**

We then tested the binding of ZIKV NS1 with the NP-anti-ZIKV/immobilized anti-ZIKV. Anti-ZIKV was immobilized on the test area and anti-Fc was on the control area and the

![Figure 25: Titration curve of the Dengue antibody pair. Scheme (left) and representative membranes (right). DENV (all serotypes combined) NS1 concentration spiked in human serum of 3846, 1613, 385, 161, 38.5, 16.1 and 0 ng/ml in strips 1 to 7, respectively.](image-url)
test was run with NP-anti-ZIKV. We titrated ZIKV NS1 spiked into human serum at concentrations 3846, 1613, 385, 161, 38.5, 16.1 and 0 ng/ml (Fig. 26 right, lanes 1-7 respectively). For ZIKV NS1 at 3846 ng/ml (Fig. 26, right, strip 1), a spot appeared at the test area, suggesting successful binding of the NS1 with immobilized antibody and the antibodies on the NP. A spot appeared at the control line, indicating a positive test. As ZIKV NS1 concentration decreased (Fig. 26, right, lanes 2-6), the test spot decreased in intensity, and no spot appeared in the test band for no NS1 in solution (Fig. 26, right, strip 7), but the control spot still appeared, confirming that the nanoparticles were able to run in the test.

Figure 26: Titration curve of the Zika antibody pair. Scheme (left) and representative membranes (right). ZIKV NS1 concentration spiked in human serum of 3846, 1613, 385, 161, 38.5, 16.1 and 0 ng/ml in strips 1 to 7, respectively

TESTING CHIKV E2 DETECTION FOR THE MULTIPLEXED RAPID TEST
We tested the ability of NP-anti-CHIKV/immobilized CHIKV for CHIKV E2 (Fig. 27). We titrated CHIKV E2 spiked into human serum at concentrations 286, 154, 32.8, 6.7, and 0 μg/ml (Fig. 27 right, lanes 1-5, respectively). The strip run with CHIKV E2 at 286 μg/ml showed spots at the test spot and control spot (Fig. 27, right, strip 1), indicating successful recognition of the NP-anti CHIKV/immobilized anti-CHIKV for E2 and therefore a positive test spot. Decreasing E2 resulted in a less intense test spot line, and only the control spot appeared at the test line when there was no E2 present (Fig. 27, right, strip 5).
These results showed that the antibody pairs could successfully recognize individual antigens DENV, ZIKV, and CHIKV, without false positive signals in the blank. We then used ImageJ to quantify the signals from each of the test bands proceeded to test each of the antibody pairs with high concentrations of the three antigens, to test if any of the tests would show a false positive signal. The limits of detection were calculated by fitting a Langmuir equation on the titration curves and the LoD was considered as the concentration that yielded a signal in the test band that would be higher than 5-times the standard deviation of the blank.

We first tested running spiked DENV NS1 in human serum on the Dengue, Zika and Chikungunya tests (Fig. 28, left, DENV). We observed that the signal from DENV NS1 could only be observed on the DENV test (Fig. 28, left, DENV, blue squares), but no significant signal was obtained in either the Zika or Chikungunya tests when running DENV NS1 at 3486ng/ml (Fig. 28, left, DENV, green circles and orange triangle,
respectively), showing that only the Dengue test was capable of detecting Dengue NS1, and there was no Dengue cross-reactivity in the other two diagnostics. The limit of detection of Dengue in the DENV test was 12.18ng/ml, which is similar to previously reported results in our lab (see chapter 3, Fig. 13, DENV and Fig. 21, ZIKV). This value is lower than reported values of NS1 in patient serum, which can be up to 15 µg/ml. We then proceeded to test running spiked Zika NS1 in human serum in the three tests. We observed that Zika NS1 was detected only in the Zika test (Fig. 28, center, green circles), and we obtained a calculated limit of detection of 1.39ng/ml at 5-times the standard deviation of the blank. ZikV NS1 at concentrations of 3486ng/ml in spiked human serum showed no significant binding with either the Dengue pair of antibodies (Fig. 28, center, blue squares), nor with the Chikungunya test (Fig. 28, center, orange triangles). Therefore it showed that the Zika test was capable of detecting Zika NS1 at low concentrations and that Zika NS1 did not cross-react in either the Dengue or the chikungunya tests.

Finally, we ran Chikungunya E2 protein in the Dengue, Zika and Chikungunya tests. We observed that CHIKV E2 was detected by the anti-Chikungunya pair of antibodies (Fig. 28, right, orange triangles), and the limit of detection at 5-times the standard deviation of the blank was 4.77µg/ml. Cross reactivity of CHIKV E2 against NP-anti-DENV/anti-DENV and NP-anti-ZIKV/anti-ZIKV at concentrations of 150µg/ml E2 spiked in human serum (Fig. 28, right, blue squares and green triangles, respectively) showed no spots at the test areas, confirming that no cross reactivity was present. Therefore, we observed that the Dengue, Zika and Chikungunya tests were selective in detecting the antigens of interest and did not show any cross-reactivity with the other diseases that were tested.

**Differential Multiplexed Detection of Zika, Dengue, and Chikungunya Antigens**

Based on the results from the individual tests, we designed a single strip that could provide multiplexed detection of antigens. On a single strip we immobilized anti-CHIKV-2 at test area a, anti-ZIKV-2 at test area b, anti-DENV-2 at test area c, and anti-Fc at the control area d. Strips were run with a mixture of NP-anti-CHIKV-1, NP-anti-ZIKV-1, NP-anti-DENV-1 (Scheme in Fig. 29). This design relied on non-cross-reactive antibodies.
being immobilized on different locations on the nitrocellulose, in order to obtain the
diagnostic of the three diseases in one test.

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Figure 29: Scheme of the triplex test. Antibody pairs (top), and expected results (bottom)

Each of the pure antigens in human serum were first tested individually (Fig. 30, strips 1-3). For DENV NS1 from all serotypes at 3.8 μg/ml Dengue NS1 (strip 1), spots at test area c and the control area appeared, indicating a positive test for DENV. No spots appeared at test areas a and b, confirming no cross reactivity in the multiplexed test. For ZIKV NS1 at 3.8 μg/ml (Fig. 30, strip 2), a spot appeared at test area b and the control area, but not in a or c, indicating a positive test for ZIKV and no cross-reactivity. For CHIKV E2 at 123 μg/ml (Fig. 30, strip 3), a spot appeared at test area a and the positive control area, but not areas b or c, indicating a positive test for CHIKV and no crossover reactivity. When the strips were tested with human serum with no biomarker present, areas a-c did not show a spot, but the anti-Fc control area showed a signal, confirming the negative control. Next, the ability of the multiplexed test to detect mixtures of the antigens was tested. For ZIKV NS1 and DENV NS1 at 1:1 (Fig. 30, strip 4), spots appeared at b and c and the control area, but not at test area a, indicating successful recognition of ZIKV and DENV biomarkers with no cross reactivity for Chikungunya. For ZIKV NS1 at 3.8 μg/ml and
CHIKV E2 at 123 μg/ml (Fig. 30, strip 5), signal appeared at a and b and the test area and not c, indicating successful detection of this mixture. For DENV NS1 at 3.8 μg/ml with CHIKV E2 at 123 μg/ml (Fig. 30, strip 6) spots appeared at test areas a and c and the control area, but not b, indicating successful detection of the mixture. For a mixture of all three antigens (ZIKV NS1 and DENV NS1 at 3.8 μg/ml, and CHIKV E2 at 123 μg/ml, Fig. 30, strip 7) spots appeared at areas a-c and the control, indicating successful detection of all 3 biomarkers. Thus, the multiplexed strip could detect each biomarker individually an in a mixture with no cross-reactivity.

Both ZIKV and DENV belong to the flavivirae family, which includes other dangerous pathogens such as Yellow Fever, Japanese Encephalitis, West Nile, or Tick Borne Encephalitis. Flaviviruses have a high homology among them, so we tested the cross reactivity of the multiplexed strip with NS1 of other flaviviruses at 3.8 μg/ml: Japanese Encephalitis virus, (JEV, Fig. 30, strip 8), Tick Borne Encephalitis (TEBV, Fig. 30, strip 9), West Nile Virus (WNV, Fig. 30, strip 10), and Yellow Fever virus (YFV, Fig. 30, strip 11). When the strips were run with all of these biomarkers in human serum, no spots appeared on test areas a-c, but control areas still showed a spot. Thus, the multiplexed strip did not exhibit cross-reactivity with these other flavivirus biomarkers.

![Figure 30: Results of the triplex test with spiked purified proteins in human serum. Different combinations were tested: Dengue (1), Zika(2), Chikungunya(3), Dengue+Zika(4), Zika+Chikungunya(5), Dengue+Chikungunya(6), Dengue+Zika+Chikungunya(7). Negative controls of similar flaviviruses did not show a signal. Japanese Encephalitis (8), Tick Borne Encephalitis (9), West Nile Virus (10), Yellow Fever (11). Uninfected (blank) serum (12)](image-url)
MULTIPLEXED DETECTION USING INFECTED CELL CULTURES

We also tested the ability of the strips to recognize the presence of biomarkers from infected cells (Fig. 31). Because the NS1 of the different serotypes of DENV have slight differences in sequence, the strip was run with each of the Dengue serotypes (Fig. 31). Vero cells were infected with DENV of each of the 4 serotypes and the cell supernatants were assayed in the strips. Strip 1 was run with the supernatants of cells infected with DENV1-4 all mixed together, which resulted in a spot at test area c, confirming recognition by the NP-anti-DENV/immobilized anti-DENV pair. Spots did not appear at test areas a and b, confirming no cross reactivity. Similar results were observed for DENV NS1 from serotypes 1-4 (Fig. 31, strips 2-5, respectively). Supernatants of uninfected Vero cells showed no spots at test areas a-c (Fig. 31, strip 10), but did show a spot at the control area, confirming the negative control. Thus, NS1 from all Dengue serotypes could be recognized.

The supernatant of Vero cells infected with ZIKV was tested (Fig. 31, strips 6-7). ZIKV from Uganda (MR766) and also ZIKV from a patient in Brazil (PER243) resulted in spots on the test area b, indicating successful recognition of both ZIKV infections. Supernatants of Vero cells infected with CHIKV also showed a positive test, with a spot at area a but not b and c. Finally, a mixture of the supernatants of vero cells infected with ZIKV, DENV, and CHIKV exhibited spots at test areas a-c, confirming a positive test for all of the biomarkers simultaneously. Therefore, the strips were able to detect the antigens for each of the diseases from infected cells.
**DISCUSSION**

Lateral flow immunoassays are of interest in point of care applications as they are affordable, user-friendly, do not require laboratory facilities or trained personnel and provide a rapid diagnostic in less than 20min\(^{13}\). Because dipstick immunoassays rely on capillary action to move the reagents and the sample in the test, they do not require electric power, or a laboratory environment to be operated. This is an advantage over nucleic acid-based diagnostics, which require extensive sample preparation, as well as electricity, specialized reagents and personnel and a cold chain for both patient samples and test reagents. Although POC testing is, in general, not as sensitive and specific as PCR, which is considered the gold standard, it allows for a self-screen or for the clinician to provide a fast and accurate diagnosis within the period of clinical illness. These characteristics are especially important during disease diagnostics in epidemics\(^{176}\) especially in tropical climates. Moreover, POC assays have proved to improve clinical outcomes and decrease hospitalization and financial costs in clinical settings\(^{92, 177}\).

Dengue, Zika and Chikungunya are three diseases transmitted by the same mosquito, but with very different potential complications. Chikungunya has been linked to Guillain Barré, and disabling joint pain. Dengue can lead to Dengue hemorrhagic fever in
secondary infections, which is life threatening, and a major issue in most countries in the tropics; and Zika has been recently linked to severe neurological disorders such as congenital defects, or Guillain Barre syndrome. Although the three diseases have such different potential outcomes, their initial clinical manifestations are similar: they are characterized by flu-like symptoms, such as fever, rash, muscle pain, arthralgia or nausea. Therefore, clinical diagnosis based on the patient's symptoms is not available. Moreover, because the three diseases are transmitted by the same mosquito, they typically co-circulate, hence the need to develop a multiplexed diagnostic that can detect and distinguish these three diseases. By multiplexing the diagnostic so that it can run on 30μl of sample, it is possible to ease the testing process for patients, clinicians and diagnostic centers by enabling diagnosis from only a fingerprick, without the need of venipuncture.

Here, we have described a Dengue, Zika and Chikungunya rapid test that is capable of detecting and distinguishing both purified proteins from each of the diseases spiked into human serum, as well as supernatants from vero-infected cells. In order to do that, we first selected pairs of antibodies that could detect the three diseases without cross-reactivity between the diseases, which was of special importance in the case of Zika and Dengue, as both diseases are flaviviruses, and they have high homology (see chapter 3, table 3). The key to achieve selectivity and spatial differentiation of the three diseases in the diagnostics was that the antibodies that were immobilized on the nitrocellulose were highly specific for each of the diseases, while cross-reactivity on the antibodies conjugated to the gold nanoparticles was not an issue. Therefore, cross-reactivity between Dengue and Zika was nonexistent, and moreover, we did not observe cross-reactivity with the NS1 of other similar flaviviruses, such as Yellow Fever, Tick Borne Encephalitis, Japanese Encephalitis or West Nile Virus. Secondly, it is known that false positives in lateral flow immunoassays can occur due to nonspecific binding between the antibodies on the nanoparticles and the antibodies immobilized on the nitrocellulose. Typical lateral flow tests only contain one test band and one antibody on the NPs; however, in this test there were three antibodies on the nitrocellulose and three antibodies immobilized on the membrane, hence nine possible combinations that could yield false positive signal. We engineered the test in order to avoid cross-reactivity between the
different antibodies that were immobilized on the nitrocellulose and the nanoparticles, which required considerable redesigning of the diagnostic and testing due to the large number of potential false-positive combinations that were possible with the multiplexed assay. On the third place, the test was designed to run on less than 100µl of sample, which is an average amount that could be obtained in a fingerprick in other studies\textsuperscript{163}, hence the diagnostic would potentially work without the need of drawing blood through venipuncture. This multiplexed test could be easily adapted to diagnose other diseases as well, provided that at least one non-cross-reactive antibody for each disease could be immobilized on the nitrocellulose.

Nevertheless, the multiplexed assay presented has some limitations. First, due to the difficulty in obtaining patient samples to test, it is still not possible to report information on the sensitivity and specificity of the multiplexed assay. To overcome this limitation, we tested supernatants of vero cells infected with the three viruses extracted from febrile patients, and observed that the assay was successful in diagnosing the cell culture supernatants, which is a robust proxy to compare with clinical patient samples. Moreover, we spiked human serum with purified antigen for each disease, and also observed that the immunoassay was capable of detecting the proteins with low limits of detection in a mixture with human serum, as well as being capable of detecting the native protein generated after virus infections on eukaryotic cells. On the second place, in the current setup, the diagnostic requires patient serum, and not whole blood, though this could easily be adapted in lateral flow assay where sample pads have been used routinely to separate out red blood cells from the assay. The limits of detection reported for the multiplexed diagnostic appear to be low enough to enable the detection of the three diseases at low enough concentrations of virus. Dengue is present in patient's serum at concentrations of around 15µg/ml, which is much higher than the limit of detection reported for the Dengue antibody pair. NS1 is detectable in patient serum between days 1-7 of disease, which are the days in which the presented diagnostic could be used. Moreover, the same Dengue antibody pair has been tested with patient samples in chapter previous (PAN antibody pair), and it had a high sensitivity and specificity with clinical samples. Currently the concentration of NS1 ZIKV and CHIKV in patient serum is not available in literature. In the case of Chikungunya, although the antibody pair has a limit of detection that seems
high, as compared to Dengue or Zika, the test has successfully detected Chikungunya in supernatants from vero cells, as well as in patient samples (data not shown). Regarding the Zika diagnostics, no data has been published on the NS1 concentration present in Zika-infected patients, however, we estimate from ELISA measurements that the concentration is roughly 10-times lower than in Dengue patients. This value correlates well with the lower viremia that has been observed in Zika patients\(^{169}\); and is higher than the limit of detection of the antibody pair used for Zika detection in the multiplexed diagnostic. Nevertheless, it is possible that Zika might be difficult to diagnose in this multiplexed test without the concentration/centrifugation step that was used in chapter 3. We estimate that a test that could handle a larger volume of patient sample would not require the centrifugation step.

Although we have not developed an ImageJ algorithm to analyze the diagnostic presented in this chapter, the analysis of the strips could indeed be automated, which would be important in order to generate accurate and objective information when diagnosing patient samples, and could help in detecting signal in the first and last days of the disease. Moreover, the approach of automating the analysis could potentially aid in reporting the epidemiology of the diseases in real time.

CONCLUSIONS

Here we showed that by using a combination of spatial separation, together with specific antibodies it is possible to provide differential diagnosis of closely-related diseases. In particular, the immunoassay presented in this work is capable of distinguishing Dengue, Zika and Chikungunya. The three diseases are spread by the same mosquito and have similar initial symptoms. Nevertheless, it is necessary to be able to distinguish among them, as the clinical outcomes of each of the diseases are very different. Because Zika and Dengue are similar flaviviruses, it is challenging to obtain antibodies capable of distinguishing among them. Moreover, we confirmed that the test was specific for Dengue, Chikungunya and Zika by testing other similar flaviviruses and showed that any of them was causing cross-reaction with the test.

The presented paper-based immunoassay is low-cost and easy to use. It has promise as a point of care, provided it can be tested in patient samples, which are currently difficult
to obtain despite the emergency situation. Future work includes testing at clinical sites using human patient samples.

CONTRIBUTIONS

Work by researchers from the Gehrke lab other than myself has been necessary for the compilation of this chapter. First, Dr. Irene Bosch has grown the hybridoma cells that were used to obtain the antibodies. Marc Carré helped in performing some experiments. Chikungunya monoclonal antibodies were from Prof. Michael Diamond’s group.
The recent Zika virus (ZIKV) and Ebola (EBOV) crises have highlighted the need for the development of rapid diagnostic tools for outbreak situations. These are especially needed when the pathogens causing the epidemic co-circulate with other pathogens that cause similar initial symptoms, and therefore clinical diagnosis based on medical observation of the patient’s symptoms is not possible. Low-cost, rapid point of care (POC) diagnostics for the pathogens causing the outbreak are critically needed for rapid response in patient treatment, resource allocation and control of epidemics. The time to manufacturing of new POC devices, even in emergency situations, is a time-consuming process, of at least one year after the outbreak starts. Producing new POC devices requires the selection and growth of new antibodies specific for the diseases of interest, which may not have been generated before the outbreak starts. Both patients and epidemics control would greatly benefit from access to rapid diagnostics especially during the initial stages of an outbreak. To address this unmet need, I present an approach that exploits the use of already existing, out of the shelf, cross-reactive antibodies that have been originally generated against a different pathogen of the same species. This allows to provide a multiplexed diagnostic for two closely-related pathogens, such as Dengue and Zika; or Marburg and Ebola by using antibodies originally raised against only one protein of the related viruses. I obtain cross-reactivity in the diagnostic because I rely on cross-reactive antibodies. Hence, to solve this issue I exploit gold nanoparticles of different colors to distinguish between the related viruses, as well as co-infections. By using this approach, I here present a test that can discriminate the four Dengue serotypes by using cross-reactive antibodies raised against NS1 of Dengue serotype 3. I also show a multiplexed Dengue and Zika diagnostic developed with antibodies raised against Dengue NS1, as well as a diagnostic that can distinguish Ebola and Marburg by using antibodies originally raised against Marburg envelope protein. Moreover, I show that we
can use the same approach to enhance a commercially available, widely used, Dengue diagnostic that cross-reacts with Zika, hence converting at a very low cost, a nonspecific diagnostic into a multiplexed, specific test for both Dengue and Zika.

INTRODUCTION

Accurate pathogen identification is critical for patient care and control of epidemics, especially when co-circulating diseases present similar non-specific clinical symptoms. Typically, screening for infectious agents relies on transcription polymerase chain reaction (PCR), which has high sensitivity and specificity. However, PCR tests are costly, slow and require a cold chain for both samples and reagents. In outbreak situations such as the last Ebola epidemic, this resulted in time-to-diagnosis of almost a week during the peak of the epidemic in Sierra Leone. This issue was critical, because diagnosis delays led to longer hospitalizations, increasing both bed demand and the likelihood of hospital-acquired disease. Point-of-care (POC) diagnostics offer the potential to substantially reduce these delays. Thus, they have gained considerable attention for disease reporting especially in emergency situations, as they can be run by non-experts, are cheap, portable, and deliver results within minutes.

Point of care immunoassays can detect a viral antigen though the accumulation of antibody-coated nanoparticles. They are ideal for field diagnosis and can also improve patient care when used as a first screening method. However, years of research are needed to produce one new rapid diagnostic. One of the reasons for their slow time to manufacturing is the requirement to produce, test and select specific antibodies against the pathogen of interest. Antibody selection is done by first immunizing mice with a viral antigen, then selecting B cells capable of binding to the antigen, fusing the B cells with myelomas to produce hybridomas and finally screening the hybridomas by ELISA and flow cytometry to evaluate the binding properties of each generated antibody. These processes are time-consuming and costly, where the production of one new antibody can take between 16-24 months and can cost ~100M, and need to be done even before testing in a lateral flow format. Without the need to produce new, specific antibodies, the time to manufacturing of the rapid diagnostic devices could be faster. Out of the shelf, cross-reactive antibodies originally generated against a closely related pathogen to the
one causing the outbreak could be used in lateral flow assays to reduce the time-lag of producing new antibodies.

Figure 32: Number of Zika (top) and Ebola (bottom) cases increased exponentially by weeks after the outbreak, highlighting the need for the rapid development of diagnostics against new outbreaks.

Antibody cross-reactivity is common, and it is exploited for vaccine development\textsuperscript{180} and in the production of therapeutic neutralizing antibodies\textsuperscript{181}. In the development of immunoassays, specific antibodies are typically preferred in order to favor disease-specific tests. However, it is not rare to find commercially available cross-reactive diagnostics\textsuperscript{182,183}, due to the difficulty in testing for all possible antigens that may induce cross-reactivity. Moreover, antibody production against specific antigens is challenging, and it typically results in the generation of a large percentage of antibodies that exhibit cross-reactivity with related pathogens. For example, when immunizing mice with purified Zika NS1 by the spleen B cell hybridoma method (B cell isolation from B cells and cell
fusion at 105 days post-immunization), we only obtained 54% Zika-specific antibodies, while the rest cross-reacted with Dengue (25%) or other flaviviruses (22%). Similar trends were observed when performing individual mice immunizations with purified NS1 from each of the Dengue serotypes, where the percentage of serotype-specific antibodies obtained were 31%, 10%, 35%, and 43%, for DENV-1, DENV-2, DENV-3 and DENV-4, respectively. Moreover, disease-specific antibodies do not guarantee a good performance in lateral flow assays, as the antibodies may lead to false positives or negatives in the assembled diagnostic or they could be too specific — when antibodies may bind a strain-specific epitope -.

Here, I exploit cross-reactive monoclonal antibodies and avoid the issue of nonspecific diagnosis by strategically using nanoparticles of different shapes and therefore distinct colors. By doing that, two NPs of different colors and the cross-reactive antibodies can be used to rapidly develop a new assay based on the already existing antibodies. I exploit the size- and shape-dependent properties of gold NPs to identify multiple pathogens and co-infections and show that this approach can be used to discriminate between diseases with varying homologies: I show that we can identify the similar four serotypes of Dengue (69-80% identity) by using DENV-3 antibodies, only. We can also distinguish Dengue and Zika (54-55% identity) by using antibodies originally raised against Dengue. Moreover, more distant pathogens such as Ebola and Marburg (35% identity) can as well be discriminated with Marburg cross-reactive antibodies. Finally, I show that we can enhance a Dengue widely available, cross-reactive POC assay and convert it into a specific Dengue and Zika diagnostic.

