

**Development of nanoparticle-based biosensing
for molecular diagnostics**

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Abstract

Ebola virus disease causes widespread and highly fatal epidemics in human populations. During the latest outbreak in West Africa only 60% of the affected people underwent diagnosis, emphasising the unmet need for suitable diagnostics tools. This thesis harnesses the intrinsic optical properties of nanoparticles for the development of two nanoparticles-based biosensing systems for the detection of Ebola virus.

The first system is a serological point-of-care test for humoral immune response to Ebola virus. This test combines in-house made capture ligands, lateral flow technology with a