RESULTS

BASIC PRINCIPLE OF THE LATERAL FLOW ASSAY

As explained in the previous chapters, immunochromatography assays (IA) rely on ligand-target binding to provide a fast detection of disease-specific biomarkers. In a typical IA test, NP-conjugates mixed with a patient sample flow through capillarity in a porous nitrocellulose membrane which has antibodies specific for the antigen immobilized on the test area. If the antigen of interest is in the sample, it binds to the antibodies on the NPs, and the complex migrates though the nitrocellulose. The NP-antibody-antigen
complex accumulates at the test line via binding of the antigen to the immobilized antibodies, resulting in a visible dot, indicating a positive test. The control area on the test strip has antibodies that can bind to the antibodies on the NP, a signal in the positive control indicates proper migration of the sample. Therefore, if the antigen is present in the test solution, the gold NPs will accumulate in both the positive control and the test band. Cross-reactive antibodies are common, especially for diseases with high homology. Some reports using them as neutralizing antibodies are in the literature\textsuperscript{181}. Cross-reactive antibodies can also be used in lateral flow assays. In this case, several diseases induce the same signal. For example, the antibody 136 (chapter 3, Table 6 and Table 8) was cross-reactive with both Dengue and Zika. When 136 is paired with itself to form a lateral flow test, we can observe signal when testing both Dengue and Zika (Fig. 32).

![Diagram](image)

Figure 33: The cross-reactive antibody 136 shows a signal for both Dengue and Zika when tested in lateral flow immunoassays. Scheme (left), expected results (middle), strips with the results (right).

D=Dengue, Z=Zika

Antibody cross-reactivity is also observed in commercial lateral flow assays. Commercial Dengue diagnostics can show cross-reactivity with other flaviviruses\textsuperscript{182}. An example is Allere’s Dengue test, which detects Dengue NS1, but also cross-reacts with many other flaviviruses such as Yellow Fever, Zika or West Nile Virus (Fig. 33). This is problematic because Dengue and Zika currently co-circulate, they share the same initial symptoms and differential diagnosis is necessary due to their distinct risk profiles.
MULTIPLEXED IMMUNOASSAY USING CROSSREACTIVE ANTIBODIES
When using cross-reactive antibodies to distinguish the different infections, it is necessary to multiplex the test and use more than one combination of antibodies with different affinities for the two antigens that cross-react. One solution to achieve that would be to prepare two side by side tests, having one of them specific for one disease, and the other cross-reactive for the two pathogens. I tested eleven antibodies originally raised against Dengue for cross-reactivity with Zika. To do that, I conjugated one antibody on gold nanoparticles and spotted the other onto the nitrocellulose. I checked each of the 121 combinations for binding with purified NS1 of each of the four Dengue serotypes and Zika, as well as uninfected human serum as a negative control (see chapter 3, full table in Table 6). I obtained some antibody pairs that could detect Zika as well as Dengue, and some combinations that could detect Dengue, but not Zika (subset table: Table 9).

Table 9: Antibody pairs matrix used develop a Dengue/Zika test based on Dengue antibodies

I observed that the antibody 136 could detect Zika and Dengue NS1 when paired with itself(Fig. 32, and table 9). Therefore, I designed the diagnostic with two parallel nitrocellulose lanes, immobilized the cross-reactive antibody 136 on membrane 1, and
used 136-conjugated gold nanoparticles in order to detect Dengue and Zika. On membrane 2, I immobilized the Dengue-specific antibody 323, and flowed nanoparticles conjugated with a PAN mixture of antibodies (271, 243, 626 and 411) to guarantee that all Dengue serotypes would be detected (Table 9). By doing this, we expect that a Zika sample would lead to a positive signal on the cross-reactive membrane 1 only, and a Dengue sample would result in a positive signal in both membranes: the Dengue-specific and the Dengue cross-reactive (Scheme in Fig. 34).

Figure 35: Scheme of the preliminary Dengue/Zika test based on Dengue antibodies. Scheme of antibody pairs (top). Expected results when testing a Zika sample (only lane 1 is positive, bottom, ZIKV). Expected results when testing a Dengue sample (both membranes are positive, bottom, DENV)

I then calculated the limits of detection of each membrane when flowing both purified Dengue or Zika NS1 spiked in human serum. First, in lane 1, I immobilized the cross-reactive antibody 136 on the nitrocellulose and conjugated the same 136 antibody on the nanoparticles and observed that 136 paired with itself and was capable of detecting both Dengue and Zika NS1. I ran serial dilutions of Zika (Fig. 35, green squares) and Dengue(Fig. 35, blue squares) NS1 with the 136/136 pair of antibodies.
Figure 36: Titration curves of cross-reactive membrane 1. Scheme (left), Green squares and top membranes Zika. Blue squares and bottom membranes, Dengue. DENV or ZIKV NS1 spiked in human serum at 38.5, 16.1, 3.9, 1.6 and 0 pg/ml (membranes, left to right).

After, in lane 2, I immobilized the specific antibody 323 on the nitrocellulose, and tested running the nanoparticles conjugated with the PAN mixture of antibodies. We can observe that this combination was able to detect Dengue at low concentrations (Fig. 36, blue squares), but not Zika NS1 (Fig. 36, green squares). This result was expected from ELISA results that showed that the antibody 323 immobilized on the nitrocellulose of lane 2 was specific for Dengue, hence, Zika could not be detected in this test membrane.

Figure 37: Titration curves of Dengue-specific membrane 2. Scheme (left), Green squares and top membranes Zika. ZIKV NS1 spiked in human serum at 38.5, 3.9, 1.6 and 0 pg/ml (left to right). Blue squares and bottom membranes, Dengue. DENV NS1 spiked in human serum at 3.9, 1.6, 0.4, 0.2, 0.05 and 0 pg/ml.
I then tested if the immunoassay showed a false positive signal when testing purified NS1 of other closely related flaviviruses. In order to do that, I ran purified NS1 of the negative controls spiked in human serum, namely Japanese Encephalitis, Tick Borne Encephalitis, West Nile Virus and Yellow Fever (Fig. 37, left to right, respectively). None of them showed a cross-reactive signal in any of the two membranes, indicating that only Dengue and Zika were detected.

Figure 38: NS1 spiked in human serum at 16.1µg/ml from similar flaviviruses (Japanese Encephalitis, Tick Borne Encephalitis, West Nile Virus, Yellow Fever virus, left to right) was used against membranes 1 (cross-reactive Dengue+Zika) and 2 (Dengue-only)

In order to confirm the performance of the diagnostic I tested the native NS1 protein released from virus-infected cells by running supernatants from Dengue and Zika infected vero cell cultures. We observe, as expected, that when flowing Dengue-infected supernatants, both the Dengue cross-reactive spot and the Dengue-specific spot light up (Fig. 38, left), and when flowing Zika-infected supernatants only the cross-reactive antibody pair shows a signal (Fig. 38, middle). For uninfected vero supernatants, no signal is observed in any of the test bands (Fig. 38, right).

Figure 39: Supernatants from infected vero cell cultures were used to check that the two lane test could detect native NS1 generated during infections. Dengue (left), Zika (middle) and uninfected supernatant (right)
Therefore, I was able to exploit the cross-reactivity of the antibody 136, to detect and distinguish between the two diseases, without false positive signal for similar flaviviruses. Both purified NS1 spiked into human serum, as well as Dengue and Zika vero-cell infected supernatants were possible to diagnose. An issue that appeared was that co-infections of Dengue and Zika could show the same result than a Dengue-only sample—as the two membranes would also show a signal-. Currently, Dengue and Zika co-circulate and double infections have been reported in patients\(^{157}\), thus, I needed to add a new variable to allow diagnosing double infections. I chose to include nanoparticles of two different colors in order to enable the diagnosis of co-infections.

**MULTIPLEXING BASED ON NANOPARTICLE COLORS**

Gold nanoparticles (NPs) are attractive for lateral flow immunoassays because they are strongly colored, due to their high extinction coefficient in the visible wavelengths of light. Their absorption spectra can be tuned by changing their shape through different synthesis protocols, permitting the synthesis of nanoparticles of varying colors. Gold nanospheres (Fig. 39, left NS), for example, typically absorb light around 520nm, and appear like red-colored solutions, and their absorption does not change much with different synthesis conditions. Gold nanostars, on the other hand, can be easily synthesized with different arm lengths and thus with different colors that range from magenta to blue to green (Fig. 39, middle ten tubes). Furthermore, they can be readily conjugated with antibodies.

![Figure 40: Picture of vials of different synthesis of gold nanospheres (NS), nanostars (NStar) and nanorods (NR)](image)

Here, the idea was to use two NPs conjugated to two different Dengue antibodies. I conjugated the red spherical nanoparticles to the cross-reactive Dengue and Zika antibody -136-; and the blue-colored nanostars with the PAN-Dengue-specific antibodies.
On the nitrocellulose, I spotted the positive control, and two test bands, one with the Dengue-specific antibody 323, and the second test band contained the cross-reactive antibody 136. By doing that, we observe that if Zika flows on the nitrocellulose, only the red nanoparticles should bind to the 136 test band (Fig. 40, top). When Dengue flows on the nitrocellulose, both nanoparticles could bind to both lanes, with stronger binding of the blue nanoparticles on the 136 test band (Fig. 40, bottom). Finally, mixtures of Dengue and Zika should yield different colors in the test bands as we vary the relative concentrations of Zika or Dengue NS1 (Fig. 40, right).

**Figure 41: Scheme of the Dengue/Zika diagnostic based on two nanoparticle conjugates.**

**NANOPARTICLE-ANTIBODY CONJUGATES**

In order to assemble this diagnostic I first synthesized and characterized the nanoparticles. Star-shaped gold nanoparticles, nanostars, have a strong absorbance peak in the visible that can be tuned by tuning the synthesis conditions, resulting in nanoparticles of different colors that can range from magenta to green. I synthesized blue-colored gold nanostars (Fig. 41, right), using a reduction of gold salts in HEPES buffer (see methods, chapter 2). Red-colored gold nanospheres (Fig. 41, right) were purchased from Innova Biosciences. Nanosphere and nanostar size, shape, and monodispersity were probed by TEM imaging (Fig. 41, left NStar and middle, NS). The nanospheres had a diameter of 38 ± 4 nm, and the nanostars had an internal diameter of 13 ± 3 nm, and a maximum length of 26 ± 5 nm.
Antibodies were conjugated to the nanostars by non-covalent adsorption, and to the nanospheres by following the manufacturer’s instructions. NPs exhibited strong surface plasmon resonance peaks in the visible wavelengths of light (Fig. 42), confirming their color characteristics. Gold nanostars had a surface plasmon resonance peak at 607 nm before antibody conjugation, and after conjugation, the surface plasmon resonance peak red-shifted to 631 nm, which is a typical phenomenon after biomolecule conjugation on nanoparticles. Absorbance was used to calculate the concentration of nanostars (see Materials and Methods, and Chapter 6 for the calculation of the extinction coefficient). After synthesis, I measured 1.75 nM gold nanostar, and 170 nM after antibody conjugation and a concentration step. 1 µl of the concentrated NStars were used in the lateral flow experiments. The commercially available gold nanospheres showed a strong plasmon resonance peak at 529 nm after antibody conjugation, 3 µl of the commercial NS preparation were used in the lateral flow experiments.

Conjugation was also probed by gel electrophoresis. Gold nanoparticles ran in agarose gels, confirming their stability. Before conjugation (Fig. 43, lane 3), NStar-Ab ran in a tight band in agarose gels, and the nanostars shifted to slower mobilities after conjugation and mPEG backfill (Fig. 43, lane 2), due to the change in size and shape caused by the antibody and mPEG addition. Although mPEG is a neutral molecule, the nanoparticles still maintained mobility in the gels, as the mPEG backfill did not completely displace the
antibodies already conjugated to the nanoparticles. Commercially available gold nanospheres had mobility in agarose gels similar to that of the conjugated nanostars (Fig. 43, lane 1).

In addition, dynamic light scattering (DLS) measurements reported an increase in hydrodynamic diameter on the nanostars from 39.8±3.5 nm to 80.4±18.8 nm, suggesting successful antibody and mPEG conjugation (Fig. 44, top). Commercially available nanospheres showed a hydrodynamic diameter of 77.6±1.3nm after antibody conjugation. Zeta potential after antibody conjugation decreased from 36.2±4.1 mV to -37.3±1 mV. The zeta potential of the commercial nanospheres was -26.1±1.9mV. By conjugating fluorescent antibodies on the nanoparticles and by calculating the concentration of nanoparticles, Ab coverage was determined to be 17.6 ± 1.2 Abs/NStar, equivalent to an Ab footprint of 56.8 nm^2 per nanostar, and the commercial nanospheres had a coverage of 9.8±0.7 Ab/NS, equivalent to an Ab footprint of 463 nm^2 per nanosphere.

Figure 44: Gel of conjugated nanospheres (1) and of nanostars before(3) and after(2) antibody conjugation.

Figure 45: DLS(top) and zeta potential (bottom) of nanostars (blue) and nanospheres (red)
DETECTION OF DENGUE AND ZIKA

LODs OF NP-AB CONJUGATES FOR INDIVIDUAL ANTIGENS

I then tested the limits of detection of the antibodies by conjugating one of the antibodies (either PAN or 136) on each of the nanoparticles, spotting either 136 or 323 on the nitrocellulose and spiking Dengue or Zika NS1 in human serum in increasing concentrations from 0 μg/ml to 3 μg/ml. First, I tested the titration curves with DENV-1 NS1 with 136 spotted on the nitrocellulose (Fig. 45, a, NStar-PAN and NS-136, in blue and red, respectively). We observe that both NPs were able to sandwich with DENV-1 NS1 and that the signal was similar for both blue and red nanoparticles, moreover, there was no signal in the blank, indicating binding occurred due to the presence of DENV-1 NS1. In contrast, when I tested the same pairs of antibodies with Zika solutions, we observe that only NS-136 were able to bind, and NStar-PAN did not show a signal even at high concentrations of Zika NS1 (Fig. 45, c). I then spotted 323 on the nitrocellulose, and observed that both NStar-PAN and NS-136 were able to bind with DENV-1 NS1 (Fig. 45, b). When running either NS-136 or NStar-PAN with ZIKV NS1 and 323 on the nitrocellulose (Fig. 45, d), no binding was observed, as expected from the pairwise analysis, that showed that 323 was a specific, Dengue-only antibody.

Figure 46: Individual titration curve of antibody pairs against ZIKV (right) and DENV (left) NS1 spiked in human serum at concentrations 3125, 1250, 625, 312, 125 and 0 ng/ml. NStar-PAN (blue) and NS-136 (red) paired with 136 (a, top) or 323 (b, bottom) against DENV-1. NStar-PAN (blue) and NS-136 paired with 136 (c, top) or 323 (d, bottom) against ZIKV.
The same analysis was done for the three remaining Dengue serotypes DENV-2-DENV-4. I calculated the limits of detection and dissociation constants of all the pairs and NS1 of the four Dengue serotypes and Zika by fitting a Langmuir equation (Table 10), the limits of detection were between 4-200ng/ml, which are in the range of NS1 concentrations found in Dengue patients\textsuperscript{130}. We observe that the Ab 136 did not bind to DENV-4 NS1, and that ZIKV NS1 was only detected by the 136/136 antibody pair.

Table 10: Calculated limits of detection (LoD) and effective dissociation constants (Kd) of each DENV serotype and ZIKV with each of the four antibody pairs

<table>
<thead>
<tr>
<th>Ab NP</th>
<th>Ab NC</th>
<th>ng/ml</th>
<th>D1V</th>
<th>D2V</th>
<th>D3V</th>
<th>D4V</th>
<th>ZV</th>
</tr>
</thead>
<tbody>
<tr>
<td>136</td>
<td>136</td>
<td></td>
<td>83.3</td>
<td>62</td>
<td>36.3</td>
<td>N/A</td>
<td>217.3</td>
</tr>
<tr>
<td>136</td>
<td>323</td>
<td></td>
<td>9.6</td>
<td>4.4</td>
<td>4.3</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>PAN</td>
<td>136</td>
<td></td>
<td>121.8, 215.8, 48.3</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAN</td>
<td>323</td>
<td></td>
<td>415.9, 369.2, 237.5</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DIFFERENTIAL MULTIPLEXED DIAGNOSIS

I achieved multiplexed detection by running a mixture of NS-136 and NStar-PAN on a strip that had the antibody 323 on test area 2, the antibody 136 on test area 3 and the positive control antibody (anti-Fc) on area 4 (Fig. 40). The test area 1 was used as a blank for normalization. I exposed the test to DENV-1 NS1, ZIKV NS1 and different mixtures of the two diseases, with a total concentration of spiked NS1 of 1 μg/ml in human serum. When only DENV-1 was present in the solution, signal was achieved on both test areas 2 and 3, and the signal was due to binding of both the blue NStar-PAN and NS-136, as expected (Fig. 46, lane 5). When only ZIKV NS1 was present, signal was achieved only in test area 3 (Fig. 46, lane 1). Mixtures of DENV-1 and ZIKV at increasing ratios of DENV-1:ZIKV of 1:2, 1:1 and 2:1 (Fig. 46, lanes 2 to 4, respectively) showed intermediate intensities. The same experiment was repeated with the three remaining serotypes of Dengue achieving similar results, except DENV-4, which as expected from table 6 (and subset table 9), did not bind to the 136 antibody, resulting in an easier test to interpret.
DATA PROCESSING AND QUANTIFICATION

I used ImageJ to measure the RGB contributions to both spots in test areas 2 and 3, after running DENV-1 NS1, ZIKV NS1 and the mixtures of DENV-1 and ZIKV. After, I trained an LDA with the six predictor variables (red, blue and green intensities of each test band) to distinguish three clusters: Dengue, Zika and the mixtures of Dengue and Zika at different ratios of NS1. The resulting confusion matrix (Fig. 47, right) showed that LDA was able to successfully distinguish DENV-1 from ZIKV and the mixtures of DENV-1 & ZIKV (green squares in the diagonal, Fig. 47), except from one test that was incorrectly-classified, which was a mixture of DENV-1 and ZIKV at a ratio of 2:1 (red square, Fig. 47). The classifier accuracy was 96%. Principal component analysis was used to plot the results from six to two dimensions to help visualizing the separate clusters of DENV-1, ZIKV and the mixtures of DENV-1-ZIKV at different ratios of Dengue to Zika NS1 (Fig. 47, left).

Figure 47: Membranes showing DENV only (1), ZIKV only (5) or mixtures of the two at ratios of DENV:ZIKV 2:1, 1:1, 1:2, lanes 2, 3 and 4, respectively. Total NS1 concentration 1250ng/ml.

Figure 48: Clustered data of the Dengue/Zika diagnostic plotted in its principal components and confusion matrix with results classification. In green, diagonal values correctly classified, in red, out of diagonal value incorrectly classified.
I also probed the IA behavior with mixtures of NS1 of each of the four Dengue serotypes, and obtained 92% classification accuracy with all the Dengue serotypes and different ratios of mixtures of Dengue-Zika (Table 11). For the LDA analysis, because each of the Dengue serotypes had slightly different binding profiles, I considered that each serotype would be part of only one cluster, and considered the mixtures of DENV-1, DENV-2, DENV-3 with Zika to be part of only one group. The mixtures of DENV-4 and Zika were considered as a different group, as DENV-4 had very different binding characteristics than the other serotypes. When classifying the tests, we observed that although the classification accuracy was acceptable (92%), some tests were incorrectly classified: Matlab was not able to separate one DENV-2 only and a DENV-3, as well as a total of four DENV and ZIKV mixtures that were incorrectly classified as Dengue only tests. These incorrectly classified mixtures corresponded to mixtures of DENV and ZIKV NS1 that contained the lowest concentrations of ZIKV NS1, at a DENV to ZIKV ratio of 2:1.

Table 11: Confusion matrix of the DENV/ZIKV test with classification of all DENV serotypes and ZIKV, as well as mixtures of the two diseases. In green, diagonal values correctly classified, in red, out of diagonal value incorrectly classified.

<table>
<thead>
<tr>
<th>True class</th>
<th>Z</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>3+4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Z+D123</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Z+D4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Thus, we observe that it is possible to distinguish double infections of Dengue and Zika by using only antibodies originally generated against Dengue NS1, in a combination of specific and cross-reactive antibodies. Taking advantage of the different colors of gold nanoparticles can provide a differential readout in the event of mixed infections.

Enhancing an existing commercial Dengue diagnostic

After the Zika outbreak, several reports appeared showing that Alere’s Dengue NS1 diagnostic was cross-reactive with Dengue and Zika. Interestingly, this indicated that the antibodies used for Alere’s SD NS1 Ag diagnostic were cross-reactive, suggesting that I could use the same approach with two colored NPs in order to convert a nonspecific
commericially available diagnostic into a specific, multiplexed test that could diagnose specifically both Dengue and Zika (Scheme in Fig. 48). In order to do that, I used the red cross-reactive nanospheres (RT-NS) of the commercial diagnostic, and I added blue nanostars conjugated to a Dengue-specific PAN antibody mixture. I then added another Dengue-specific antibody (323) on the nitrocellulose by manually pipetting on the commercial diagnostic. Finally, I used the cross-reactive RT Ab that was already printed on the commercial immunoassay, as the cross-reactive antibody on the nitrocellulose.

![Diagram](image)

*Figure 49: Scheme of the test to enhance a cross-reactive commercially available diagnostic that can be used to distinguish DENV and ZIKV.*

I ran tests by spiking NS1 of Dengue, Zika and a 1:1 mixture of the two in human serum. As expected, we observe that Dengue is able to bind to the spotted 323 mAb (Fig. 49, lane 1), as well as to the test band in the commercial diagnostic. We observe that when DENV-1 NS1 ran, the NStar-PAN had a high affinity on the commercial test band, which resulted in the formation of a blue test band. When only ZIKV NS1 ran in the membrane, signal appeared on the commercial diagnostic test band, and the signal was red, indicating that the commercial nanospheres were binding to Zika NS1 at the commercial antibody test band, and no signal was observed from either the spotted 323 dot (Fig. 49, lane 3). When mixtures of Dengue and Zika were tested, the signal in the commercial diagnostic at the test band turned from blue to purple, while the blue dot characteristic of DENV-1 was visible, showing that a higher number of red commercial NPs were binding to the commercial diagnostic's test band, due to the presence of ZIKV NS1 (Fig. 49, lane 2).
Figure 50: Pictures of the enhanced diagnostic tested with DENV (1), ZIKV (2) and a 1:1 mixture of the two diseases (2). NS1 total concentration was 1250ng/ml

DATA PROCESSING AND QUANTIFICATION

I used ImageJ to measure the RGB contributions of the spotted 323 and the commercial diagnostic test band. After, in a similar manner than the previous tests, I trained an LDA to distinguish the different Dengue and Zika tests, and plotted the results in a confusion matrix, in which the diagonal values represent the correctly classified tests (green squares, Fig. 50, right), we see that LDA was capable to successfully distinguish DENV NS1 from ZIKV NS1 and from the mixtures of DENV and ZIKV. Principal component analysis was used to plot the results from six to two dimensions that could separate the clusters of DENV-1, ZIKV and the mixtures of DENV-1-ZIKV at a 1:1 ratio of Dengue to Zika NS1 (Fig. 50, left).

Figure 51: Clustered data of the enhanced diagnostic plotted in its principal components and confusion matrix with results classification. In green, diagonal values correctly classified, in red, out of diagonal value incorrectly classified.

DISTINGUISHING ALL SEROTYPES OF DENGUE WITH DENV-3 ANTIBODIES

I next attempted to use the same approach of using cross-reactive antibodies together with nanoparticles of different colors in a different application, to confirm that the same
principle could be applied. In this case, the objective was to multiplex a Dengue serotype-specific test that could detect and distinguish the four different serotypes in only one nitrocellulose strip, and by using DENV-3 antibodies, only. This approach has several advantages: First, it could allow to diagnose the serotype of patients by using only 30µl of serum instead of the 150µl that were required in the Dengue serotype-specific diagnostic presented in chapter three, which in turn permits the use of a fingerprick as the method of obtaining the serum, instead of the venipuncture that is required to obtain volumes higher than 100µl\[^{163}\]. Moreover, by using antibodies generated against only one of the serotypes of Dengue, the device could be greatly simplified, the cost of production of the antibodies would be \(\frac{1}{4}\) of the cost of producing antibodies for each of the serotypes in individual mice injections, and in turn, because less antibodies are used, the cost of production of the antibodies would as well be reduced.

**Antibody Pairs**

By following the same approach that was used in chapter 3, we generated three antibodies by immunizing mice with NS1 from Dengue 3, as the antigen. I tested the different combinations of the antibodies in pairs by conjugating one antibody on the nanoparticles, immobilizing the other antibody on the membrane, and testing all the combinations against NS1 from the four Dengue serotypes, as well as blank and Zika NS1 as negative controls. We observe that most of the antibodies were able to bind to more than one Dengue serotype (green squares Fig. 51, left). The pair combinations show which antibodies exhibited crossover reactivity. For example, 411 did not bind to either DENV-2 not DENV-4 NS1, the antibody 55 was able of binding to DENV-1, DENV-3 and DENV-4 NS1, and the antibody 323 was able of binding to the four Dengue serotypes. By analyzing the pair-wise tests I designed a diagnostic in which the antibodies 411 and 323 were spotted on the nitrocellulose (Fig. 51, right, scheme), and they provided spatial localization. In order to mitigate the crossover reactivity, I used two different NPs to enable differential readout; else, it would not be possible to distinguish DENV-2 from DENV-4, nor DENV-3 from DENV-1. Thus, strategic design of the antibody pairs on the NPs and the nitrocellulose led to an arrangement where the red nanospheres were conjugated to the antibody 323, the blue nanostars to the antibody 55, and the antibodies 323 and 411 were spotted on positions 2 and 3 of the nitrocellulose, respectively. The
positive control was spotted on location 1, and location 4 was used as a blank for test normalization (Fig. 51).

**Figure 52:** Matrix of pairwise signal from antibodies raised against D3V(left), green indicates that signal was observed in the immunoassays. Scheme of the diagnostic built to detect the four serotypes of Dengue in one test, using antibodies raised against DENV-3.

**LODs of NP-Ab conjugates for individual antigens**

I next tested the limits of detection of all the antibody pairs with each of the Dengue serotypes. The titrations of the individual antibody pairs were done by conjugating one antibody on the nanoparticles, spotting the other antibody on the nitrocellulose and running increasing concentrations of NS1 of each of the four Dengue serotypes spiked into human serum from 0 μg/ml to 3 μg/ml. First, I tested the titration curves for DENV-1, DENV-2, DENV-3 and DENV-4 NS1 with NS-323 (red empty circles, Fig. 52 a, c, e, g, respectively) and NStar-55 (blue empty squares, Fig. 52 a, c, e, g) and paired each of the nanoparticles with the antibody mAb 411 on the nitrocellulose. We observe that neither DENV-2 NS1 nor DENV-4 NS1 were able to bind to the spotted Ab 411 (Fig. c and g) even at high concentrations of NS1; as expected from the pairwise analysis (Fig. 51). Moreover, we observe that both DENV-1 NS1 and DENV-3 NS1 were able to bind to the Ab-323 spotted on the nitrocellulose, and that NS-323 showed a stronger signal at high concentrations than NStar-55 (Fig. 52 a and d, respectively). It was expected that DENV-1-NS1 and DENV-3-NS1 would be most cross-reactive, as the antibodies were raised
against DENV-3 NS1, and these two NS1 sequences have the highest sequence identity measured in BLAST (79%, Table, 3, Chapter 3) among the four serotypes. Moreover, when no NS1 was added, at 0 μg/ml NS1, none of the antibody pairs showed a signal, indicating that binding was due to the presence of NS1, and not due to nonspecific binding.

I then spotted the antibody Ab-323 on the nitrocellulose and tested the limits of detection curves for DENV-1, DENV-2, DENV-3 and DENV-4 NS1 with NS-323 (red circles, Fig. 52 b, d, f and h, respectively) and NStar-55 (blue squares, Fig. 52 b, d, f and h, respectively). We observe that NPs were able to bind to the 323 antibody spotted on the nitrocellulose. For DENV-1 NS1, both NS-323 and NStar-55 were able to bind with similar intensities to the nitrocellulose mAb-323 (Fig. 52 b, red circles and blue squares, respectively), DENV-2 NS1 was only detected with NS-323 (red circles, Fig. 52 d), but NStar-55 was not able to bind (blue squares, Fig. 52 d), DENV-3 NS1 was able to bind with higher intensity to NS-323 than to NStar-411 (Fig. 52 f) and DENV-4 was able to bind to NStar-411 (Fig. 52 g, blue squares).

![Figure 53](image-url)

Figure 53: Individual titration curve of antibody pairs against all Dengue serotypes, NS1 spiked in human serum at concentrations 3125, 1250, 625, 312, 125 and 0ng/ml. NStar-55 (blue) and NS-323(red) paired with 411 (a, top) or 323(b, bottom) against DENV-1. NStar-55 (blue) and NS-323 (red) paired with 411 (c, top) or 323(d, bottom) against DENV-2. NStar-55 (blue) and NS-323 (red) paired with 411 (e, top) or 323(f, bottom) against DENV-3. NStar-55 (blue) and NS-323 (red) paired with 411 (g, top) or 323(h, bottom) against DENV-4.
I calculated the limits of detection and dissociation constants of each of the antibody pairs and for each of the four Dengue serotypes by fitting a Langmuir equation (Materials and Methods), the limits of detection were between 4-400ng/ml (Table 12), which are in the range of NS1 concentrations that are found in Dengue patients.

Table 12: Calculated limits of detection (LoD) and effective dissociation constants (Kd) of each DENV serotype with each of the four antibody pairs

<table>
<thead>
<tr>
<th>Ab NP</th>
<th>Ab NC</th>
<th>ng/ml</th>
<th>NS1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>D1V</td>
</tr>
<tr>
<td>Nstar-55</td>
<td>411</td>
<td>LoD 129.1</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kd 501.9</td>
<td>N/A</td>
</tr>
<tr>
<td>NS-323</td>
<td>411</td>
<td>LoD 8.9</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kd 92.2</td>
<td>N/A</td>
</tr>
<tr>
<td>Nstar-55</td>
<td>323</td>
<td>LoD 79.8</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kd 252.7</td>
<td>N/A</td>
</tr>
<tr>
<td>NS-323</td>
<td>323</td>
<td>LoD 229.6</td>
<td>379.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kd 240.9</td>
<td>424.8</td>
</tr>
</tbody>
</table>

DIFFERENTIAL MULTIPLEXED DIAGNOSIS

Multiplexed detection was achieved by using a strip that had the antibody 323 on test area 2, the antibody 411 on area 3, and the positive control antibody (anti-Fc) on area 4 (Fig. 51). Test area 1 was used as a blank for normalization. A mixture of the two NP-antibody conjugates was mixed in the solution (NS-323 and NStar-55). When exposed to the individual antigens at 1 µg/ml in human serum, the tests showed the expected patterns (Fig. 53). When DENV-2 NS1 was present, test area 2 showed a red spot along with the control area 4 (Fig. 53 lane 2). This suggests that the NS-323/323 could form a sandwich with ZIKV NS1 among the mixture of the NP-antibodies, and NStar-411 did not bind to either 323 nor 411 on the nitrocellulose. When DENV-4 NS1 was present, a blue dot appeared in test area 2, indicating that the antibody 55 conjugated to the nanostars was able to form a sandwich with 323 on the nitrocellulose (Fig. 53 lane 4). When DENV-1 was present in the solution, a blue dot formed in position 2, due to the binding to NStar-55 and a red/purple dot formed in position 3, indicating binding from both NS-323 and NStar-55 (Fig. 53 lane 1). DENV-3 NS1 was able to bind to both locations, 2 and 3 and formed a purple dot in position 2 and a red/blue dot in position 3, due to the stronger
binding of NS-323 to 411, than NStar-55 to 411 with the antibody 323 (Fig. 53 lane 3). For serum with no NS1, only the positive control area 4 showed a spot (Fig. 53 lane Ø).

![Image of gel electrophoresis with lanes labeled 1 to 4 and a blank (Ø).]

Figure 54: Pictures of serotype-specific membranes with D1V(1), D2V(2), D3V(3), D4V(4) and blank (Ø)

**DATA PROCESSING AND QUANTIFICATION**

I next used ImageJ to measure the red, green and blue contributions of the signals in the two spots for the NS1 of each Dengue serotype and trained a linear discriminant analysis (LDA, cross-validation of 10) with six predictor variables (RGB of both spots) to distinguish the four Dengue serotypes. A confusion matrix was used to plot the results from the LDA, where the diagonal values show the responses that were correctly classified, and the out of diagonal values would show miss-classified values. In this case, LDA was able to successfully separate the four Dengue serotypes and the blank (Fig. 54, right), with 100% accuracy. Principal component analysis was used to plot the results from six to two dimensions that could separate the clusters of the four Dengue serotypes and blank (Fig. 54, left).

![Image of principal component analysis with PC2 and PC3 axes showing clusters for D1V, D2V, D3V, and D4V, with a confusion matrix showing correct classification.]

Figure 55: Clustered data of the serotype-specific diagnostic plotted in its principal components and confusion matrix with results classification. In green, diagonal values correctly classified.
I also probed the IA behavior with mixtures of NS1, and obtained a 81% classification accuracy with all the combinations of one, two, three and four Dengue serotypes (Table 13).

Table 13: Confusion matrix with results classification of all Dengue serotypes and possible mixtures. In green, diagonal values correctly classified. In red, incorrectly classified out of diagonal values.

The misclassified tests were a DENV-3 confused with a mixture of 1 and 3, a DENV-1-DENV-3 mix confused with a DENV-3 only, a mixture of 2 and 3 confused with a mixture of 2 and 3, which was expected due to the similarity in signal of DENV-3 and DENV-1, these two are also the serotypes with highest homology. A mixture of DENV-2-DENV-3 and a mixture of DENV-3-DENV-4 were confused by a DENV-3 only, which was expected as the DENV-2 and DENV-4 had a lower signal than the DENV-3. Two DENV-1-DENV-2-DENV-3 mixtures were misclassified as DENV-1-DENV-2-DENV-4 and a DENV-1-DENV-2-DENV-3-DENV-4. One mix of DENV-1-DENV-3-DENV-4 was misclassified as a DENV-1-DENV-2-DENV-4 and one DENV-2-DENV-3-DENV-4 was misclassified as a DENV-1-DENV-2-DENV-4. As expected, the DENV-1 and DENV-3 were the serotypes that in the mixtures would show the most misclassification probability, due to their similarity in the individual tests. Moreover, as expected, the mixtures of three or more serotypes had a larger probability of misclassification; fortunately, co-infections with more than one serotype of Dengue are not common, albeit possible. Hence the classification algorithm could be improved by accounting for the probabilities in a general population of suffering from infection by more than two, three or the four Dengue serotypes.
simultaneously. On the other hand, in practical terms, a solution for this issue could be either adding in the diagnostic one DENV-1 antibody or DENV-1 cross-reactive with had less cross-reactivity; or a third nanoparticle with a different color (preferably green, as it is an orthogonal color to red and blue), in order to add one more dimension in the analysis.

**DETECTION OF EBOLA AND MARBURG USING MARBURG ANTIBODIES**

Finally, I tested if viruses of other species, other than the flaviviruses Dengue and Zika, could as well be diagnosed with the same approach of using cross-reactive antibodies. Therefore, I tested cross-reactive mAb originally generated against Marburg, in order to produce a diagnostic that could detect the related Ebola (EBOV), Marburg (MARV) and mixtures of the two diseases. In the case of EBOV and MARV, the viruses share only 30% identity. For the EBOV and MARV diagnostic I chose two antibodies of the five that were tested, and both were cross-reactive for EBOV and MARV. Hence, the diagnostic pattern was slightly more complicated, as the relative binding of one pair of antibodies versus the other in each of the two spots in the nitrocellulose was the cause of the different signals for EBOV, MARV and the mixtures of the two, without using a selective antibody immobilized on the nitrocellulose, as I had in the previous two examples.

**ANTIBODY PAIRS**

Antibodies raised against MARV GP were produced by immunizing mice with purified MARV glycoprotein. In order to proceed with testing the antibody pairs, because MARV and EBOV are highly pathogenic, I used replication-competent vesicular stomatitis viruses bearing the glycoprotein of Ebola virus\(^{185}\) (VSV-rGP-EBOV) or the glycoprotein of Marburg virus (VSV-rGP-MARV) instead. We produced VSV-rGP-EBOV and VSV-rGP-MARV-infected viral supernatants allowing the VSV viruses to replicate by infecting BHK cells (Baby Hamster Kidney fibroblasts). I tested five antibodies raised from the immunizations with the supernatants containing VSV-rGP-MARV and VSV-rGP-EBOV, by conjugating one antibody on the gold nanoparticles and immobilizing the other on the nitrocellulose. We observe that out of the 25 combinations of antibody pairs, 1G11 was cross-reactive and capable of pairing with itself with both the VSV-rGP-MARV and the VSV-rGP-EBOV; moreover, the antibody 2G12 was cross-reactive as well with 1G11, but showed a much lower signal when VSV-rGP-EBOV was the antigen (Fig. 55, left). Therefore, in order to detect both Ebola and Marburg, I conjugated the cross-reactive
1G11 on the gold nanospheres and 2G12 on the gold nanostars; and immobilized 2G12 on test area 2 and 1G11 on test area 1. Test area 4 contained the positive control (anti-Fc), and test area 1 was used as a negative control (Fig. 55, scheme on the right).

<table>
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<tr>
<th>NPs</th>
<th>2D10</th>
<th>1G11</th>
<th>2D6</th>
<th>1F1</th>
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Figure 56: Matrix of pairwise signal from antibodies raised against Marburg, green indicates that signal was observed in the immunoassays. Scheme of the diagnostic built to detect Ebola and Marburg based on cross-reactive antibodies.

LODs of NP-Ab conjugates for individual antigens
I then tested the limits of detection by conjugating one of the antibodies (1G11 or 2G12) on the nitrocellulose and running the infected (VSV-rGP-EBOV or VSV-rGP-MARV) supernatants at different dilutions with uninfected media. First, I tested the titration curves of VSV-rGP-EBOV with 1G11 immobilized on the nitrocellulose and NStar-2G12 and NS-1G11. We observe that both NPs were able to bind strongly to the VSV-rGP-EBOV (Fig. 56, a). I then tested VSV-rGP-EBOV against 2G12 spotted on the nitrocellulose and NStar-1G11 and NS-2G12 (Fig. 56, c), and observed very low binding even at high concentrations of NStar-2G12 and NS-1G11, which was expected, as 2G12 spotted on the paper had a very low binding to the VSV-rGP-EBOV.

I then tested the titrations of VSV-rGP-MARV. We observe a strong binding when spotting 1G11 on the nitrocellulose and using either the NStar-2G12 or NS-1G11 (Fig. 56, b). Moreover, we observe that when 2G12 was absorbed on the paper and VSV-rGP-MARV was used as the antigen, signal from both NStar-2G12 and NS-1G11 was observed (Fig. 56, e).
Hence, I expected to observe a difference in the diagnostic between EBOV and MARV, especially in test area 2 of the nitrocellulose.

DIFFERENTIAL MULTIPLEXED DIAGNOSIS

I achieved multiplexed detection by running NS-1G11 and NStar-2G12 on a strip that had the antibody 2G12 on test area 2, the antibody 1G11 on test area 3 and the positive control antibody (anti-Fc) on area 4 (Scheme in Fig. 55, right). The test area 1 was used as a blank for normalization. I exposed the test to VSV-rGP-EBOV, and observed the formation of one purple dot in test area 3, and slight background in test area 2 (Fig. 57, lane 5). After, I exposed the test to VSV-rGP-MARV and observed the formation of two dark spots of purple-color, which resulted from the binding of both the nanostars and the nanospheres in both test areas 2 and 3. Mixtures of VSV-rGP-EBOV and VSV-rGP-EBOV at EBOV to MARV ratios of 1:2, 1:1 and 2:1 yielded intermediate colors (Fig. 57, lanes 2
to 4, respectively). When uninfected BHK cell supernatant was tested, no signal was observed in any of the test bands, and only the positive control anti-Fc showed a signal (Fig. 57 lane Ø).

Figure 58: Membranes showing Marburg only (1), Ebola only (5) or mixtures of the two at ratios of MARV:EBOV 2:1, 1:1, 1:2, lanes 2, 3 and 4, respectively; or Blank (Ø).

DATA PROCESSING AND QUANTIFICATION

I measured the RGB contribution of both spots by ImageJ in test areas 2 and 3 after running VSV-rGP-EBOV, and VSV-rGP-MARV, and mixtures of the two viruses at different ratios. After, I trained an LDA to distinguish the two diseases and plotted the results in a confusion matrix (Fig. 58, right). We observe that LDA was able to distinguish and accurately classify VSV-rGP-EBOV, VSV-rGP-MARV and the mixtures of the two diseases with a 100% classification accuracy. Principal component analysis was used to plot the results from six to two dimensions in order to visualize the separate clusters of VSV-rGP-EBOV, VSV-rGP-MARV and the cluster containing mixtures of the two viruses at different ratios of VSV-rGP-EBOV to VSV-rGP-MARV (Fig. 58, left).

Figure 59: Clustered data of the Ebola/Marburg diagnostic plotted in its principal components and confusion matrix with results classification. In green, diagonal values correctly classified.

The results presented here demonstrate that we are able to screen, identify and exploit cross-reactive and specific antibodies raised against one virus in order to diagnose...
another closely-related virus. I have tested this setup with diseases of different identities, from 30% in the case of Ebola and Marburg, to close to 80% for the four serotypes of Dengue. In this setup, the cross-reactive antibodies can give a positive signal for the two related diseases, which is typically a negative outcome in lateral flow tests –false positives-. However, we see that it is possible to overcome this negative effect and use the cross-reactive antibodies to multiplex tests against similar diseases. In order to distinguish between the two viruses and double infections I use gold nanoparticles of different extinction spectra. By doing that, it is possible to extract which virus is being tested from knowing the nanoparticle or relative combination of nanoparticles that is binding on the test band.

After testing several scenarios, we observe that cross-reactive antibodies together with gold nanoparticles of different extinction were able to distinguish closely-related diseases. In summary, I have shown that we can use cross-reactive antibodies raised from immunizing mice with Dengue serotype 3 NS1 in order to distinguish the four serotypes of Dengue. I also showed that it is possible to use cross-reactive antibodies raised against Marburg, and use them, together with gold nanoparticles of different extinctions, to detect Ebola in VSV-infected supernatants. Moreover, I showed that we can design and test a diagnostic capable of detecting Dengue, Zika and co-infections of the two diseases. Finally, I showed we can enhance a commercial Dengue diagnostic that is cross-reactive with Zika and turn it into a multiplexed Dengue and Zika diagnostic. The limits of detection of each of the antibodies presented in this chapter has been characterized. This test requires the use of image post-processing and further classification by LDA. I have performed the image analysis and test classification on a computer, for objective data analysis and accurate classification, with the disadvantage that the analysis was not delocalized. However, the codes written could be done on a phone, which would give capabilities for geolocating the diagnostics, as well as accurate analysis.

**DISCUSSION**

In emergency outbreaks, inexpensive diagnostics that can deliver results within the hour are increasingly needed for patient treatment and control of epidemics. Accurate pathogen identification is especially needed for diseases that have different risk profiles,
especially, when clinical diagnosis of the epidemic is not possible, due to co-circulation of other diseases with the same symptoms. POC diagnostics can provide rapid detection of antigens where molecular tests such as PCR are not available, slow, expensive, require trained personnel and a cold chain for the reagents and patient samples. This is especially true in outbreak situations, where rapid diagnostics have been an important asset to treat patients and control the spread of the disease\textsuperscript{81, 179}. Moreover, the highest impact of POC in outbreak situations is during the initial stages of the outbreak, when they can most contribute to early triaging and treatment of patients, thus having an impact on reducing the overall size of the outbreak. The diseases presented in this chapter, Dengue, Zika, Ebola and Marburg, all have similar non-specific flu-like initial symptoms, such as fever or headache. Because these diseases have a major impact on countries in the tropics, we have developed rapid diagnostic tests that can be affordable, specific, sensitive, rapid, equipment free and easily delivered to those in need. One issue when developing rapid diagnostics for outbreak situations is that although the tests can be designed to be rapid and easy to use, constructing the diagnostics can take a long time, especially due to the need of developing new antibodies against specific antigens of the new pathogen causing the outbreak, which is at least 4 months.

This chapter describes a method to rapidly screen for out of the shelf, cross-reactive antibodies that were originally developed against a pathogen of the same species to the one being tested. We can build rapid tests that can exploit the cross-reactive antibodies to specifically detect and distinguish both the new pathogen and the disease that was used originally to raise the antibodies. Because we are able to use the cross-reactivity of already existing antibodies, we can reduce the time to develop the new, disease-specific antibodies, and therefore can deliver specific rapid diagnostics when they can have the highest impact: during the initial stages of the outbreak. Moreover, there is no need to perform new immunizations, thus reducing the cost of the lateral flow immunoassays. Because the diagnostics run on capillary action to move the reagents and the sample, no electric power is needed, therefore, tests can be run at the bedside or in rugged environments with limited lab infrastructure. The tests are self-contained, thus the risk of infection by laboratory personnel during sample handling is also reduced. In this chapter,
I exploited cross-reactive antibodies to develop specific diagnostics and I tested this approach using four different examples:

First, we can identify the four Dengue serotypes by using antibodies originally raised against DENV-3 NS1 which could cross-react with the four serotypes of Dengue. In order to do that, I performed an unbiased parallel screening of all the anti-DENV-3-raised antibody combinations against the four different Dengue serotypes, which is an approach that can be used as well for other related diseases. I used gold nanoparticles of different extinction spectra in order to distinguish the four serotypes. These results are significant for several reasons: (1) First, although the homology of the four Dengue serotypes is high (85-90), the combination of gold nanoparticles of different extinction with cross-reactive antibodies can be a powerful tool to distinguish these closely-related diseases. This test could identify the four Dengue serotypes by using only 30μl of patient serum. This could be achieved by a finger prick, without the need of venipuncture, thus simplifying the diagnostic. (2) Secondly, the serotype-specific diagnostic can be used for epidemiological surveillance, which is critical in both patient care and public health preparedness. The serotype-specific Dengue test described could be used in medical patient care as well as an inexpensive test to screen for asymptomatic patients. Most importantly, the diagnosis of the four serotypes of Dengue has important implications in public healthcare policy. The introduction of a new Dengue serotype previously unknown to an area can result in epidemics of Dengue hemorrhagic fever because the individual serotypes do not provide long-lasting cross-protection against the four Dengue serotypes, and the infection by a new serotype increases the patient’s risk of developing Dengue hemorrhagic fever. Rapid testing of the four serotypes can, therefore aid public health authorities to predict and respond early to epidemics of Dengue hemorrhagic fever. (3) On the third place, the use of cross-reactive antibodies for serotype-specific Dengue detection is critical for preparedness against the emergence of a new Dengue serotype. Although there are four Dengue serotypes causing large epidemics in humans in urban settings, there is no barrier for the emergence of a new sylvatic Dengue serotype that can infect the human population. Each of the four known human Dengue serotypes represents an independent cross-species transmission of a sylvatic Dengue into humans. Although sylvatic Dengue transmission typically occurs in the forests in cycles between monkeys and Aedes
mosquitoes, these viruses can cause disease in humans as well as outbreaks in urban settings. A Dengue serotype 5 has been reported\textsuperscript{20}, and different sylvatic Dengue serotypes exist and have shown to cause Dengue hemorrhagic fever in humans\textsuperscript{39}, therefore, the re-emergence of sylvatic DENV in the human transmission cycle is a realistic prospect. Serotype-specific Dengue tests that can use cross-reactive antibodies to diagnose and distinguish the four Dengue serotypes could be used as a screening tool in the event of the emergence of a new Dengue serotype. (4) Finally, the serotype-specific diagnostic is significant for DENV vaccine clinical trials. The different vaccine candidates to not show equal protection against all the Dengue serotypes. There has been a lot of discussion in the literature about the risks of these Dengue vaccines and their potential to increase the epidemics of Dengue hemorrhagic fevers, as these are caused by heterologous infections of Dengue. Hence, in Dengue vaccine clinical trials, knowledge on the circulating serotypes is of most importance to inform the trial outcomes.

The second example of diagnosis of different diseases by using cross reactive antibodies is the case of the Dengue and Zika diagnostic. Dengue and Zika viruses share between 72-73 homology. Here, I performed screening of several antibodies originally raised against Dengue and selected one of them that could as well detect Zika in lateral flow assays. I then designed and tested an assay that used specific and cross-reactive antibodies, together with different nanoparticles to distinguish the two diseases as well as mixed infections. This diagnostic is significant for several reasons: (1) First, although Dengue and Zika share the same initial symptoms, the potential outcomes of each of them are different, hence the need of a specific diagnostic. Dengue can lead to hemorrhagic fevers in about 1% of the infected individuals, whereas Zika can lead to neurological disorders such as Guillain Barré syndrome or microcephaly of newborns. (2) 80% of Zika patients do not show symptoms against the disease\textsuperscript{186}. The diagnostic presented can be used for epidemiology surveillance in both symptomatic and asymptomatic patients that might carry the disease. Detection of Zika NS1 can be more challenging than Dengue as Zika leads to lower viremia\textsuperscript{169}. A solution for this issue could be concentrating the serum 5-times, in a similar approach to chapter 3, or by using larger volumes of patient serum. (3) Due to the similarity of the two viruses, there is still the need of a serological rapid diagnostic that can distinguish between them. IgG and IgM tests
have not been successful, as the immune responses of individuals against the viruses can be non-specific, leading to false positives in both lateral flow and ELISA serological assays. Therefore, a NS1 test could aid in the early diagnosis of the virus infections by providing a more specific response. (4) The diagnostic used antibodies that were only Zika cross-reactive, therefore, potential outbreaks of Yellow Fever of West Nile Virus would not yield false positives in the diagnostic, as the NS1 of these diseases is not detected by our test. In order to detect other flaviviruses, a new unbiased screening of the antibodies would be needed. (5) The Dengue and Zika NS1 diagnostic could also be used to detect the pathogen in mosquitoes, hence detecting the diseases before the large outbreak occurs and in turn alerting public health officials and allowing them to take action by reducing the mosquito population.

The third example I showed is the detection of Dengue and Zika by enhancing Allere’s widely available Dengue diagnostic that is cross-reactive with Zika\(^{182}\). Therefore, showing that it is possible to update an existing diagnostic originally developed against one similar disease and convert it into a specific test for the two co-circulating pathogens. This is significant for several reasons: (1) It indicates that in the point of care, where the commercial Dengue tests are currently widely available, one can easily and rapidly hack an available diagnostic to detect the new pathogen, as well as co-infections by simply adding a new test band and additional gold nanoparticles at a very low cost. This is especially significant in some economically-challenged tropical countries where Zika and Dengue outbreaks occur, where the cost of the diagnostic is an important factor. (2) This same approach can be used as a platform to detect the emergence of new pathogens in the future. There are approximately 70 viruses of the Flaviviridae genus alone\(^{147}\). Allere’s Dengue rapid test is cross-reactive with NS1 of all the flaviviruses that were tested in the lab, which included Yellow Fever, West Nile Virus, Japanese Encephalitis, Tick Borne Encephalitis, the four serotypes of Dengue and Zika. Therefore, in the case that any of these flaviviruses emerged, they would also show as positives in Allere’s rapid test and the same methodology that I described could be applied to enhance the commercially available rapid test and in turn diagnose the new pathogen. This is not an unrealistic prospect. Many flaviviruses other than Zika and Dengue are dangerous pathogens for humans and can cause outbreaks. For example, Yellow Fever has in the past caused
large epidemics that disrupted economies and decimated populations. Recently, infections with Yellow Fever have been reported both in monkeys and humans in several areas in Brazil, and it is of most importance to control its transmission before a new Yellow Fever outbreak starts. The West Nile outbreak in New York in 1999 resulted in 7 deaths and 62 confirmed infections a few weeks after its introduction. Outbreaks of Japanese Encephalitis, which can cause neurologic or psychiatric sequelae in 30-50% of the symptomatic patients, are common in South-East Asia and Western Pacific. The introduction of any of these flaviviruses in new areas could have serious consequences, as they would be introduced in a non-immunized population and could easily cause an outbreak. Hence, enhancing an existing, cross-reactive diagnostic as a platform to detect and distinguish new pathogens could help during the first weeks of an outbreak to screen, treat, identify and isolate infected patients.

Finally, I showed an Ebola and Marburg rapid test developed with cross-reactive Marburg antibodies. Ebola and Marburg only share 31% identity. I obtained two antibodies originally raised against Marburg that were cross-reactive for the two viruses, and used their relative binding affinities for Ebola or Marburg combined with nanoparticles of different colors in order to distinguish the two viruses or co-infections. There have been previous reports using cross-reactive antibodies raised against Marburg as potential therapeutics for neutralizing Ebola Hashiguchi, 2015, Structural Basis for Marburg Virus Neutralization by a Cross-Reactive Human Antibody, as well as research to obtain a vaccine for the two viruses by taking advantage of their similarity. Hence, it was not surprising that we could develop a diagnostic for the two diseases based on the cross-reactive antibodies even though the lower homology, as compared to the aforementioned flavivirus examples. This showed that the approach of using cross-reactive antibodies for diagnostics can be used for pathogens of varying homologies. The results on the Ebola/Marburg test were also significant because even though the antibodies were originally raised against the purified Marburg glycoprotein, we were able to design and test the diagnostic with the less pathogenic replication-competent vesicular stomatitis viruses bearing the glycoprotein of Ebola virus (VSV-rGP-EBOV) or the glycoprotein of Marburg virus (VSV-rGP-MARV), showing that the diagnostics could also detect the cloned protein of the viruses on the membranes of VSVs. In addition, the development of
rapid diagnostics against Ebola and Marburg is of most importance for patient treatment and disease surveillance in outbreak situations, as was seen in the 2014-2015 Ebola outbreak. Although the Ebola outbreak is thought to have first started on December 2013, when a 2-year old and his family died, the case was mis-diagnosed, Ebola spread, and it was not until March 2014 when the WHO released its first report on the West African Ebola Virus outbreak. It took 11 months from then, until the 24th of February 2015 for the approval of an emergency use authorization for the ReEBOV rapid diagnostic, which was the first rapid test approved for use in patients. During the Ebola outbreak, the rapid response in delivering rapid, POC diagnostics would have helped in containing the diseases for several reasons: (1) POC devices could have helped in identifying potential contacts, reaching patients in their homes and testing both symptomatic and asymptomatic individuals. On average, each Ebola patient is estimated to transmit the disease to 1.8 people leading to the exponential growth of infections. The average time from symptoms offset to diagnosis is around 5 days. Mathematical modeling shows that by using POC devices together with PCR for Ebola screening, the size of the epidemic could be reduced between 32-42%, as compared to the PCR-only approach. Hence the capacity to diagnose early and isolate infectious patients could lead to a rapid reduction of the transmission of the disease. (2) However, during most of the outbreak, POC devices were not available in the field, and the diagnosis of Ebola relied on polymerase-chain reaction. PCR tests are slow and expensive, costing around $100 per test, and taking 2-6 hours to perform. Although PCR testing can be done in under 6 hours, the time to diagnosis was much longer during the Ebola outbreak due to limited laboratory and logistic infrastructure. Reports of delays of up to 1 week to diagnosis during the Ebola outbreak were reported, with an average of 1.3-2.3 days to diagnosis after measures to reduce laboratory processing time were implemented. This issue was critical, because delays in diagnostics lead to longer hospital stays, increasing not only bed demand, but also the risk of hospital-acquired disease. (3) Also, PCR testing requires for both samples and reagents to be kept cold or frozen, which is particularly difficult in the affected countries due to the limited infrastructure, frequent power outages and hot climate. (4) Moreover, Ebola and Marburg pose significant risks for health-care workers and require high levels of biosafety not only when manipulating the biological fluids for
testing the diseases, but also when collecting venous blood for the PCR, which needs to be performed by trained medical personnel. (5) Moreover, PCR is an imperfect reference standard\textsuperscript{192, 193}. Reports of different Ebola PCR assays used during the Ebola outbreak showed that the Altona PCR failed to diagnose some Ebola positive patient samples that were positive for both another PCR diagnostic (Trombley) as well as in rapid tests (ReEBOV). In order to help solving these difficulties, we developed one Ebola and Marburg rapid diagnostic that can be used at the point of care, and which requires minimal laboratory facilities or medical training, without the need of extensive handling of pathogenic biological fluids. Moreover, the test could run after collecting blood through a finger prick and return results at the bedside, within minutes instead of days for patients suspected of Ebola or Marburg.

Image analysis of the diagnostics was used in order to ensure objective and quantitative data. Machine vision was very sensitive and capable of classifying the different diseases tested through an LDA algorithm. Although I used a computer for the analysis and classification of the results, image processing and analysis could be done in a mobile phone or through a small reader machine, which could as well provide geolocation of the diagnostic data, and thus capacity to inform and control the outbreak spread in real time.

The four tests described in this chapter show great promise for the development of new diagnostics that can strategically use out of the shelf cross-reactive antibodies for disease detection. Nevertheless, the tests have some limitations. I have tested the devices with purified proteins or supernatants from infected cells, but I have not tested the devices with clinical patient blood samples due to the difficulty of obtaining and handling them, which would require a team located in endemic areas testing the samples in real time before the red blood cells lysed. Although the current design of the device could not be successful with whole human blood, lateral flow devices that incorporate a red blood cell separators have been developed and tested in the lab, and therefore, this does not seem an obstacle for this research. Moreover, sensitivity and specificity testing in human serum has not been performed for the tests presented in this chapter, however, the Zika and Dengue tests use similar antibodies to the ones presented in chapter three, and therefore I do not expect the results to differ significantly. Finally, the rapid tests described here, as well as PCR, perform best when analyzing samples collected during the acute phase of the
infection, when the virus is detectable, while serological assays such as IgG and IgM tests perform best when evaluating patients after the virus has been cleared. Therefore, the ideal scenario would be to use a combination of several diagnostic techniques in order to better inform and treat the patients.

This paper serves as a proof of concept of how cross-reactive antibodies can be used to rapidly develop POC diagnostics. In an ideal scenario, the rapid tests presented in this chapter could be rapidly developed at a low cost by using out of the shelf cross-reactive antibodies and deployed in the field. These would serve as a rapid initial screening tool to isolate high and low risk patients while a second confirmatory screening by PCR is developed. The ability to rapidly manufacture and test POC rapid diagnostics is needed to treat patients and control epidemics.

CONTRIBUTIONS

Work by researchers from the Gehrke lab and the Hamad-Schifferli lab, other than myself has been necessary for the compilation of this chapter. First, Dr. Irene Bosch has grown the hybridoma cells that were used to obtain the antibodies. Marc Carré and Cristina Rodríguez helped in performing some experiments.
ENGINEERING IMMUNOCHROMATOGRAPY ASSAYS

ABSTRACT

The point-of-care (POC) diagnostics that have been explained in this thesis exploit gold nanoparticle (NP)-antibody conjugates for visual readout. Hence, the sensitivity and specificity of the devices depend on many factors that include the architecture of the test, the nanoparticle technology, or even the media in which the test runs. The main focus of the chapter is to test methodologies that allow increasing the sensitivity and specificity of lateral flow devices by optimizing their main components. In order to achieve that, there are three main areas in which this chapter will focus. On the first place, the nanoparticles that give the colorimetric readout in the diagnostic. Within this framework, the subchapter “the Extinction Coefficient of gold nanostars, nanospheres and nanorods” provides an understanding of the optical properties of several gold nanoparticles, such as nanostars, nanospheres and nanorods, and measures their optical extinction coefficient which is a necessary parameter to calculate the concentration of the nanoparticles based on their optical properties, as well as to understand the synthesis methods that show a darker signal, and therefore ultimately a darker readout in the lateral flow assays. On the second place, in the subchapter “Effect of the protein corona on antibody-antigen binding in nanoparticle sandwich immunoassays” I investigate the effect of the protein corona on the function of nanoparticle antibody conjugates in dipstick sandwich immunoassays. We observe that sandwich immunoassay formation was influenced by whether the strip was run in corona forming conditions, i.e., in human serum. Strips run in buffer or pure solutions of bovine serum albumin exhibited false positives, but those run in human serum did not. Serum pretreatment of the nitrocellulose or the conjugates eliminated false positives. Langmuir binding analysis determined how the immobilized Ab affinity for the NP-Ab/NS1 was impacted by corona formation conditions, quantified as an effective dissociation constant, $K_{D}^{\text{eff}}$. Results show that corona formation mediates the specificity and sensitivity of the antibody-antigen interaction of Zika NS1 in immunoassays, and develops strategies to reduce nonspecific binding in different buffers by taking advantage of the protein corona that naturally forms around gold colloids. Finally, on the third place, in the subchapter “Factors that influence the detection limit of lateral flow immunoassays”
I investigate the effects of NP size and shape, conjugation and surface chemistry, nitrocellulose pore size, antibody thermodynamic properties and antigen cross-reactivity on the overall dipstick performance. We observe that the assay performance depends significantly on the conjugate properties, as well as on the antibodies used in the test. These approaches are completely general and can be used to optimize immunochromatographic detection for a range of biomarkers.

The Extinction Coefficient of Gold Nanostars, Nanospheres and Nanorods

Gold nanoparticles (Au NPs) are attractive for interacting with biological systems due to their small dimensions, surface chemistry and optical properties. They can be synthesized in a variety of sizes and shapes, resulting in different chemical and physical properties. While gold nanospheres (NS) and gold nanorods (NR) have tunable optical properties by modifying their shape and size through synthesis procedures, Au nanostars (NStars), highly branched gold nanocrystals\textsuperscript{194} or nanoflowers\textsuperscript{195}, are of particular interest because minor shape modifications enable easily manipulating their optical properties. Gold colloids possess a surface plasmon resonance (SPR) peak that is tunable throughout the visible and near IR spectrum, resulting in different extinction profiles and therefore distinct colors\textsuperscript{196-199}. In the case of gold nanostars, due to their sharp tips they have a narrow SPR, facilitating selective excitation with a laser\textsuperscript{194,200,201}. Furthermore, their synthesis is facile and can be done in an aqueous nontoxic buffer\textsuperscript{194}, making NStars amenable for biological applications in targeted photothermal therapy and theranostics\textsuperscript{198,199,202,203}, imaging\textsuperscript{204,205}, sensors\textsuperscript{206}, and Surface Enhanced Raman Spectroscopy (SERS)\textsuperscript{195,200,203,207}, as well as lateral flow immunoassays\textsuperscript{114}. Because of the increasing interest in the optical and physical properties of NStars, a variety of synthesis approaches have been developed using different surfactants and reducing agents\textsuperscript{200,201,208-210}, which can yield to the formation of NStars of different symmetries. Nevertheless, many applications of nanostars are complicated by the lack of a simple manner to quantify their concentration. Au NStars absorb strongly in the visible spectrum. Their molar extinction coefficient, $\varepsilon$, is a fundamental parameter that allows for quantifying their concentration, and is thus critical for characterizing their behavior in therapeutic and sensing applications. $\varepsilon$ is also essential for bioconjugation, as it allows for quantification of the biomolecule surface
density and footprint on the NStars. Most importantly, understanding the relationship between $\varepsilon$ and the NStar size/shape opens new avenues for the design of NStars with desired optical properties for particular applications, such as SERS\textsuperscript{211}, two photon luminescence\textsuperscript{212}, surface enhanced fluorescence, localized surface plasmon resonance spectroscopy\textsuperscript{206}, or as conjugates in lateral flow immunoassays\textsuperscript{114}.

For spherical Au NPs and Au nanorods (NRs), $\varepsilon$ has been widely studied and characterized\textsuperscript{209, 210, 213}, and its dependence on nanoparticle physical dimensions is well understood. $\varepsilon$ of nanospheres can be explicitly described as a function of nanosphere diameter, and thus can be calculated based on particle geometry. For NRs, both their SPR position and $\varepsilon$ can be written as a function of their volume and aspect ratio. Agreement between experimental and computational models is generally good. One model that has been widely used is the discrete dipole approximation (DDA), which relies on approximating a NP volume as an array of point dipoles, and calculates the interaction of electromagnetic radiation with the dipoles. This allows prediction of the extinction, absorption and scattering of light by metallic NPs of arbitrary shapes. In particular, the Fortran code DDSCAT has gained increasing interest as a reliable tool for modeling the optical properties of gold NPs. However, differences between computational and experimental observations arise due to the polydispersivity in NP dimensions and shapes in solution, as well as interactions between NPs, which might not be accounted for in the computational model\textsuperscript{194, 209, 210, 213, 214}.

Here I present results quantifying $\varepsilon$ as a function of size and shape for gold NStars, nanospheres and nanorods, comparing experimental and computational measurements. First, I synthesized the nanoparticles of different sizes and shapes, which result in different SPRs. I measured the extinction coefficient of the synthesized NStars at their maximum surface plasmon resonances (SPR), which range from $5.7 \times 10^8$ to $26.8 \times 10^8$ M$^{-1}$cm$^{-1}$, and compared them with the extinction coefficient of gold nanorods (range from $3.7 \times 10^8$ to $57.6 \times 10^8$ M$^{-1}$cm$^{-1}$) and nanospheres (range from $0.7 \times 10^8$ to $52.5 \times 10^8$ M$^{-1}$cm$^{-1}$). Measured values correlate with those obtained from theoretical models of the NStars using the discrete dipole approximation (DDA), which I use to simulate the extinction spectra of the nanostars. We observe that $\varepsilon$ correlates with the NStar volume and SPR position, and similar trends are observed both experimentally and numerically.
Finally, because of the growing interest of NStars for biological applications and in lateral flow immunoassays in particular, I conjugated antibodies (Abs) and ssDNA aptamers onto the NStars, and use the experimentally measured \( \varepsilon \) values to quantify Ab and DNA surface coverage and footprint on the NStar surface. We observe that DNA surface coverage and footprint results agree with the measurements obtained for other well-established NPs, such as NRs and nanospheres. Ab coverage is lower in comparison, which could be attributed to a shape effect. This simple method for determining nanostar concentration has the potential to facilitate the use of Au NStars in biological and chemical applications.

RESULTS AND DISCUSSION

SYNTHESIS AND CHARACTERIZATION OF GOLD NANOPARTICLES

GOLD NANOSPHERES

Gold nanospheres were synthesized following the turkevich\(^{112} \) citrate reduction method (see Materials and Methods for individual nanosphere synthesis). By reducing the concentration of citrate present in the final solution it was possible to increase the size of the nanospheres, producing nanospheres with slightly different red colors (Fig. 59).

![Figure 60: Vials of nanospheres. NS8nm, NS18nm, NS32nm (left to right)](image)

TEM imaging was used to measure the sizes of the gold nanospheres (Fig. 60). The diameters of the nanospheres increased from 8.4 ± 0.7nm for the NS8nm (Fig. 60, a, left), 18.4 ± 2.3nm for the NS18nm (Fig. 60, b, middle) and 32.7 ± 5.5nm for the NS32nm (Fig. 60, c, right).
GOLD NANORODS

Cetyltrimethylammonium bromide (CTAB)-coated gold particles that absorbed at four different wavelengths were synthesized. Rod-shaped short nanorods (NR23nm) with an aspect ratio (AR) = 1.8, rod-shaped nanorods with an AR of 2.94 (NR30nm), long capsules with an AR of 3.25 (NR45nm) and long nanobones with an AR of 2.2. Solutions of different colors could be achieved by changing the synthesis conditions and thus, the dimensions of the nanorods (Fig. 61).

TEM imaging was used to measure the sizes of the gold nanorods (Fig. 62). The length of the nanorods increased from the short nanorods until the nanobones. The short nanorods (NR23nm), had a length of 22.7± 5.2nm and a width of 12.7 ± 3.3nm, the nanorods (NR30nm) had a length of 30 ± 5.8nm with a width of 10.2 ± 2.4nm, the nanocapsules (NR45nm) had a length of 45.4 ± 18.5nm and a width of 14 ± 4.7nm and finally, the longer nanobones (NB57nm) had a length of 57.2 ± 25.97 and a width of 26± 6nm.
GOLD NANOSTARS

Au salts can be reduced in certain Good's buffers to form NStars. HEPES, in particular, is able to act as both a Au reducing agent and growth directing agent. HEPES reduces the Au$^{3+}$ ions into Au$^{0}$, leading to the formation of gold NStars. The piperazine moiety of HEPES is thought to be responsible for the anisotropic growth of NStars$^{194}$. I modified the HEPES reduction approach to tune the SPR across a broad wavelength range by varying the Au/HEPES ratio, a strategy that has been utilized in other NStar syntheses$^{196,197}$. Tuning the Au/HEPES ratio changes the shape of the produced NStars, and thus results in NStars with different extinction spectra (see Methods for individual NStar synthesis procedures). This produced suspensions with colors that ranged from magenta to blue to green (Fig. 63).

Hydrodynamic diameter, $D_H$ and zeta potential ($\zeta$) of the NStars (Fig. 64, top and bottom, respectively) were measured by dynamic light scattering (DLS), and by Ferguson analysis and indicated similar characteristics between different preparations of the nanoparticles.
Gel electrophoresis of the synthesized NStars showed that the NPs did not aggregate or smear when running in the gels (Fig. 65 indicating that BPS-coated NPs were negatively charged and not aggregated. The different NStars had similar $D_H$ values as measured by Ferguson analysis.

TEM imaging (Fig. 66) was used to measure the size of the NStars.
Increasing the HEPES/Au ratio on the geometry of the particles resulted in NStars with increasing arm lengths, while the core diameter remained mostly uniform (Fig. 67, left). Eccentricity, defined as the distance from the middle of the longest axis to the intersection of the two longest axes of the NStar, increased with increasing HEPES concentrations (Fig. 67, right).
Figure 68: (Left) TEM measurements of the dimensions of the nanostars (longest, middle and shortest axes), and the diameter of the largest inscribed circle in Au NStars: NStar200, NStar300, NStar350, NStar400, NStar500, NStar600, NStar750, NStar900 and NStar1000. Right: TEM measurements of the eccentricity of the NStars, measured as the distance between the center of the longest axis to the intersection of the two longest axes.

QUANTIFYING THE EXTINCTION COEFFICIENT

In order to quantify $\varepsilon$ of the NPs, the extinction spectrum of each synthesis was first measured. To quantify the amount of Au for a given NP solution, the NPs were separated from unreacted gold by centrifugation and then dissolved in aqua regia. The concentration of Au ions, quantified by ICP-AES, together with the measured NP volume -as extracted from the TEM 2D projections-, and the crystal structure of gold was used to calculate $\varepsilon$ of the different NP preparations.

GOLD NANOSPHERES

The extinction spectrum of each synthesis of nanospheres was measured (Fig. 68), the 8nm NS had a SPR peak at 520nm, the 18nm NS had a SPR peak at 522nm, and the 32nm NS had a SPR peak at 531nm. Therefore, it was possible to observe, as expected, an increase on the SPR with increasing diameters of nanospheres.

Figure 69: UV-vis extinction spectra of NS of increasing diameters.
After synthesis, the NPs were centrifuged and dissolved in aqua regia in order to quantify the number of gold atoms in each solution by ICP-OES and the extinction coefficient of the gold nanoparticles was calculated (Table 14). The extinction coefficient of the nanospheres increased with the nanoparticle diameter, and the results were similar to the values available in literature210.

<table>
<thead>
<tr>
<th>Synthesis</th>
<th>Geometric factors</th>
<th>Optical properties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diam. circle</td>
<td>Volume (nm³)</td>
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<tr>
<td>NS 8nm</td>
<td>8.4±0.7</td>
<td>0.3x10⁴</td>
</tr>
<tr>
<td>NS 18nm</td>
<td>18.4±2.3</td>
<td>3.4x10⁵</td>
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<tr>
<td>NS 32nm</td>
<td>32.7±5.5</td>
<td>19.8x10⁶</td>
</tr>
</tbody>
</table>

Table 14: Extinction coefficient of gold nanospheres

GOLD NANORODS
In order to quantify ε of the NR, the extinction spectrum of each synthesis was first measured (Fig. 69).

![UV-VIS extinction spectra of NR of increasing lengths.](image)

Table 15: Extinction coefficient and geometric parameters of gold nanorods.
Gold Nanostars

In order to quantify $\varepsilon$ of the NStars, the extinction spectrum of each synthesis was first measured (Fig. 70, a, left). The SPR maximum redshifted with increasing HEPES concentration (Fig. 70, b, right). To quantify the amount of Au for a given NStar solution, the NStars were separated from unreacted Au and then dissolved in aqua regia. The concentration of Au ions, quantified by ICP-AES, ranged from 127-149 mg/l. Based on an original concentration of Au$^{3+}$ of 158 mg/ml, 80-95\% of the Au$^{3+}$ ions were reduced in NStar formation. Therefore, the synthesis reaction yield is intermediate between NRs (15\%, in a seed-mediated synthesis approach) and spherical Au NPs (>$95\%$, by citrate boiling)$^{213}$.

![Figure 71: a) UV-vis extinction spectra of NStars synthesized with increasing HEPES concentration, b) SPR maximum wavelength as a function of the HEPES reaction concentration in the reaction](image)

TEM images of NStars were used to calculate the volume of an individual NStar. An average of 6 arms in each NStar was determined by tilting the TEM stage $\pm$ 30°. ImageJ was used to draw boundaries around the NStars to obtain the diameter of the maximum inscribed circle (cyan line and circle, Fig. 71, a, bottom); and the longest, middle and shortest arm lengths (red, green and blue lines, respectively, Fig. 71, a, top). Because TEM images show 2D projections of the NStars, which could adopt a variety of configurations on the TEM grid, the volume of the NStars was measured if they were positioned in different configurations, obtaining a maximum difference in volume of 5\% with different configurations. Therefore, NStars were modeled using the measured arm distances as the maximum lengths of the NStar arms, and the diameter of the maximum inscribed circle as the internal diameter of the NStars (Fig. 71, b). For each synthesis, at least 100 NStars were measured for statistical significance. The measured NStar
dimensions were used to draw the 3D models of the NStars in AutoCAD (Fig. 71, b); AutoCAD's functions _MASSPROP and AREA were used to calculate the volume and area of individual NStars (Table 16).

Consequently, knowing the volume of individual NStars, the extinction spectrum of each NStar solution, and the total Au ion concentration in each solution, it was possible to determine $\varepsilon_{\text{expt}}$ for each NStar sample (Table 1). $\varepsilon_{\text{expt}}$ values at the SPR maximum ranged from $5.7 \times 10^8$ to $26.8 \times 10^8$ M$^{-1}$ cm$^{-1}$. These values were on the same order of magnitude as measured values for NRs and NPs of similar volumes.$^{213}$

Simulating the extinction spectra of nanostars by the discrete dipole approximation (DDA) To gain further insight into the nature of the NStars, $\varepsilon$, the extinction cross section of the NStars was simulated using the discrete dipole approximation (DDA) with the freely-available DDSCAT package. DDA approximates target particles of arbitrary geometries and complex refractive indexes, as an array of polarizable points located in a cubic lattice. I divided each NStar volume into at least 20000 dipoles, as suggested by other reports.$^{215}$ I averaged the extinction cross section between light interacting with the particle at the three major perpendicular axes by rotating the nanoparticle with respect to the incident light. This allowed calculation of the extinction ($Q_{\text{ext}}$), absorption ($Q_{\text{abs}}$) and scattering ($Q_{\text{scat}}$) cross-sections (Fig. 72).
I used $Q_{ext}$ obtained from the simulations to calculate theoretical extinction coefficients (in units of $M^{-1} cm^{-1}$) of the NStars ($\alpha_{\text{theory}}$) using the formula $^{214}$:

$$
\alpha = \frac{C_{\text{ext}} N_A}{\ln 10} = N_A \left( \frac{10^{-17}}{\ln 10} \right) \left( \frac{9 \pi}{16} \right)^{1/3} V^{2/3} Q_{\text{ext}}
$$

where $Q_{ext}$ is the maximum extinction cross-section of the NStars, $V$ is the NStar volume and $N_A$ is Avogadro's number. $\alpha_{\text{theory}}$ values at the SPR maximum ranged from $7.8 \times 10^8$ to $35.3 \times 10^8 M^{-1} cm^{-1}$ (Table 16).
The simulated extinction cross-sections spectra of the NStars (Fig. 73) showed that SPR red-shifted with increasing arm length, analogous to the experimental results. Simulated spectra had narrower extinction full-width at half maximum (FWHM) compared to the experimentally measured spectra (Fig. 74), which has also been observed for nanospheres, NRs\textsuperscript{214,216,217}, and other metallic NPs\textsuperscript{218}, and can be explained by the fact that DDSCAT approximates an ideal and monodisperse particle shape that does not interact with other NPs in solution.

*Table 16: Experimental and Simulation results of the extinction coefficient and geometric parameters of gold Nanostars.*

<table>
<thead>
<tr>
<th>Synthesis</th>
<th>Geometric factors</th>
<th>Optical properties</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>TEM size (nm)</td>
<td>AutoCAD</td>
</tr>
<tr>
<td></td>
<td>Max Length</td>
<td>Diam. circle</td>
</tr>
<tr>
<td>NStar 200</td>
<td>21±4</td>
<td>15±3</td>
</tr>
<tr>
<td>NStar 300</td>
<td>24±5</td>
<td>15±3</td>
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<td>NStar 350</td>
<td>26±6</td>
<td>12±3</td>
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<tr>
<td>NStar 400</td>
<td>26±5</td>
<td>13±3</td>
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<tr>
<td>NStar 500</td>
<td>31±7</td>
<td>12±4</td>
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<td>NStar 600</td>
<td>30±9</td>
<td>11±3</td>
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<tr>
<td>NStar 750</td>
<td>41±11</td>
<td>16±6</td>
</tr>
<tr>
<td>NStar 900</td>
<td>46±12</td>
<td>16±3</td>
</tr>
<tr>
<td>NStar 1000</td>
<td>55±16</td>
<td>16±5</td>
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</table>

Moreover, simulation results showed a SPR maximum red-shifted compared to the experimental values (Fig. 74). This difference could be due to polydispersity of the NStar samples, where NStars with different eccentricities can be obtained during the synthesis, interactions leading to aggregation, slight truncations of the NStar edges\textsuperscript{217}, or a difference in the refractive index of the medium around the particles because of the capping BPS\textsuperscript{219}.

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I studied if lack of symmetry could explain the difference between experimental and simulation SPR maxima. Cluster analysis (Matlab, mkeans, with normalized Euclidean lengths) was used to separate the NPs in two different clusters. The inputs for the clustering were the dimensions of the NStars and the eccentricity values. The analysis revealed that two clusters with different eccentricities could be observed in the data. For instance, Figure 75 a, for NStars-500 two species with high and low eccentricities could be observed, green triangles and red squares, respectively. Similar results were obtained for the different synthesis of NStars, I observed that all NStar synthesis could be clustered in two different species with high and low eccentricities. Figure 75 b, The Silhouette Values of the clustering analysis (most of them higher than 0.8) of NStar500, indicated that most NPs were well separated from neighboring clusters.

From the analysis of the TEM projections of the NStars (Fig. 76, a and Table 17), I created 3D models of the different NStars Figure 76 b, for each of the two clusters with low eccentricity (red) and high eccentricity (green) in order to compare them with NStars of average eccentricity (blue).
Table 17: Cluster analysis results of the NStar TEM measurements. Two clusters with low (C1) and high (C2) eccentricities were obtained for each of the NStar synthesis.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cluster</th>
<th>Long axis (nm)</th>
<th>Middle axis (nm)</th>
<th>Short axis (nm)</th>
<th>Diameter (nm)</th>
<th>Eccentricity (nm)</th>
<th># NP</th>
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<tr>
<td>NStar1000</td>
<td>C1</td>
<td>54.2±16.2</td>
<td>36.1±10.7</td>
<td>29.5±9.3</td>
<td>16.3±4.5</td>
<td>5.4±3.6</td>
<td>162</td>
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<tr>
<td></td>
<td>C2</td>
<td>57.5±13.9</td>
<td>36.1±11.8</td>
<td>30.7±9.9</td>
<td>16.5±4.5</td>
<td>20.7±6.5</td>
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<td>NStar900</td>
<td>C1</td>
<td>47.4±12.9</td>
<td>31.3±9.1</td>
<td>30.0±8.9</td>
<td>15.9±3.7</td>
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<tr>
<td></td>
<td>C2</td>
<td>44.2±11.2</td>
<td>30.5±8.1</td>
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<td>14.5±5.5</td>
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<td>NStar750</td>
<td>C1</td>
<td>57.5±11.2</td>
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<td></td>
<td>C2</td>
<td>43.1±10.8</td>
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<td>15.9±7.1</td>
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<td>11.1±3.4</td>
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<tr>
<td></td>
<td>C2</td>
<td>30.2±9.7</td>
<td>21.1±5.9</td>
<td>20.2±5.3</td>
<td>10.9±3.2</td>
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<td>NStar500</td>
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<tr>
<td></td>
<td>C2</td>
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<td>C2</td>
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<tr>
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<td>20.2±3.4</td>
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<tr>
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<td>C2</td>
<td>19.8±3.8</td>
<td>18.4±3.9</td>
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<td>14.3±3.1</td>
<td>4.4±2.6</td>
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</table>
Figure 77: a) TEM image of nanostars and b) autocad model with different eccentricities. Simulation results for nanostars with different eccentricities NStar200, NStar300, NStar350, NStar400, NStar500, NStar600, NStar750, NStar900 and NStar1000 (c-k) for each of the clusters with low (red) and high (green) eccentricities, and compared the results to the average NStar simulation (blue line), and experimental data (black lines).

I performed DDA simulations of the optical properties of the NStar200, NStar300, NStar350, NStar400, NStar500, NStar600, NStar750, NStar900 and NStar1000 (Figure 76, c-k, respectively) for each of the clusters with low (red) and high (green) eccentricities, and compared the results to the average NStar simulation (blue line), and experimental data (black lines). As expected, we observe that NStars with average eccentricity showed an extinction spectra intermediate between the extinction of NStars with high and low eccentricities.

Polydispersity analysis
The polydispersity of the nanostar samples was evaluated in the simulations. First, I determined what would be a feasible number of bins, or clusters. I investigated what the minimum number of clusters would be by evaluating the silhouette factor. The silhouette value for each point is a measure of how similar that point is to points in its own cluster, when compared to points in other clusters. The silhouette value for the $i^{th}$ point, $S_i$, is defined as

$$S_i = \frac{(b_i - a_i)}{\max(a_i, b_i)}$$
where \( a_i \) is the average distance from the \( i^{th} \) point to the other points in the same cluster as \( i \), and \( b_i \) is the minimum average distance from the \( i^{th} \) point to points in a different cluster, minimized over clusters. The silhouette value ranges from -1 to +1. A high silhouette value indicates that \( i \) is well-matched to its own cluster, and poorly-matched to neighboring clusters. If most points have a high silhouette value, then the clustering solution is appropriate. If many points have a low or negative silhouette value, then the clustering solution may have either too many or too few clusters. The average silhouette values resulting from splitting the NStars in two clusters were 0.80, 0.89, 0.87, 0.85, 0.78, 0.79, 0.77, 0.82 and 0.82 for NStar200, NStar300, NStar350, NStar400, NStar500, NStar600, NStar750, NStar900 and NStar 1000, respectively. These average silhouette values were higher than the values obtained for three, four or five clusters, indicating that no more than two clusters were necessary to represent the NStars. We observe that the closest results to experimental data were obtained from using an average NStar model. Therefore, the average eccentricity values have been used to compare to experimental results.

In summary, \( \varepsilon_{\text{theory}} \) exhibited a linear dependence on the SPR maximum (open squares, Fig. 77, a), as was observed for \( \varepsilon_{\text{expt}} \). Moreover, we observe a linear dependence of \( \varepsilon_{\text{theory}} \) on \( V^{2/3} \) (open squares, Fig. 77 b), which has been previously reported for NRs\(^{220}\). We observe that values for \( \varepsilon_{\text{theory}} \) tend to be lower than \( \varepsilon_{\text{expt}} \), which has also been seen for NRs\(^{221,222}\). Also, we can observe that the SPR maximum is linearly dependent on the \( V^{2/3} \) of the NStars (Fig. 77 c). Figure 77 shows that the SPR maximum can be used to approximate the NStar \( \varepsilon_{\text{expt}} \) if the NStar dimensions or the plasmon absorbance data are available, and thus can be used to determine the NStar concentration in solution. The SPR maximum peak showed a linear correlation with the maximum arm length to core diameter\(^{221,222}\).
Figure 78: $c_{\text{theor}}$ (blue open squares) and $c_{\text{expt}}$ (black filled squares) as a function of a) SPR max and b) NStar volume $V^{2/3}$. c) SPR maximum as a function of volume, $V^{2/3}$. Linear dependence of the SPR with $c_{\text{theor}}$ has been observed.

**NSTAR BIOCONJUGATION TO DNA AND PROTEINS**

Because NStars are attractive for biological applications, I covalently conjugated them to ssDNA aptamers and also antibodies that recognize mouse immunoglobulin G. I used the $c_{\text{expt}}$ values obtained above to quantify the Ab and DNA coverage on the NStars. Both the antibodies and aptamers conjugated to the NStars were fluorescently tagged to enable quantification of their loading (See Methods). ssDNA thrombin binding aptamer (TBA) was conjugated to the NStars via covalent conjugation to a 5′ thiol. TBA loadings on the NStar200, NStar350, NStar500 and NStar750 were 8.8 ± 0.4 x10^{12}, 11.6 ± 0.5 x10^{12}, 7.7 ± 0.3 x10^{12} and 1.9 ± 0.1 x10^{12} DNA molecules-cm^{-2}, respectively calculated from supernatant-loss measurements of unbound DNA. Loadings are similar in order of magnitude to published values observed for TBA on NRs (a loading of 5.6x10^{12}-12.3x10^{12} for NRs with 1256 nm^2 area), and spherical NPs of similar surface areas (loadings of 17 x10^{12} for NPs with 1766 nm^2 area). Extinction spectra of NStar-DNA conjugates were slightly red-shifted relative to unconjugated NStars, but not significantly.
broadened, showing that they were stable in solution (Fig. 78 a-d), similar to NRs and nanospheres\textsuperscript{225-227}.

![Image of extinction spectra](image)

**Figure 79: NSStar bioconjugation to DNA and antibodies.** Extinction spectra of a) NSStar200, b) NSStar350, c) NSStar500, d) NSStar750 conjugated to TBA DNA (red) and antibodies (blue) compared to bare NSStars (black dotted).

Ferguson analysis of gel electrophoresis showed that NSStar $D_H$ increased $\sim$7 nm upon DNA conjugation (Fig. 79 a). This increase in $D_H$ correlates with the secondary structure of the TBA, which is a folded 15mer with a 15mer spacer\textsuperscript{228}, suggesting that Ferguson analysis is accurate in quantifying NSStar size after bioconjugation. However, DLS measurements showed a larger increase in $D_H$ of 30-90 nm upon DNA conjugation (Fig. 79, d-k), which suggests aggregation of the NSStar-DNA in solution as opposed to in an electric field. This difference in $D_H$ is somewhat expected given the different experimental conditions used to measure $D_H$ for gel electrophoresis vs. DLS. Ferguson analysis showed a decrease in NSStar $\zeta$ (Fig. 79, b) due to the negative charge of DNA. Conjugated gold NSStars were retarded in gel electrophoresis relative to free NSStars (Fig. 79, c), revealing that the bound DNA affected both NSStar size and charge.
I also explored bioconjugation of NStars to fluorescently labeled antibodies (IgG) via covalent attachment (see Methods). IgG loading on the NStars were 3.0±0.6, 5.7±1.2, 31.3±1.5 and 3.3 ± 0.1 IgG molecules per NStar for NStar200, NStar350, NStar500 and NStar750 respectively, measured from supernatant-loss of unbound antibodies. Ab loadings were calculated assuming a 67.5 nm^2 top view footprint of an IgG antibody and were 3-10x lower than described for nanospheres. The lower loading of Abs could be due to the irregular surfaces of the NStars, where curvature effects could potentially be undesirable for conjugation to the relatively large Abs. Extinction spectra were not significantly broadened after bioconjugation, suggesting that NStars were stable in solution after Ab conjugation (Fig. 78 a-d). Ferguson analysis showed an increase in $D_H$ of 7 nm upon conjugation (Fig. 79, a), which correlates well with the sizes and loadings.
of Abs on the NStars\textsuperscript{175}. However, DLS showed a larger increase in average $D_H$ (52 nm) (Fig. 79, d-g), which could be due to aggregation. Ferguson analysis showed that $\zeta$ of the NStars decreased upon antibody binding due to the negative charge of the Abs (Fig. 79, b). These results show that the NStars can be conjugated to DNA and antibodies and that the $c_{\text{expt}}$ can be used to quantify biomolecule footprint on the NStar surfaces.

In summary, Au NStars are promising for a broad range of biological applications due to their strong and highly tunable extinction in the visible. One of the challenges in characterizing NStars is that their asymmetry and irregular shape complicate volume estimation, thereby presenting challenges for determining concentration. The molar extinction coefficient, $c_{\text{expt}}$, of NStars can be used to measure their concentration, and it is a critical parameter when using NStars in biological applications. I have quantified the molar extinction coefficient $c_{\text{expt}}$ for NStars of different geometries and sizes, by correlating their extinction with the concentration of Au particles, which was measured by analyzing TEM images of the NStars, and counting Au ions by ICP-OES. The results correlate with DDSCAT computational models of the NStars. I use the experimental values of $c_{\text{expt}}$ to quantify NStar concentration, and to quantify biomolecule coverage measurements on NStar-Ab and NStar-DNA conjugates. DNA aptamers and antibodies were conjugated successfully to the Au nanostars. Nanostar curvature effects do not appear to hinder DNA conjugation to the NStars, but do seem to hinder conjugation to the relatively larger antibodies. Future work includes modification of NStar surface chemistry to increase the efficiency of antibody conjugation to NStars. These results are promising in order to design better conjugates for lateral flow immunoassays that are capable of higher absorption, hence ultimately achieving higher signals at lower concentrations.
EFFECT OF THE PROTEIN CORONA ON ANTIBODY-ANTIGEN BINDING IN
NANOPARTICLE SANDWICH IMMUNOASSAYS

It is widely known that when nanoparticles (NPs) are introduced into biological environments such as blood, serum or intracellular fluid, the proteins present in these fluids form a protein corona around the NPs\textsuperscript{232}. Protein coronas mediate the interactions between NPs and their environment, influencing processes such as association with cell surfaces and intracellular uptake\textsuperscript{233-237}. Often protein coronas have negative impacts on these processes, as the corona around the NP can actually obscure its targeting ligand, reducing the specificity of interaction of the targeting species on the NP surface\textsuperscript{238-240}. The impact of the protein corona has been studied predominantly for aspects of nanomedicine concerned with the transport, blood circulation, and cell interactions of nanomaterials such as targeting and cell uptake. It is now known that the protein corona mediates interactions of NPs with cell receptors and other binding species, influencing the specificity of cell uptake\textsuperscript{239, 241-243}, and can have both negative and beneficial side effects\textsuperscript{244-247}. However, another situation where the protein corona has a significant impact is in point of care immunoassays used to detect pathogens and other biomarkers in blood, serum, and other body fluids. In lateral flow immunoassays (Fig. 80)\textsuperscript{248, 249}, a biological fluid is added to the sample pad where it wicks to the conjugate pad through capillary action\textsuperscript{250, 251}. In the conjugate pad, the biological fluid is mixed with gold nanoparticles conjugated to antibodies that are selected against the biomarker of interest. Then, the NP-Ab suspended in serum continue wicking through the nitrocellulose membrane where two antibodies have been immobilized, an antibody against the biomarker of interest in the test line, and an antibody against the antibody on the gold nanoparticles in the positive control band. If the biomarker of interest is present in the fluid, it binds simultaneously to antibodies conjugated to the NP (NP-Ab), as well as to the specific antibodies immobilized on the test line. Due to the optical properties of the NPs, a color appears on the test line due to the accumulation of the NPs. In the positive control band, there is NP-Ab binding to an anti-IgG antibody immobilized on the paper. For the assay to be successful, the biomarker must be able to bind to the antibodies on the NP and also those on the test line. This binding must be accomplished in the presence of all the proteins, ions, and small molecules that are present in the biological fluid. These
proteins are present at high concentrations and thus can form coronas that can potentially obscure the critical antibody-antigen interactions and prevent binding events (Fig. 80, b). If the corona prevents binding, it can result in false negatives, which can have serious consequences for a diagnostic assay. On the other hand, the corona composition could also lead to false positives, leading to a non-specific diagnostic. This can appear when other proteins in the sample can bind to the NP-Ab. Several methods, including the addition of sucrose, tween or adding capture negative bands in the nitrocellulose have been used to remove false positives. However, false negatives typically remain a major challenge for dipstick and lateral flow immunoassays. Surface effects\textsuperscript{252,253} from both the NP and the nitrocellulose substrate and the capillary flow regime of the entire system can further complicate the environment of the test. Lateral flow and dipstick point of care immunoassays have been utilized on a wide range of biological fluids, not only blood and serum, but also urine, saliva, semen, and breast milk, which can result in coronas with different compositions and physical properties. Despite its importance, the protein corona has been largely overlooked in the context of lateral flow and dipstick immunoassays. Thus, there is a need to understand how the protein corona impacts antibody-antigen binding in these sandwich immunoassays.

Here, I studied how the protein corona impacts antibody-antigen binding in sandwich immunoassays for the Zika virus (ZIKV) biomarker nonstructural protein 1 (NS1) in human serum. I probed the properties of coronas that form around the NP-Ab conjugates, both with covalent (NP-Ab-C) and passive (NP-Ab-P) conjugation methods, and quantified the ability of the NP-Ab to bind to Zika NS1. I found that the protein corona resulting from the serum influences the outcome of the test, and is dependent on the conjugation strategy that was used to prepare the NP-Ab conjugates. Moreover, I found that the covalently conjugated NPs were less susceptible to the media changes than the passively conjugated NPs. I present an approach to quantify the binding affinity of the Ab to the ligand NS1 using a modified Langmuir isotherm, and show that the binding affinities are modified by the protein coronas. Results show that the corona that forms around the NP-Ab conjugates can have important ramifications, and is a positive side effect. Furthermore, these results underscore the importance of the protein corona in LF/dipstick assays that require the use of serum and other bodily fluids.
RESULTS AND DISCUSSION

Au star shaped nanoparticles (NPs) were synthesized using literature methods, resulting in nanostars with short arms\textsuperscript{194, 223}. TEM imaging showed that the NPs had a mean diameter of 14.5 ± 3.1 nm (Fig. 81, a). Optical absorption spectra of the NPs showed an SPR peak at 536 ± 5 nm (Fig. 81, b, black). Measurement of the hydrodynamic diameter ($D_H$) by dynamic light scattering (DLS) showed that the NPs had $D_H = 32.6 ± 2.6$ nm (Fig. 81, c, black line), and zeta potential measurements determined that the NPs were negatively charged with a zeta potential of -28.3 ± 1.8 mV (Fig. 81, d). NPs have a known molar extinction coefficient\textsuperscript{113} ($\varepsilon_{536} = 5.7\times10^8$ M$^{-1}$ cm$^{-1}$) which allowed quantification of the NP concentration from the optical absorption spectrum.
NPs were conjugated to monoclonal Abs selected for Zika virus nonstructural protein 1 (NS1), a biomarker for the disease (see chapter 2, Materials and Methods and chapter 3). Antibodies were selected by injecting mice with Zika NS1 protein, and immortalizing the B cells capable of binding to the Zika NS1, but not to NS1 from other similar viruses (such as Dengue, Yellow Fever, Japanese Encephalitis, West Nile Virus or Tick Borne Encephalitis). The anti-NS1 antibodies were conjugated to the NPs by a covalent attachment method, or by passive absorption (Chapter 2: Materials and Methods). Briefly, for the conjugates bound by covalent attachment (NP-Ab-C), I used a heterobifunctional linker with a hydrazide on one end and a dithiol on the other, linked by a short polyethylene glycol (PEG) chain (hydrazide dithiolalkane aromatic PEG$_6$-NHNH$_2$)$_{184}$, which served as a link between the NPs and the antibodies. For the passively conjugated nanoparticles (NP-Ab-P), Abs were added to the NPs at an incubation ratio of 100:3, and
the Abs were allowed to adsorb by electrostatic interactions onto the surface of the NPs\textsuperscript{254-256}.

After bioconjugation following both conjugation strategies, the NP surfaces were modified with thiolated PEG of a MW = 5 kDa as a backfill, which is used to reduce non-favorable Ab-NP and Ab-Ab interactions on the NP surface\textsuperscript{120, 257, 258}. Following this, free Abs were separated by centrifugation. DLS showed that the covalently bound NPs-Ab-C conjugates had a \(D_H = 71.6 \pm 6.2\) nm (Fig. 81, c, blue), and passively conjugated NPs-Ab-P had a \(D_H = 124.9 \pm 7.2\) nm (Fig. 81, c, red), and both were larger than the bare NPs (Fig. 81, c, black). The charge of the covalently conjugated NPs, showed a zeta potential of \(-30.1 \pm 1.5\) mV, where they became more negative (Fig. 81, d) relative to the plain NPs, while the zeta potential of the passive conjugation was \(-17.6 \pm 1.9\) mV (Fig. 81, d), more positive. Changes in the zeta potential indicate a change of charge of the NPs, and hence surface modifications. For the covalent conjugation, gel electrophoresis showed a mobility shift (Fig. 81, e, lane 3) relative to the NP (lane 1). The NP SPR red shifted and broadened slightly upon Ab conjugation for both the covalent (Fig. 81, b, blue) and passive conjugation strategies (Fig. 81, b, red).

Previous work from our lab has found that PEG backfills improve Ab functionality of the NP-Ab conjugate and reduce non-specific binding of the conjugate in lateral flow assays and other uses of NP-biomolecule conjugates\textsuperscript{175, 259}. PEG backfill of the covalently-conjugated NPs did not result in a mobility shift in gel electrophoresis (Fig. 81, e, lanes 2 and 3) due to the mild backfill conditions, confirming that the Abs were not displaced from the NP.

In addition, fluorescence spectroscopy and ELISA of the supernatant after PEG backfill also confirmed that there was negligible Ab in the supernatant, confirming that the Abs were not displaced (Fig. 82). The Ab coverage on the NPs was determined by measuring the change in concentration of free Ab in solution after conjugation to the NPs using fluorescence spectroscopy. Ab coverage was determined to be \(4.3 \pm 2.2\) Abs/NP and \(17.6 \pm 1.2\) Abs/NP for the covalent (NP-Ab-C) and passive (NP-Ab-P) conjugation strategies, respectively. This was equivalent to an Ab footprint of 232.6 and 56.8 nm\(^2\) per Ab using the calculated surface area of a NP of 1000nm\(^2\), for the covalent and passive conjugation strategies, respectively\textsuperscript{113}. The dimensions of a typical IgG antibody of 14.5,
8.5 and 4\textsuperscript{260}nm, which has an average footprint of 81.3nm\textsuperscript{2}, so this suggests that the coverage was less than a monolayer during the covalent conjugation strategy. On the other hand, the passive conjugation strategy yielded more than a monolayer, which correlated well with the DLS information that showed larger conjugates for the electrostatic conjugation strategy. However, it is of note that even if the Ab had a high or low coverage resulting more or less than a multilayer on the NPs, it does not preclude the NP-Ab from being able function in an immunoassay.

I also measured whether or not the Ab would be displaced from the NP-Ab conjugates upon incubation with the various solutions used in the experiments. I measured the amount of Ab in the supernatant by fluorescence spectroscopy and ELISA after exposure to HS, HSA, BSA, and PBS and found that the displaced Abs to be negligible for both covalently attached and non-covalently attached Abs on NPs (Fig.82).
First, the strips were run with the conjugates by both passive (NP-Ab-P) and covalent (NP-Ab-C) methods in different biological fluids: human serum (HS), pure bovine serum albumin (BSA), and phosphate buffered saline (PBS). The dipstick assay consisted of a nitrocellulose strip onto which anti-Zika NS1 monoclonal antibodies were immobilized on the test area (Fig. 83, scheme). Also, a control antibody (anti-Fc) was immobilized on the control area. The conjugates were mixed with NS1 at varying concentrations in solution, into which the nitrocellulose strip was partially submerged. Upon contact with the nitrocellulose, the fluid migrated up the strip by capillary action to a wick (not depicted for clarity).

For the strip run only in PBS (Fig. 84, a, bottom), the test line intensity was present even when there was no NS1 in the sample (Fig. 84, a and b, covalent and passive binding, respectively, blue). This indicates that both the NP-Ab-C and the NP-Ab-P conjugates non-specifically bound to immobilized Abs on the nitrocellulose. The conjugates did not bind to the nitrocellulose outside of the test line, so this was not simply due to interactions between the nitrocellulose and the conjugates, but evidently the PBS modifies electrostatic screening in a way that affects the specificity of protein-protein or protein-NP interactions. The rightmost strip in Fig. 84, a and b, in PBS constitutes a false positive, which were observed in both covalent conjugation (Fig. 84, a, bottom) and electrostatic conjugation (Fig. 84, b, bottom).
Figure 85: Dipstick immunoassay results as a function of ZIKV NS1 concentration (L-R): 91.6 nM, 38.4 nM, 9.2 nM, 3.8 nM, 0.9 nM, 0 nM for a) NP-Ab-C, covalent conjugation in PBS, BSA, and HS, b) NP-Ab-P, passive conjugation in PBS, BSA, and HS. Top dot: control line. Bottom dot: test line. Test line intensity vs. NS1 concentration for NP-Ab-C (c) or NP-Ab-P (d) in PBS (blue triangles), BSA (red circles), and HS (black squares), and fits to $K_{D}^{\text{eff}}$ (lines).

ImageJ$^{128}$ was used to analyze the test line intensities as a function of NS1 concentration, and the test line intensity showed a concentration dependence that did not reach baseline at 0 nM NS1 (Fig. 84, blue triangles, c and d, covalent and passive, respectively, also Fig. 85 shows the intensity at 0nM for both passive conjugation, gray and covalent conjugation, white). At 0nM we observed that for the passive conjugation NP-Ab-P, the signal was saturated. For the covalently bound antibody, on the other hand, the signal at 0nM was lower than the signal with high concentrations of NS1, indicating that the covalently bound antibody helped in reducing the non-specific interactions that resulted from the presence of PBS.

Figure 86: Background at 0ng/ml observed with passive (grey) and covalent(white) conjugations in different media
The strips were then run in a 10 mg/ml solution of BSA in PBS, which is widely used as a blocking agent for non-specific adsorption. Again, a band at the test line was present for no NS1 present (Fig. 84 a and b, middle, covalent and passive conjugation respectively), indicating that the NP-Ab was non-specifically binding to the immobilized antibodies on the test line. Like with PBS, the rightmost strip in Fig. 84a and 84b in BSA constitutes a false positive. For passive conjugations, NP-Ab-P, the intensity was high at all NS1 concentrations, indicating that the signal was not dependent on the concentration of NS1. For the covalently bound conjugates, NP-Ab-C, the intensity decreased with NS1 concentration but did not reach baseline at 0 nM NS1 (Fig. 84c and d covalent and passive, respectively, red circles, also Fig. 85 shows the intensity at 0nM for both passive conjugation, gray and covalent conjugation, white). We also observed that the background for the BSA solution, in the case of the covalently-bound conjugates NP-Ab-C, was lower than in the case of PBS, indicating that covalent conjugation helped in reducing nonspecific binding and that BSA was able to partially reduce nonspecific interactions compared to PBS alone, albeit not completely.

When the strips were run with HS (Fig. 84 a (top) and b (top), for covalent conjugation and passive conjugation, respectively), the test line intensity decreased with decreasing NS1 concentration (Fig. 84 c and 84 d, for active and binding, respectively, black squares). The intensity was zero for no NS1 present (Fig. 85), indicating no false positives and that there was no non-specific adsorption on the test line. This shows that the proteins present in HS influence the Ab-NS1 interactions, and impact the specificity of the sandwich formation. This effect was observed for both, the passive and the covalent conjugations. Because pure BSA does not remove the non-specific interactions, the removal of false positives is due to the exposure to the complex mixture of HS.

In summary, similar trends were observed by both covalent and passive conjugations. We observe that covalent (Fig. 85 white bars) conjugation showed lower background than the passive conjugation (Fig. 85 grey bars) at 0ng/ml, by running in both PBS and BSA, indicating that the performance of covalently-conjugated NPs was less dependent on the media where the tests were performed.

I performed a control experiment to probe whether the positive test lines were a result of NS1 adsorbing to the NP-Ab conjugate through NS1-Ab specific binding, or if NS1, on the
other hand, was absorbing on the NP-Ab coronas. I changed the Ab on NP to an anti-Dengue NS1 antibody, which cannot bind to Zika NS1 (Fig. 86 a). For strips run with NP-anti-Dengue Abs, the test line intensity remained at the baseline for all Zika NS1 concentrations, indicating that the NP-Ab is not interacting with the NS1 nonspecifically via adsorption to cause the NP-Ab to accumulate at the test line (Fig 86 c, black squares). In addition, I confirmed that the NP-anti-Dengue was indeed functional by using it in an assay with Dengue NS1 and anti-Dengue immobilized Abs (Fig. 86 b). Here, the NP-anti-Dengue Ab did bind to the Dengue NS1 and exhibited an expected concentration dependence (Fig. 86 c, red circles). This shows that the NPs are not accumulating at the test line regardless of the type of Ab on its surface, suggesting that the test lines are not due to the NS1 non-specifically adsorbing to the NP. I repeated this for NP-Abs that were non-covalently conjugated, and the results were the same (Fig. 86 d).

![Diagram](image)

**Figure 87:** Control experiment of assays with Dengue antibodies and Dengue NS1 probing involvement of NS1 in corona formation. A) dipstick assay with immobilized anti-Zika antibodies and NPs conjugated to anti-Dengue antibodies run with Zika NS1, b) dipstick assay with immobilized anti-Dengue antibodies and NPs conjugated to anti-Dengue antibodies run with Dengue NS1, c) Test line intensity of the NP-anti-DV run with Zika NS1 (black squares) and NP-anti-DV run with Dengue NS1 (red circles) for covalently attached anti-DV antibodies, d) Test line intensity of the NP-anti-Dengue run with Zika NS1 (black squares) and NP-anti-Dengue run with Dengue NS1 (red circles) for non-covalently attached anti-Dengue antibodies, e) KDs from fits of the binding curves.
This shows the NS1 causing a positive test line, confirming that the specific Ab-NS1 interaction is necessary for formation of a sandwich immunoassay. Furthermore, this confirms that the Abs are not displaced from the NP in the HS, because if the Abs were displaced from the NP, then the behavior in both of these assays would be identical, where the NP would accumulate at the test line regardless of the type of Ab.

**Properties of Protein Coronas that Form Around the NP-Ab**

In the strips run with HS, the NP-Ab is in an environment with a high concentration of proteins and also many small molecules, favoring formation of a protein corona. Because corona formation is most likely responsible for modifying the Ab-NS1 interactions in the sandwich immunoassays, I probed the properties of protein coronas that form around the NP-Ab when in HS. Both covalently bound NP-Ab-C and passive Ab conjugations NP-Ab-P, were incubated with HS to form protein coronas. The NP-Ab with the coronas were separated from the free serum proteins in HS by spin centrifugation. In the case of the covalently bound NPs, NP-Ab-C, DLS showed that the $D_H$ increased after incubation in HS from $71.6 \pm 6.2$ nm to $128.9 \pm 11.2$nm (Fig. 87, a, orange, covalent conjugation), suggesting formation of a large and multilayered protein corona. For passive conjugation we observed similar results, where the $D_H$ increased after incubation in HS from $87.1 \pm 7.2$ nm to $156.7 \pm 12.9$nm. Zeta potential measurements of the NP-Ab-C with the coronas (Fig. 87 c) showed a change to a less negative zeta potential (black compared to grey), suggesting that the charge of the species changed due to corona formation. NP-Ab-C exposed to BSA showed a shift to a larger $D_H$ (Fig. 87 a, pink), but the increase in size was not as large as for the NP-Ab-C in HS. Similar results were observed for the passive conjugation.
Then, I examined the behavior of the NP-Abs with pre-formed coronas in the different fluids. NP-Ab were incubated in HS and allowed to form a protein corona using literature methods (Chapter 2: Materials and Methods)\textsuperscript{247}. DLS showed a $D_{H}$ was larger compared to NP-Ab-C ($71.6 \pm 6.2 \text{nm}$), and was $172.8 \pm 15.0$, $106.5 \pm 15.6$, and $123.3 \pm 18.1$ in PBS, BSA, and HS (Fig. 87 b, blue, pink and orange, respectively). For the passive conjugation, we observe similar results, where DLS showed a $D_{H}$ was larger compared to NP-Ab-P ($87.1 \pm 12.9 \text{nm}$), and was $141.8 \pm 7.1$, $128.9 \pm 11.8$, and $164.2 \pm 8.2$ in PBS, BSA, and HS. The zeta-potential of the NP-Ab with coronas (Fig. 87 c, black) showed a change upon corona formation, though the net trend in charge was not systematic. These results show that the pre-formed corona is larger than the NP-Ab-C, by as much as $57.3 \text{nm}$, suggesting a multilayer structure, which is relatively stable in PBS, BSA, and HS. The zeta potential of the NP-Ab with a corona was less negative for both PBS and BSA, and more negative in HS. In the case of the passive conjugation, similar results were observed, except that the zeta potential of the NP-Ab-P with a corona was more negative for both PBS and HS, and less in BSA. Showing that although the corona formation seemed similar for both passive and active conjugations, the charge changes could differ slightly.

The effect on the SPR was negligible, as the absorption spectrum for NP-Ab-C and NP-Ab-C showed no major change after incubation in HS after 1 hr (Fig. 88, NP-Ab-C). The PEG backfill was not able to avoid protein corona formation. Some reports in the literature have shown that PEGylation can prevent coronas, though there is evidence that
PEGylated NPs still form coronas\textsuperscript{257, 261, 262}. However, given the mild conditions for backfill, corona formation was still expected. Similar results, where the effect of corona formation on the SPR of the nanoparticles, were obtained for passive conjugation of Ab on the nanostars.

![Optical absorption spectra of NP-Ab-C conjugates after incubation in the running buffers for 1h.](image)

Figure 89: Optical absorption spectra of NP-Ab-C conjugates after incubation in the running buffers for 1h.

I filtered the HS with different molecular weight cutoff (MWCO) filters to roughly determine which proteins were responsible for removing non-specific adsorption in the test line. The main components of HS are albumins (MW 67 kDa), globulins and regulatory proteins of various molecular weights. Strips were run in HS that had been passed through MWCO filters of 1 MDa, 300 kDa, 50 kDa, 10 kDa (covalent conjugation, only), and 5kDa (passive conjugation, only), and the test line intensity as a function of NS1 concentration was examined (Fig. 89 a and c for covalent and passive conjugations, respectively).

Observing the covalently bound NP-Ab-C, as well as the passively bound NP-Ab-P, the HS that had been passed through the 1 MDa filter (red circles) resembled the strip run in the full HS (Fig. 89 a and Fig. 89 c, black squares, for covalent and passive conjugations respectively), and did not have a false positive at 0 nM NS1 (Fig. 89 b and d, for covalent and passive conjugations, respectively). However, for the strips run in HS that had been passed through smaller filters -300 kDa, 50 kDa, 10 kDa or 5kDa-, the concentration dependence increasingly resembled the PBS and BSA curves in Fig. 89, c and d, and the test line intensity at 0 nM NS1 increased, indicating a false positive signal. Therefore, the high molecular weight proteins or multiprotein complexes in the HS are responsible for mediating non-specific adsorption and contribute to minimizing the false positive binding observed at 0 nM NS1. Similarly, the passive conjugation (NP-Ab-P) was more affected by the buffer in solution (Fig. 89 c and d), while the covalent antibody conjugation behaved in a more robust manner, and showed a lower background at 0nM (Fig. 89 a and b)
Figure 90: Treatment of HS with Molecular weight cutoff (MWCO) filters. Test line intensities of dipstick assays run with HS that has been passed through MWCO filters of 1 MDa (red circles), 300 kDa (green triangles), 50 kDa (blue triangles), 10 kDa (orange diamonds) and no filter, full HS (black squares). Fits to $K_D^{\text{eff}}$ were performed for the full HS (black dotted line) and 1 MDa HS (red dotted line) for the covalent conjugation (a), and passive conjugation (c). Test line intensity at 0 nM ZIKV NS1 for test strips run in HS and HS after passing through MWCO filters, for the covalent conjugation (b) or passive conjugation (d).

Error bars indicate average of three independent measurements.

**KINETICS OF CORONA FORMATION**

The behavior of protein coronas is dynamic as their formation occurs over time, and their composition and size evolves. The dipstick sandwich assays used here have running times of ~20 minutes, which is a typical timescale for POC assays. To investigate the kinetics of corona formation, I probed corona formation as a function of time to see if it would be changing over the timescale of the sandwich immunoassay. NP-Ab-C and NP-Ab-P were incubated with HS for different times and the size of the resulting corona was measured as a function of time by DLS (Fig. 90 top, covalent conjugation). The $D_H$ was larger than the original NP-Ab-C (71.6 ± 6.2 nm) and NP-Ab-P (87.1 ± 7.0 nm), and similar at all times measuring, for the covalent conjugation, NP-Ab-C: 181.5 ± 15.0 nm, 116.9 ± 10.6 nm, 116.9 ± 10.6 nm and 111.3 ± 9.6 nm in measurements of 1 min, 5 min, 15 min and 1 hr, respectively. For the passive conjugation, NP-Ab-P, $D_H$ was also larger and similar at all times, measuring 185.8 ± 10.6 nm, 168.5 ± 10.6 nm, 156.7 ± 11.6 nm and 172.8 ± 13.4 nm in measurements of 1 min, 5 min, 15 min and 1 hr, respectively, indicating that for the two conjugation strategies, the corona formed faster than minutes. Zeta
potential measurements of the NP-Ab-C exposed to HS at different times (Fig. 90, bottom) showed that the zeta potential was roughly the same (-12.1 ± 0.4 mV, -14.0 ± 0.2 mV, -13.8 ± 0.4 mV and -14.6 ± 0.4 mV measured at 1 min, 5 min, 15 min and 1 h, respectively), and similar results were obtained for the zeta potential of the passive conjugation, NP-Ab-P (-16.2 ± 0.5 mV, -16.4 ± 0.4 mV, -14.8 ± 0.4 mV and -16.2 ± 0.3 mV measured at 1 min, 5 min, 15 min and 1 h, respectively), also supporting that corona formation for both passive and covalent conjugation strategies, was faster than the timescale of the measurement. This corroborates reports from the literature that protein coronas form rapidly around NPs, and apparently here is occurring faster than the timescale of the sandwich immunoassay. Thus, in the case of both the covalent and passive conjugations, by the time the NP-Ab/NS1 has migrated to the test line, it most likely already has a corona formed around it. Consequently, the protein corona is unavoidable in an immunoassay that requires at least a few minutes to run. The first step in a dipstick assay is NS1 binding to the nanoparticle-antibody conjugates, which for the dipstick assay here occurs in the serum solution. However, this time scale is most likely on the order of seconds, and we were not able to conduct DLS and gel assays faster than this timescale to probe how corona formation impacts the NS1 binding to the nanoparticle-antibody conjugates.

![Figure 91: DLS of NP-Ab-C exposed to HS as a function of time (top). Zeta potential of NP-Ab-C exposed to HS as a function of time (bottom).](image-url)
I then investigated the effect of making pre-formed coronas around the NP-Ab on the sandwich immunoassay. This experiment was done for both, passive conjugations of NP-Ab-P as well as covalently conjugated NPs, NP-Ab-C. In both cases, preformed coronas exhibited markedly different behavior in the immunoassays than without protein coronas. For assays run in PBS and pure BSA with no NS1, the test line intensity was zero above baseline, showing that there was no non-specific adsorption (Fig. 91 a and b, bottom and center). Image analysis showed that the test line intensity increased with NS1 concentration and showed saturation after 30 nM NS1, similar to the strips run in HS (Fig. 91 c and d, red circles and blue triangles, for PBS and BSA, respectively). Assays for nanoparticle-antibody conjugates with pre-formed coronas run in HS showed behavior similar to the nanoparticle-antibody conjugates with no preformed coronas (Fig. 91 c, and Fig. 84, black squares). This shows that forming a corona around the nanoparticle-antibody conjugates, regardless of the conjugation strategy, can reduce false positives, and the passivation is similar to when the test is run in HS.

**Figure 92:** Dipstick immunoassay results as a function of ZIKV NS1 concentration (L-R): 91.6 nM, 38.4 nM, 9.2 nM, 3.8 nM, 0.9 nM, 0 nM for a) NP-Ab-C, covalent conjugation in PBS, BSA, and HS, b) NP-Ab-P, passive conjugation after pre-forming a protein corona on the conjugates and then running them in PBS, BSA, and HS. Top dot: control line. Bottom dot: test line. Test line intensity vs. NS1 concentration for NP-Ab-C(c) or NP-Ab-P (d) in PBS (red circles), BSA (blue triangles), and HS (black squares), and fits to $K_D^{\text{eff}}$ (lines). Background at 0ng/ml observed with passive (grey) and covalent (white) conjugations in different media.
Pre-treating the paper with NP-Ab affects test line assay

In addition, the serum could be impacting the surface effects of the nitrocellulose to mitigate the non-specific adsorption occurring in the assay. To investigate the effect of protein adsorption on the nitrocellulose, I pretreated the nitrocellulose strips with HS before running the assays. Strips were first put in contact with HS, which was allowed to run through the whole strip. Excess HS was washed by running PBS through the strips. This experiment was done for both, passive conjugations of NP-Ab-P as well as covalently conjugated NPs, NP-Ab-C. Strips were put into a tube with either the NP-Ab-P or NP-Ab-C conjugates with NS1 at different concentrations spiked into HS, PBS, or BSA. The strip was not allowed to dry between the solution transfer, so the kinetics of the migration were slightly different from when dry strips are put in fluid contact with the NP-Ab + antigen solution. However, this difference in kinetics was assumed to have a minor effect on test line intensity. Test line intensity for all running solutions (PBS, BSA, HS) increased with increasing NS1 concentration (Fig. 92a, b, c, d), though there were slight differences in the dose response curve for the three solutions. Assays run with BSA no longer showed false positives when no NS1 was present, though there was a residual intensity for the passive conjugation (NP-Ab-P) in the strip run in PBS. However, in comparison to no serum pretreatment (Fig. 84), the non-specific adsorption on the test line was mitigated. This shows that the nitrocellulose also introduces non-specific adsorption effects and impacts the Ab-NS1 interactions due to exchange between proteins in the nitrocellulose to the NP-Ab complexes, or due to HS corona formation around the nitrocellulose surface. Thus, both the NP and nitrocellulose introduce surface effects that can impact sandwich immunoassays.
Figure 93: Dipstick immunoassay results as a function of ZIKV NS1 concentration (L-R): 91.6 nM, 38.4 nM, 9.2 nM, 3.8 nM, 0.9 nM, 0 nM for a) NP-Ab-C, covalent conjugation in PBS, BSA, and HS, b) NP-Ab-P, passive conjugation after pre-forming a protein corona on the nitrocellulose and then running them in PBS, BSA, and HS. Top dot: control line. Bottom dot: test line. Test line intensity vs. NS1 concentration for NP-Ab-C(c) or NP-Ab-P (d) in PBS (red circles), BSA (blue triangles), and HS (black squares), and fits to $K_D^{off}$ (lines). Background at 0ng/ml observed with passive (grey) and covalent(white) conjugations in different media.

**Langmuir Model Analysis of Test Line Intensities**

Clearly, both the NP and the nitrocellulose introduce surface effects that influence the Ab-NS1 binding interaction. To quantify the impact of protein corona formation and serum pretreatments on the immunoassays, I utilized a modified Langmuir model to determine a binding affinity of the Ab-NS1 interaction$^{120, 268}$. The model utilizes the surface adsorption model where the free species is the NP-Ab/antigen complex, and the binding event is described by the binding to the immobilized Ab on the test line, or successful sandwich immunoassay formation. Thus, for a free species $A$, to bind to a surface, $S$, to form a surface immobilized species, $SA$ (Fig. 93 a):

$$S + A \leftrightarrow SA$$  \hspace{1cm} (1.)

the fraction of occupied sites, $\Theta$, can be measured as a function of the concentration of $A$ to obtain an expression containing $K_D$, the equilibrium dissociation constant:

$$\Theta = \frac{nK_D[A]}{1 + K_D[A]}$$  \hspace{1cm} (2.)
where n is the total number of surface sites, and $K_D$, is the equilibrium dissociation constant defined as:

$$K_D = \frac{[S][A]}{[SA]}$$  \hspace{1cm} (3.)

which describes the affinity of A for the surface S. In a dipstick or lateral flow assay, there are two binding events. First, the antigen binds to the NP-Ab. Then, the NP-Ab-antigen complex binds to the immobilized antibody on the test line. Here, the Langmuir surface binding model can describe the second binding event, so A is taken to represent the NP-Ab/NS1 complex and S is the immobilized antibody. When the NP-Ab/NS1 binds to immobilized antibody, the sandwich immunoassay represents the species SA (Fig. 93 b). The concentration of SA is proportional to the test line intensity, which is proportional to the number of NPs at the test line due to Ab-NS1 binding.

It should be of note that this model makes several assumptions. The Langmuir model assumes that surface bound species SA are independent and do not influence binding of adjacent sites, and that the surface binding can result only in a monolayer. This model also assumes equilibrium binding conditions, which is a large approximation as the conditions are under flow and occur over relatively short timescales.42 The binding affinity constant here is an effective binding constant, $K_D^{eff}$, as it lumps together the binding of the NS1 to the NP-Ab with the binding of the NP-Ab/NS1. With these caveats, I use $K_D^{eff}$ only to compare how the binding affinity changes with the different corona conditions, and do not use it as a way to obtain absolute binding affinities. To obtain $K_D^{eff}$ values, test line intensities vs. NS1 concentration were fit to Equation 3 (Table 1). $K_D^{eff}$ for NP-Ab run in PBS and BSA (Fig. 84, red circles and blue triangles) were not fit as they would not yield a meaningful value because their test intensity was nonzero at 0 nM NS1, and had no NS1 concentration dependence.
Figure 94. Langmuir binding model. a) Schematic of the free species A adsorbing onto a surface S, forming a surface bound species, SA. b) Analogous schematic with the NP-Ab conjugate bound to NS1 binding to surface bound antibody on the test line.

For the strip run in HS with no pretreatments (Fig. 84 black squares) had $K_D^{\text{eff}} = 12.7$ nM (NP-Ab-C), and $K_D^{\text{eff}} = 9.2$ nM (NP-Ab-P). Comparing the values to antibody-antigen interactions for NS1, this shows that the affinity of the NP-Ab-NS1 interactions was similar to Ab-NS1$^{259}$. For NP-Abs-C with premade coronas, the $K_D^{\text{eff}}$ was 11.0 nM when in PBS and 31.1 nM in BSA. For NP-Ab-P, with pre-made coronas, the $K_D^{\text{eff}}$ was 6.7 nM when in PBS and 5.0 nM in BSA. With both conjugations, the $K_D^{\text{eff}}$ was on the same order of magnitude as the NP-Ab conjugates run in HS with no preformed corona. This shows that the preformed corona can restore the specificity of the Ab-NS1 interactions to an affinity similar to the native antibody-antigen interactions.

For the HS pretreated paper, the $K_D^{\text{eff}}$ for strips run in PBS was 19 nM and BSA 10.2 nM for covalently-conjugated NP-Ab-C; while for NP-Ab-P I obtained $K_D^{\text{eff}}$ of 5.1 and 3.1, for PBS and BSA, respectively, again showing that the specificity of the Ab-NS1 interaction
could be restored by passivating the surface of the nitrocellulose with serum proteins. \( K_D^{\text{eff}} \) differs slightly between PBS vs. BSA vs. HS, showing that small differences in binding affinities remain even in pretreated paper.

Limits of detection (LODs) were also obtained from the fits, and were found to in the 1-20 ng/ml NS1 order of magnitude range\(^{175,270}\). Results are summarized in Table 18.

<table>
<thead>
<tr>
<th>Run media</th>
<th>Covalent conjugation</th>
<th>Passive conjugation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>LOD (ng/ml)</td>
<td>( K_D^{\text{eff}} ) (nM)</td>
</tr>
<tr>
<td>Bare-NP-Ab</td>
<td>HS</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
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<tr>
<td>Preformed coronas on NP-Ab</td>
<td>HS</td>
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</tr>
<tr>
<td></td>
<td>BSA</td>
<td>15.9</td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>1.1</td>
</tr>
<tr>
<td>Paper pretreatment</td>
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</tr>
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<td></td>
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<td>2.3</td>
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<td></td>
<td>PBS</td>
<td>0.2</td>
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Table 18: LOD, \( K_D^{\text{eff}} \) and \( R^2 \) values obtained from fitting the test line intensities as a function of ZIKV NS1 concentration in Figs. 84, 91 and 92 to the modified Langmuir binding isotherm model for bare NPs, preformed coronas around the NP-Ab, and HS pretreatment of the nitrocellulose.

Fits of the MWCO filter data (Table 19) could also yield \( K_D^{\text{eff}} \) of 12.7 nM and 9.2nM for the NP-Ab-C and NP-Ab-P and 1 MDa filtered HS, respectively. By covalently binding the NP-Ab-C, I obtained effective binding constants for strips run in HS filtered through 300 kDa, 50 kDa, and 10 kDa filters were 10.7, 10.2, 10.6 and 5.2 nM, respectively. Passive conjugation results were higher, where I obtained \( K_D^{\text{eff}} \) of 4.8nM for the 300kDa filter, and \( K_D^{\text{eff}} \) could not be calculated with smaller filters.
To sum up, in sandwich immunoassays, the antigen of interest must bind to a matched pair of Abs conjugated a NP and immobilized on the nitrocellulose substrate in a complex environment of full serum. Serum proteins result in protein corona formation around the nanoparticle-antibody conjugates and also adsorb to the nitrocellulose, and both of these phenomena impact the antibody-antigen interactions at the test line. We observe that passive and binding conjugation of antibodies on the nanoparticles lead to differences in the overall performance of the immunoassay. Covalent conjugation is the strategy that yields to lower false positive signal and is less susceptible to the media where the tests are run. Here we also observe that the serum proteins reduce false positives, which suggests that the protein corona plays a critical but beneficial role in the assay. These results highlight the importance of the protein corona in sandwich immunoassays, which are used in numerous point of care and medical lab assays. Because non-specific adsorption can influence rapid diagnostic assay results, it can impact treatment, quarantining, and therapy, and thus can have significant medical outcomes.

Furthermore, immunoassays are utilized in a broad range of different biological fluids, not just serum but also blood, urine, saliva, sweat, and others. The protein and small molecule compositions of these fluids vary greatly, and thus can result in formation of different coronas. Because of this, a better understanding of protein corona properties and formation in these different biological fluids is important, which will be the focus of future work. In addition, comparison of affinity constants of the Ab-NS1 interaction, $K_D^{\text{eff}}$, is

<table>
<thead>
<tr>
<th>MWCO filter</th>
<th>Covalent conjugation</th>
<th>Passive conjugation</th>
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<tbody>
<tr>
<td></td>
<td>LOD (ng/ml)</td>
<td>$K_D^{\text{eff}}$ (nM)</td>
</tr>
<tr>
<td>Full HS (no filter)</td>
<td>1.6</td>
<td>12.7</td>
</tr>
<tr>
<td>1 MDa</td>
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<tr>
<td>300 kDa</td>
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<tr>
<td>50 kDa</td>
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<tr>
<td>5 kDa</td>
<td>4.7</td>
<td>5.2</td>
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Table 19: LODs and $K_D^{\text{eff}}$ values obtained by Langmuir isotherm fits of test line intensities (Fig. 89) and corresponding $R^2$ values. Full HS and HS filtered with 1 MDa, 300 kDa, 50 kDa and 10 kDa MWCO filters were fit to obtain $K_D^{\text{eff}}$. 
possible via application of a Langmuir binding model, though due to the model's approximations, most likely only relative changes in $K_D^{\text{eff}}$ is prudent.

Non-specific adsorption is problematic even for relatively simple systems of NP-protein or NP-DNA conjugates in solution. In comparison, LF and dipstick assays are much more complex with many inorganic-biological interfaces, resulting in numerous opportunities for non-specific adsorption. This can have either beneficial or negative side ramifications on immunoassay outcomes. Quantification of these interface effects is important for not only understanding them, but also developing routes for eliminating negative influences of the interface. Despite their prevalent commercial and clinical use, lateral flow immunoassays face many challenges for use in the field. Not only do they suffer from non-specific adsorption, which can result in faint or smeared test lines, complicating the diagnosis, but also face complications in their stability and robustness. There are numerous efforts to improve their performance by optimizing not just the NP-Ab properties but also the assay materials, housings, and mode of operation. Hopefully, results from this work will aid in improving lateral flow immunoassays for their widespread deployment.
FACTORS THAT INFLUENCE THE LIMIT OF DETECTION LIMIT OF IMMUNOCHROMATOGRAPHY ASSAYS

In a dipstick sandwich immunoassay, NP-antibody conjugates mix and bind to a biomarker of interest while they flow by capillarity in a nitrocellulose membrane where a secondary antibody has been immobilized. If the antigen is able to bind simultaneously to the antibody on the nanoparticles and the antibody on the nitrocellulose, a visible test line color that serves as the readout to provide the diagnosis is formed, due to the accumulation of gold nanoparticles.

In order to improve the overall performance of lateral flow immunoassays many parameters can be optimized, some of them are the materials selected, the reagents, or the interfaces at the nanoscale. First, materials selection would involve the selection of the nanoparticles, nitrocellulose pore size and speed, and absorbent pad for the application. Secondly, reagents optimization includes the antibody pair against the antigen of interest with the necessary affinity, specificity and the optimization of reagents such as buffers and stabilizers. On the third place, the interface between proteins and NPs can greatly impact not only the structure but also the function of the protein, due to surface effects from the protein interacting with the NP, the nitrocellulose surface or the surface ligands on the NP.

Here, I construct a lateral flow immunoassay capable of binding to Dengue serotype 3 NS1 protein. I use this immunoassay as a model to compare different parameters that can have an effect on the sensitivity and specificity of the device: First, I synthesize different gold nanoparticles and characterize how the NP physical and optical properties affect the overall performance of the lateral flow immunoassay. Secondly, I test how the nitrocellulose pore size, and therefore the speed of the flow can affect the properties of the device. On the third place, I test the effect of different antibody pairs and observe that the antibody pair has a critical effect on the performance of the device. On the fourth place, I test the cross-reactivity of the assay by running NS1 from the other three serotypes, DENV-1, DENV-2 and DENV-4, and compare the performance of the lateral flow immunoassay when it is targeting the four different Dengue serotypes. This study offers key strategies for optimizing NP-antibody conjugates for detecting biomarkers in immunochromatographic assays.
RESULTS AND DISCUSSION

SYNTHESIS AND BIOCONJUGATION OF GOLD NANOPARTICLES

Gold nanoparticles are interesting for lateral flow sensing applications due to their tunable intense colors that arise from high extinction coefficients in the visible wavelengths of light. Gold nanoparticles can be conjugated to antibodies in order to detect antigens of interest, and the strong absorption of the nanoparticles allows for colorimetric detection by eye. Gold nanoparticles were synthesized by following literature methods (see Materials and Methods). Star-shaped nanoparticles (NStar200 and NStar400) were synthesized by reduction of gold in HEPES buffer and nanospheres (NS) were synthesized by citrate reduction, leading to the formation of NPs of different colors (Fig. 94).

Figure 95: Vials with NS (left), NStar200 (middle) and NStar400 (right)

The nanoparticles presented were chosen as they had similar volume per NP, as a method of normalization. Nanospheres had a diameter of 18.4±2nm, and a calculated volume of $3.4 \times 10^3 \text{nm}^3$/NP (Fig. 95, right, scale bar 50nm); NStar200 showed a core diameter of 15±3 core diameter, an arm length of 21±4nm and a calculated volume of $3.0 \times 10^3 \text{nm}^3$/NP (Fig. 95, middle, scale bar 50nm); finally, NStar400 had a core diameter of 13±3nm and a maximum arm length of 26±5nm, and a calculated volume per nanoparticle of $3.4 \times 10^3 \text{nm}^3$ (Fig. 95, left, scale bar 50nm).

Figure 96: TEM images of NS (right), NStar200 (middle) and NStar400 (left). Scalebar is 100nm

Optical absorption showed an SPR peak at 524nm for the NS, at 539nm for the NStar200 and at 607nm for the NStar400 (Fig. 96, red, magenta and blue solid lines, respectively).
NPs have a known molar extinction coefficient $\varepsilon_{NS} = 8.5 \times 10^8 \text{M}^{-1}\text{cm}^{-1}$, $\varepsilon_{NStar200} = 5.7 \times 10^8 \text{M}^{-1}\text{cm}^{-1}$, $\varepsilon_{NStar400} = 6.6 \times 10^8 \text{M}^{-1}\text{cm}^{-1}$, which allowed to calculate their concentrations from the optical absorption spectrum. After synthesis, the concentrations of NPs were measured: 2.1nM, 1.7nM and 1.4nM for NStar200, NStar400 and NS, respectively.

![Figure 97: Optical absorption spectra of NS (red), NStar200 (magenta) and NStar400 (blue) before (dashed line) and after (solid line) antibody conjugation](image)

DLS measurements showed that the NPs had similar hydrodynamic diameters, $D_H = 32.7 \pm 3\text{nm}$, $32.7 \pm 2.6\text{nm}$ and $38.5 \pm 1.2\text{nm}$ for the NS, NStar200 and NStar400, respectively (Fig. 97, top, red, magenta and blue patterned bars, respectively). Zeta potential showed that the nanoparticles were negatively charged. They had zeta potentials of $-33 \pm 1.6$, $-39 \pm 1$ and $-41.3 \pm 0.5\text{mV}$, for the NS, NStar200 and NStar400, respectively (Fig. 97, bottom, red, magenta and blue patterned bars, respectively).

![Figure 98: Hydrodynamic diameter (top) and zeta potential(bottom) of NStar200, NStar400 and NS before and after antibody conjugation.](image)

NPs were conjugated to monoclonal antibodies selected against NS1 of DENV-3 (mAb55, mAb323, mAb411) or DENV-1 NS1(mAb136). Antibodies were selected by injecting mice
with purified Dengue serotype 3 NS, and immortalizing the B cells that were able to produce antibodies capable of binding to the NS1 of DENV-3 or DENV-1. We selected three DENV-3 anti-NS1 antibodies, namely, mAb55, mAb411 and mAb323, and one DENV-1 anti-NS1 antibody, namely mAb136. We conjugated the antibodies on the gold nanoparticles by following a passive, electrostatic binding approach. Abs were added to the NPs at a incubation ratio of 100:3, and the Abs were allowed to adsorb onto the surface of the NPs. After bioconjugation, the NP surfaces were modified with a mPEG of 5kDa MW, which is used to reduce non-specific binding on the NP surface. Next, the unbound antibodies were separated by centrifugation.

After conjugation, UV-VIS showed that the SPR peaks of the nanoparticles broadened and red-shifted, which is indicative of biomolecule conjugation on NPs. The SPR after conjugation was at 548nm, 631nm and 527nm for the NStar200, NStar400 and NS, respectively (Fig. 96). Moreover, DLS measurements showed larger NPs after biomolecule conjugation, with a D_h of 64.9±5.4nm, 78.3±2.89nm and 43.8±3.5nm for the NStar200, NStar400 and NS, respectively (Fig. 97, top). The charge of the nanoparticles after bioconjugation did not change much, we measured zeta potential of -38±0.2 mV, -36.9±0.4 mV and -30.6±0.7 mV for NStar200, NStar400 and NS, respectively (Fig. 97, bottom).

We also checked antibody binding on the NPs by gel electrophoresis (Fig. 98). NStar400, NStar200 and NS were able to run in tight bands in the gel (lanes 2, 4 and 6 are NStar400, NStar200 and NS, respectively) before Ab conjugation, and after conjugation they shifted to lower mobility in the gels, which was expected (lanes 1, 3 and 5 are NStar400, NStar200 and NS, respectively). The antibody coverage on the nanoparticles was measured as 6Ab/NStar200, 14.2 Ab/NStar400 and 6.1 Ab/NS which were equivalent to footprints of 167nm^2, 86 nm^2 and 174 nm^2 for the NStar200, NStar400 and NS, respectively. The dimensions of a typical IgG antibody are 14.5, 8.5 and 4nm^2, with an average footprint of 81.3nm^2, therefore, indicating that less than a monolayer was achieved in NS and NStar200, and roughly monolayer coverage was achieved in NStar400. However, we should note that even if the Ab had coverage of less than a monolayer, this does not preclude the NP-Ab from performing well in lateral flow immunoassays.
Figure 99: Gel electrophoresis of NStar400, NStar200 and NS before conjugation (lanes 2, 4 and 6, respectively) and after antibody conjugation (lanes 1, 3 and 5, respectively)

GOLD NANOPARTICLE EXTINCTION INFLUENCE

First, the strips were run with different synthesis of NP-mAb. The dipstick assay, similarly to previous chapters, consisted of a nitrocellulose strip onto which the antibody mAb323 had been immobilized on the test area, and a control antibody (anti-Fc) was immobilized on the control area (Fig. 99). The conjugates were mixed with DENV-3 NS1 at varying concentrations of NS1 in solution from 0-3000ng/ml. When the fluid contacted the nitrocellulose, it started migrating through the strip into a wick, by capillary action.

Figure 100: Scheme of the tests, indicating the changes that could yield to lower limits of detection in immunoassays.

I compared the assay's overall performance when running with different synthesis of NPs (NStar-400, NStar-200 and NS), first in the HF135 nitrocellulose and by conjugating the monoclonal antibody mAb55 on the nanoparticles. I ran DENV-3 NS1 at concentrations ranging from 0-3000ng/ml in the tests. In order to normalize the NP-conjugate concentration in the tests, the same area under the absorbance curve was fixed in all experiments, although this yielded different concentrations of nanoparticles and antibodies in solution, however, in previous experiments we had observed that the area
under the absorbance curve was the value most similar to overall darkness of a spot in ImageJ, thus, we used the area under the absorbance curve as normalization. The LoD was determined as the minimum concentration that yielded an average test intensity 5-times higher than the standard deviation of the blank samples, and the $K_D^{\text{eff}}$ was the concentration that would yield 50% of the binding at saturation.

Langmuir curves were fitted on the data, and showed $R^2$ of 0.96, 0.97 and 0.95 for the NS, NStar, NStar400 (Fig. 100, left). The calculated LoD were 37.9ng/ml for the NStar-400, 73.1ng/ml for the magenta NStar-200 and 236.6ng/ml for the nanospheres. The calculated $K_D^{\text{eff}}$ were 180.7ng/ml for the NStar-400, 196.5ng/ml for the NStar-200 and 238ng/ml for the NS. We can observe that the $K_D^{\text{eff}}$ does not vary much with the different NP preparations, however, large differences in the limits of detection can be observed (Fig. 100, right): The NStar-400 had a ~8-times lower LoD compared to the NS, while NStar-200 had an intermediate behavior, of ~2-times higher LoD than the NStar-400. Between NStar200 and NStar400, the mAb coverage seemed to correlate with the LoD, where NStar400 had a 2-times lower LoD, with double the number of antibodies conjugated to its surface. For the NS, although the coverage was similar than the NStar200, we observed a higher limit of detection. This indicated that factors other than the coverage, such as curvature or surface molecules are important for the limit of detection in lateral flow assays.

![Graph](image)

*Figure 101: Testing the immunoassays with different nanoparticles. NS (green), NStar400 (black) and NStar200 (blue). Titration curves done by spiking NS1 in human serum at concentrations 3125, 1250, 625, 312, 125 and 0ng/ml (left). On the right, bar plot with the calculated LoD and effective Kd with the different NPs.*

As compared to ELISA measurements, ELISA showed a LoD of 9.5ng/ml, which was 4-times lower than the limit of detection of NStar-400. Sandwich ELISA showed an overall
binding constant of 70.1ng/ml, also lower than the $K_D^{eff}$ observed in lateral flow experiments.

**Effect of Pore-Size of the Nitrocellulose**

I next investigated the effect of the pore size of the nitrocellulose on the intensities of the test. The nitrocellulose membrane is critical in determining the sensitivity of lateral flow assays. Nitrocellulose membranes are available with different pore sizes. Because this membrane contains the immobilized antibodies where the test band and positive control band will appear, the nitrocellulose is critical in lateral flow assays. An ideal membrane can provide support and binding to the antibodies immobilized onto it, while at the same time giving low non-specific adsorption in the regions of the positive control and test bands. The flow speed of the nitrocellulose can influence the sensitivity of the assay.

In order to find the best nitrocellulose for the assays, nanoparticles (NStar400) conjugated to the antibody mAb55 were tested in dipstick assays, and mouse antibody mAb323 was immobilized on the lateral flow strips with different pore sizes by spotting (Fig. 99, scheme). Titration curves were performed by running DENV-3 NS1 at concentrations ranging from 0-3000ng/ml spiked into human serum. After the tests ran, I studied the intensities of the test bands, and fitted a Langmuir isotherm to obtain the LoD and $K_D^{eff}$ of the tests.

I tested the effect of decreasing pore sizes from HF75, HF90, HF120, HF135, HF180 until HF240, all purchased from Millipore. The numbers after the HF indicate the time in seconds that it takes for water to wick through 4cm of the nitrocellulose, in a standardized test, indicating that the HF75 was the fastest nitrocellulose to test that takes 75 seconds to run 4cm of nitrocellulose, while HF240 was the slowest, taking 240 seconds to run 4cm of nitrocellulose. We observe that the intensity of the fastest tests with larger pore sizes, had in general lower intensities at saturation (3000ng/ml) than the slower tests. I fitted a Langmuir isotherm on the titration curves and obtained $R^2$ of 0.96, 0.87, 0.98, 0.96, 0.95 and 0.97 for the HF75, HF90, HF120, HF135, HF180 and HF240 respectively (Fig. 101, left). The limits of detection were 30.3ng/ml, 60ng/ml, 20.9ng/ml, 37.8ng/ml 68ng/ml and 21.7ng/ml for nitrocellulose of flow speeds 75s/4cm, 90s/4cm, 120s/4cm, 135s/4cm, 180s/4cm and 240s/4cm respectively (Fig. 101, right, solid bars).
Figure 102: Testing the immunoassays with different nitrocellulose speeds. Titration curves done by spiking NS1 in human serum at concentrations 3125, 1250, 625, 312, 125 and 0ng/ml (left). On the right, bar plot with the calculated LoD and effective Kd with the different nitrocellulose speeds (seconds/4cm).

The effective dissociation constants were 293.9ng/ml, 170.9ng/ml, 166.59ng/ml, 138.79ng/ml, 206.39ng/ml and 242.99ng/ml for nitrocellulose membranes of flow speeds 75s/4cm, 90s/4cm, 120s/4cm, 135s/4cm, 180s/4cm and 240s/4cm respectively (Fig. 101, right, dashed bars). The highest values at saturation (highest signal at high concentrations of NS1) were observed for HF135, HF180 and HF120. The lowest effective dissociation was found to be the membrane HF135, while the lowest limit of detection was found of the membrane HF120. We observe that neither very slow nitrocellulose nor very fast nitrocellulose yielded the best results, and that those would be observed with intermediate speeds of the nitrocellulose membrane, the optimum speeds were in between 120s/4cm and 135s/4cm.

Effect of the pair of antibodies used
I then conjugated different antibodies on the NStar400 nanoparticles in order to test the effect of different antibodies with different binding properties on the overall performance of the test. I used HF135 nitrocellulose, and ran DENV-3 NS1 at increasing concentrations ranging from 0-3000ng/ml spiked into filtered human serum. I fitted a Langmuir isotherm on the titration curves and obtained $R^2$ of 0.96, 0.95, 0.99 and 0.94 for the antibody pairs of mAb323 on the nitrocellulose with mAb55, mAb136, mAb411 and mAb323 conjugated on the gold nanoparticles (Fig. 102, left). We observe that the intensity at saturation increased from NStar400-mAb323, NStar400-mAb136, NStar400-mAb55 until NStar400-mAb411, indicating that the NStar-mAb411 would be the nanoparticles that would achieve a highest signal at high concentrations of NS1 protein.
We observed a similar trend in the LoD, indicating that the pair of antibodies with highest limit of detection would be NStar400-mAb323 paired with mAb323 on the nitrocellulose, which had a limit of detection of 159.8ng/ml, followed by NStar-mAb136 with mAb323 on the membrane, with a limit of detection of 56.17ng/ml and the best would be NStar400-mAb55 paired with mAb323 on the nitrocellulose with a LoD of 37.8ng/ml and NStar-mAb411 with mAb323 on the membrane which had a LoD of 10.4ng/ml. The $K_{D}^{\text{eff}}$ effective of each of the pairs was 180.7ng/ml, 159.8ng/ml, 76.18ng/ml and 202.8ng/ml for the antibody mAb323 on the membrane and the NStars conjugated with the antibodies mAb55, mAb136, mAb411 and mAb323, respectively (Fig. 102, right).

These results were not surprising, as homo-pairs are typically regarded as less sensitive, hence the mAb323 homo-pair yielded the highest limits of detection and $K_{D}^{\text{eff}}$, which we had observed previously in the lab. mAb136- mAb323 was the second worst pair, which was also not surprising, as mAb136 is an antibody originally raised against DENV-1 NS1, and the titration curves were done by using DENV-3 NS1, so it was possible that lower binding affinity could be obtained with an antibody originally raised against another Dengue NS1 serotype. ELISA was also done with the antibody pairs, and showed similar results. The LoD in sandwich ELISA for mAb411, mAb136, mAb323 and mAb55 paired with mAb323 were 6.2ng/ml 6.5ng/ml, 12.4ng/ml and 9.5ng/ml respectively, which were between 1.7 to 12.9 times lower than the limits of detection in the lateral flow assays.
The effective dissociation constants measured in ELISA were 82.9ng/ml, 179.1ng/ml, 63.6ng/ml and 70.1ng/ml for mAb411, mAb136, mAb323 and mAb55, which were the same order of magnitude than the effective $K_D^{\text{eff}}$ measured in lateral flow assays. The antibodies mAb411 and mAb136 performed slightly better (8% and 11% improvement, respectively) at binding half of their capacity in lateral flow immunoassays than in ELISA format, showing that the surfaces of the nanoparticles and the nitrocellulose were able to aid in the binding of the NS1 to the antibodies, whereas the antibodies mAb55 and mAb323 performed better in ELISA formats than in lateral flow assays by 2.6 and 3.2-times, respectively, indicating that the binding of these antibodies was hindered by the presence of the nitrocellulose and nanoparticles, possibly due to denaturation of the antibodies or binding of the light chains to either the nitrocellulose or nanoparticles. It has also been observed that individual antibodies can perform better at different pH or salt concentrations. In the experiments presented here, the salt concentration and pH was kept the same for all antibodies, which could be one of the reasons for variability in the results.

**EFFECT OF THE TARGET ANTIGEN**

Although the pair of antibodies mAb55 and mAb323 were raised against DENV-3 NS1, 323 is capable of binding to the four Dengue serotypes, and mAb55 is able to bind DENV-1, DENV-3 and DENV-4 (Fig. 193, left). Therefore, when paired with each other, they can detect NS1 from DENV-1, DENV-3 and DENV-4. I tested the effect of varying antigens at increasing NS1 concentrations from 0-3000ng/ml and observed that DENV-2 NS1 was not detected even at high concentrations of the antigen, and therefore it was not possible to calculate a LoD, nor a $K_D^{\text{eff}}$ for DENV-2 NS1 binding on the nitrocellulose. We also observed that DENV-3 NS1 was the antigen that yielded highest intensity the maximum saturation (Fig. 103, red, left), as well as the lowest limit of detection, that was 47.2ng/ml (Fig. 103, red solid bar, right), followed by DENV-1 with a LoD of 79.8ng/ml (Fig. 103, black solid line, right). The highest limit of detection was against DENV-4. DENV-4 NS1 was only reliably detected at concentrations higher than 370ng/ml (Fig. 103, green solid line, left). Although DENV-1 and DENV-4 showed a similar intensity at saturation (Fig. 103, left), Langmuir fits showed that DENV-4 had a $K_D^{\text{eff}}$ higher 5.8-times than DENV-1,
which were 1481 ng/ml for DENV-4 and 252.6 ng/ml for DENV-1. The $K_D^{\text{eff}}$ of the binding with DENV-3 was 357.6 ng/ml (Fig. 103, right, dashed bars).

Figure 104: Testing the immunoassays with different antigens. DENV-1 (black), DENV-2 (blue) and DENV-3 (red) and DENV-4 (green). Titration curves done by spiking NS1 in human serum at concentrations 3125, 1250, 625, 312, 125 and 0 ng/ml (left). On the right, bar plot with the calculated LoD and effective Kd with the different antigens, DENV2 not included as a Langmuir curve could not be fitted.

These values could be compared to ELISA results with the same pairs of antibodies, where similarly, DENV-2 NS1 was not detected by either ELISA or IA. Nevertheless, the effective $K_D^{\text{eff}}$ and limits of detection of ELISA and IA did not follow the same trend. In an ELISA format, the LoD of DENV-4 was the lowest at 2.7 ng/ml, with a $K_D^{\text{eff}}$ of 142.4 ng/ml. The LoD of DENV-3 NS1 was 9.5 ng/ml with a $K_D^{\text{eff}}$ of 70.1 ng/ml and for DENV-1 NS1, the LoD was 27.4 ng/ml with a $K_D^{\text{eff}}$ of 200 ng/ml. The difference between ELISA results and lateral flow could be due to several reasons. First, the capillarity-flow nature of the latter, where the kinetics of binding are important for the overall performance of the test, which is not a variable in ELISA format. Secondly, in ELISA format, there are no interactions between NPs and the antibodies, nor between the nitrocellulose and the antibodies/antigens in the test.

**DISCUSSION**

Accurate laboratory diagnostics are necessary for appropriate patient treatment and management. The current methods for Dengue and Zika detection include thermal cycling amplification (PCR) and enzyme-linked immunoabsorbent assays (ELISA). However, these methods require specialized equipment, reagents, and highly trained personnel, have a complex methodology, and a slow turnaround for readout, challenging their
suitability in the field. Immunochromatography assays, also known as lateral flow tests, on the other hand, are simple, easy to use, low-cost diagnostic tools that provide fast detection of antibodies or antigens.

Lateral flow is an ideal method for diagnosing disease in remote areas because the need for refrigerated storage can be obviated; moreover, specialized chemicals and expertise are not needed. However, one challenge of LFI is that their limit of detection, sensitivity and specificity depend on many factors, such as the antibodies/antigens used in the test, the nanoparticle properties and conjugation methods, the flow rates of the nitrocellulose or the media in which the assay runs, among others. In this chapter I focused on some central elements of lateral flow devices, in order to shed light in their effect on the overall performance of lateral low immunoassays.

First, gold nanoparticles are critical to ensure that the devices have enough sensitivity to detect the illness even at low concentrations of target protein, such as in early or late stages of the disease. In order to better understand the effect of gold nanoparticles in lateral flow immunoassays, I synthesized and calculated the molar extinction coefficient of several nanoparticles typically used for biological applications. We observed from this chapter that the extinction coefficient, hence the absorbance of the nanoparticles at constant concentration, increased with increasing the volume of the nanoparticles. This result was significant, as it allows to calculate the concentration of the nanoparticles based on only their absorbance in solution. Moreover, the results compared well with other types of NPs in the literature, and they also corresponded well with simulations performed in DDA.

On the second place, I tested two different conjugation strategies to attach antibodies on gold nanoparticles: passive, electrostatic conjugation and covalent conjugation. I constructed lateral flow assays by following the two conjugation strategies, and used different media to test the lateral flow assays. We observe that the performance of the covalent conjugation was less dependent on the media where the NPs were flowing, however, electrostatically bound conjugates would yield to many false positives – and very low specificities-, when tested in both PBS and BSA. We also observed that both strategies to prepare the conjugates behaved similarly when tested in human serum. This result was significant. It indicated that the conjugation strategy and post-modifications had
different importance depending on the application, where in applications in buffers/low protein media, the best strategy was to perform a HS corona pre-treatment on the nanoparticles before using them in lateral flow assays. We also observed that even though the covalent conjugation led to lower coverage in the lateral flow assays, it also led to lower nonspecific binding in buffers, while the intensity of the overall signal was maintained.

Finally, I tested different strategies to modify the lateral flow assays and try to obtain lower limits of detection, or higher signals in the device at high concentrations. These results were significant as they showed that for our application, the best nitrocellulose were the ones with intermediate pore-sizes, HF180, H135 and HF120; the best membrane to use in lateral flow assays is dependent on the type of application and architecture of the LFA, as others find different membranes to perform best in their application\textsuperscript{272}. We also observed that NStar400 had a lower limit of detection and highest overall signal in the immunoassay, leading to a LoD that was only 4-times higher than the LoD of ELISA tests, as compared to spherical nanoparticles, which had a LoD that was 25-times higher than ELISA. Hence, showing that using highly absorbing NPs can help reduce the overall limit of detection of immunoassays. We also observed the differences between ELISA and lateral flow assays when testing different antibody pairs and antigens, and observed that the best performers in ELISA were not the same as in lateral flow assays, indicating that it is necessary to select the best binders for each application.

**Contributions**

Work by researchers from the Gehrke lab and the Hamad-Schifferli lab, other than myself has been necessary for the compilation of this chapter. First, Dr. Irene Bosch has grown the hybridoma cells that were used to obtain the antibodies. Marc Carré, Diana Fandos and Cristina Rodríguez helped in performing some experiments. “The extinction coefficient of gold nanostars” and the “Effect of protein corona in lateral flow immunoassays” have been published. These chapters are partially reprinted. Reproduced with permission from de Puig, H.; Bosch, I.; Carre-Camps, M.; Hamad-Schifferli, K., Effect of the Protein Corona on Antibody-Antigen Binding in Nanoparticle Sandwich Immunoassays. *Bioconjugate Chemistry* **2017**, *28* (1), 230-238. Copyright
CONCLUSIONS AND FUTURE WORK

The main objective of this work was to design, assemble, characterize, and validate rapid immunochromatography rapid tests to achieve the serotype-specific detection of Dengue, as well as the diagnosis of co-circulating diseases, such as Zika and Chikungunya. Due to the molecular similarities shared by these viruses, the biggest challenge was in characterizing and identifying the combinations of antibodies that could allow the serotype-specific detection of the viruses while keeping a low enough limit of detection. The diagnostics were designed in such a way that images of the tests could be captured using a mobile phone and automatically analyzed by ImageJ in order to obtain standardized objective and quantitative information from the lateral flow assays. This way, by obtaining the GPS location of the pictures we have the possibility of creating map containing linked data of the diagnostic and geolocation of the patients, and thus, enabling real time epidemiology. The approaches presented are generalizable and can be applied to many applications requiring rapid diagnostic based on a fluid sample. These new, effective, low-cost devices would be very useful in developing countries, and also for developed countries, where they can contribute to lowering the overall cost of healthcare. Other applications of the improved lateral flow devices include food and water surveillance, real time-epidemiology, drug compliance and field diagnostics of disease.

Below is a summary of the main conclusions, limitations and advantages of this work, while more extended conclusions can be found in each chapter.

In chapter 3, “Development of Rapid Diagnostics to Detect and Distinguish the four Dengue virus serotypes and Zika virus”, I discuss the development of a serotype-specific viral antigen test for Dengue, as well as a specific test for Zika. These tests detect the viral NS1 protein, which is released into the bloodstream after synthesis by infected cells. The development of these diagnostics was possible through production of highly-specific monoclonal antibodies. The diagnostics developed in this chapter were first validated with purified recombinant proteins expressed in mammalian cells to facilitate proper protein folding and secondary modifications, including glycosylations. The limits of detection of the diagnostics, as measured with recombinant mammalian-expressed NS1 proteins spiked into human serum, were in the range of 1-20ng/ml for the four Dengue serotypes and Zika. Moreover, we demonstrated that the tests did not cross-react with NS1 proteins.
from a number of other related flaviviruses (West Nile Virus, Japanese Encephalitis, Yellow Fever, Tick Borne Encephalitis, and Usutu). The second validation of the diagnostics was done with first pass patient samples and virus isolates, expanded by infection in vero or c6/36 cell cultures. Supernatants from infections with the four Dengue viruses and Zika, from different epidemics were tested in the diagnostic, and we observed a good correlation between the NS1 produced by cell infection and the recombinant NS1 proteins. Finally, the diagnostics were validated using patient serum from different endemic areas in the Americas and India. We obtained sensitivities and specificities of 0.76 and 0.89 for DENV-1, 0.89 and 0.98 for DENV-2, 1 and 1 for DENV-3, 0.88 and 1 for PAN DENV and finally 0.81 and 0.86 for the ZIKV test; showing that it is possible to generate a rapid test to distinguish closely related viruses in lateral flow assays and that mobile phone images can be used to obtain quantitative information from the diagnostics. This test represents an improvement over the currently used diagnostic devices (PCR and ELISA), which are, many times, not available in endemic areas. The tests described in this chapter could be used for patient screening, as well as in asymptomatic patients, in order to provide routine patient care, as well as epidemiological surveillance. Nevertheless, the tests presented have some limitations, and opportunities for improvement remain. First, using whole blood instead of serum would simplify the use of the tests by avoiding the serum preparation step, and would be possible by using a blood separator paper. Second, for the ZIKV diagnostic we used a 5x concentration step in the patient’s serum due to the low viremia that ZIKV patients have in blood. This step would not be necessary if the diagnostic were adapted to accept larger volumes of patient sample, or by introducing a concentration step in the lateral flow device. Third, the image analysis was done on a computer, as ImageJ could not run on a phone; however, by using a different program for analysis, a phone app could be installed on the phone, which would give the results much faster to patients. Finally, the diagnostics presented in this chapter are effective only when analyzing samples collected during the acute phase of the disease, prior to clearance of the virus by the immune system, which is useful when diagnosing febrile patients, which was the objective of this work. However, serological tests for anti IgG and IgM would be useful to detect the disease during the post-acute
phase. It would be desirable to combine the two methods in order to provide better care for patients during both the febrile phase of the disease and the convalescent stage.

In chapter 4, “Multiplexed detection of Dengue, Zika and Chikungunya”, I presented an immunoassay that could be multiplexed in order to diagnose Dengue, Zika and Chikungunya, without cross-reactivity with other similar flaviviruses. This diagnostic was validated with purified NS1 (DENV and ZIKV) and E2 (CHIKV) proteins spiked into uninfected human serum, and no cross-reactivity was observed with flaviviruses NS1 proteins (West Nile Virus, Japanese Encephalitis, Yellow Fever and Tick Borne Encephalitis). Moreover, the diagnostic was also validated with supernatants from Dengue, Zika and Chikungunya-infected vero cell cultures. This diagnostic demonstrated that by using a combination of spatial separation and specific antibodies it was possible to provide differential diagnostics for closely-related pathogens. This presented the advantage that it could detect three diseases that have very similar symptoms with only 30μl of patient serum. Future work for this chapter includes validating the triplex diagnostic with human serum samples, and similarly to chapter 3, adapting it to work with whole blood.

In chapter 5, “A novel approach for rapid responses to Emerging Threats: repurposing cross-reactive antibodies with multicolored nanoparticles”, I present a method to decrease the time-to-manufacturing of lateral flow devices by using cross-reactive antibodies of closely-related pathogens, that could be readily obtained. In order to solve the issue of cross-reactivity, I exploit gold nanoparticles of different colors to distinguish between the related viruses as well as co-infections. This approach is completely general and is explained in the chapter with four different examples: (1) A test that can discriminate the four Dengue serotypes by using cross-reactive antibodies raised against NS1 of Dengue serotype 3. (2) A diagnostic that can distinguish Ebola and Marburg by using antibodies originally raised against Marburg envelope protein. (3) A multiplexed Dengue and Zika diagnostic developed with antibodies raised against Dengue NS1. (4) And finally, I show that we can use the same approach to enhance a commercially available, widely used, Dengue diagnostic that cross-reacts with Zika, hence converting at a very low cost, a nonspecific diagnostic into a multiplexed, specific test for both Dengue and Zika. The tests in this chapter were validated with purified recombinant
proteins spiked into human serum, and the Ebola/Marburg diagnostic was also validated with supernatants from BHK cells infected with replication-competent vesicular stomatitis viruses bearing the glycoprotein of Ebola virus (VSV-rGP-EBOV) or the glycoprotein of Marburg virus (VSV-rGP-MARV). The results shown in this chapter demonstrated that it is possible to screen, identify and exploit cross-reactive antibodies originally developed against one virus in order to diagnose a different closely-related pathogen. This was shown with diseases of different identities, from 80% homology, in the case of the four serotype NS1 proteins of Dengue to 30% homology in the Ebola/Marburg E2 proteins example. Although the cross-reactive antibodies could give a positive signal for the two related diseases, gold nanoparticles of different colors can be used in order to distinguish between the infections as well as co-infections. This test requires the use of image post-processing as well as LDA algorithms to classify the results, which could be done on a mobile phone.

The novelty of chapter 5 lies in the fact that it enables the use of either “out of the shelf” cross-reactive antibodies, or already existing nonspecific diagnostics, in order to diagnose outbreaks of previously unknown pathogens. This enables the rapid development of diagnostics on the first stages of an outbreak, permitting a faster response and control of epidemics. Nevertheless, the tests presented in chapter 5 have some limitations, and opportunities for improvement remain. First, the tests were not validated with patient samples because of biosafety requirements and the difficulty of obtaining patient samples during the epidemic outbreaks, and only purified proteins or vero sups were used. In order to test if the approach is valid, patient samples from both single and double infections should be tested, in order to analyze the test’s sensitivity and specificity. Second, although a mobile phone could in theory be used for the analysis of the images, all analysis was done on a computer.

In chapter 6, “Engineering immunochromatography assays”, I discuss methods that can be used to lower the detection limit of lateral flow immunoassays. This chapter is divided into three different parts. First, “The extinction coefficient of gold nanostars, nanospheres and nanorods” explores both analytical and computational methods to calculate the extinction coefficient of gold nanoparticles, and quantifies the extinction coefficient of nanostars, nanospheres and nanorods, which is a parameter that can be used to
calculate the concentration of the nanoparticles based on their optical extinction spectra. It shows that the extinction coefficient increases with the nanoparticle volume, as well as a good correlation between experimental and computational methods. The second part “Effect of the protein corona on antibody-antigen binding in nanoparticle sandwich immunoassays”, highlights the importance of the medium (serum, PBS or protein suspensions) where the particles are dispersed when running in the lateral flow immunoassays. It develops a method to reduce false positives and nonspecific binding by using protein coronas. Moreover, it compares two different strategies of binding the antibodies on the gold nanoparticles, by either covalent conjugation or electrostatic binding, and shows that covalent conjugation yields to both lower limits of detection as well as improved performance of the nanoparticles in the different buffers tested. The third part, “Factors that influence the limit of detection of immunochromatography assays”, explains different methods that can be used in order to lower the limit of detection of lateral flow assays, such as the type of NPs used, nitrocellulose pore-size or the antibodies used in the test. It shows that by using nanoparticles with increased absorbance it is possible to reduce the limit of detection of immunoassays, and it highlights the importance of using antibody pairs with low limits of detection. Together, this chapter provides techniques to improve the limits of detection, and thus, the sensitivity and specificity of lateral flow immunoassays. Opportunities for improvement would include using other nanoparticle preparations, such as polymeric nanoparticles or fluorescent nanoparticles that may allow for further decreasing the detection limit of the assays.

Taken together, this thesis has presented methods to rapidly prepare lateral flow immunochromatography devices to detect the four serotypes of Dengue, as well as Zika, Chikungunya, as well as Ebola and Marburg. Moreover, it explains methods to increase the sensitivity and specificity of the immunoassays. Nevertheless, a general opportunity for improvement over this thesis would be to enable the tests to run with whole blood of infected patients, which will require a prospective study so that tests re run concurrently with blood draws from infected individuals in endemic areas.
FUTURE PROSPECTS AND APPLICATIONS

The work presented here can enable new applications and potentially open up new research possibilities.

DEVELOPMENT OF LATERAL FLOW IMMUNOASSAYS FOR DIAGNOSIS WITH WHOLE BLOOD

Using whole blood instead of serum would simplify the use of the diagnostics as a point of care in the clinics. In order to do that, the nanoparticles would need to be dried on a conjugate pad, and the entire test should be placed inside a cassette. After testing several designs of 3D-printed cassettes, with the help of Luis Alberto Mora and Nikolas Albarran, we have already built and tested a mold to produce injection-molded housings for the multiplexed tests at mid-large scale. The injection molded cassettes and molds were manufactured in the Edgerton center machine shop. In order to use the devices, new materials such as a blood separator filter would need to be purchased and tested.

REAL TIME EPIDEMIOLOGY BASED ON RAPID DIAGNOSTIC RESULTS

The second possibility that this work introduces is, given a large enough cohort of patient samples, start understanding the geographical and temporal pathogen spread patterns based on real-time diagnostic data. This capability would be very useful to understand the spread of epidemics in endemic countries, as well as giving policy-makers and governments the possibility to make informed decisions in mosquito control and eradication, as well as vaccination initiatives.

In order to do that, two actions need to be taken in advance. First, it would be desirable to develop a phone app to analyze the diagnostics, in order to give faster results to doctors and nurses in endemic areas. Secondly, a database to store the patient’s information with mapping capabilities of the results should be built.

DISEASE-SPECIFIC SEROLOGY TESTING

The tests presented in this thesis are accurate when diagnosing disease in patients that are in the febrile phase of disease, before the virus has been cleared by the immune system. A serologic test that could detect IgG and IgM would be useful to diagnose past infections. Typically, this is done by conjugating the E protein of the virus on gold nanoparticles, spotting anti-IgG and anti-IgM on the nitrocellulose, and using the patient’s generated antibodies (which can be either IgG or IgM, depending on the maturity of the patient’s immune response) as the antigen. The similarity of the immune responses
against all flaviviruses typically leads to the generation of a mixture of cross-reactive antibodies against many flaviviruses in the patient’s serum, and therefore it may be difficult to distinguish the diseases if targeting the patient’s generated antibodies against the E protein of the virus. However, NS1 is not as conserved between the different flaviviruses, and therefore, a serologic test to target anti-NS1 antibodies could represent an advantage, as it would help in distinguishing the different infections. Following a similar approach as in chapter 5, nanoparticles of different colors could potentially be conjugated to the NS1 from different serotypes of Dengue, or different flaviviruses, in order to identify the infections that originally caused the immune response, based on the relative binding of the nanoparticles of different colors. This approach would be useful to diagnose past infections of patients, and moreover, it could be used to inform on the efficacy of vaccines. The currently licensed DENV vaccine, Dengvaxia, only contains the E protein, thus vaccinated patients would have anti-E antibodies in their system, but no anti-NS1 antibodies. Therefore, the presence of anti-NS1 antibodies in the patient’s blood would indicate that the patient was infected by Dengue.
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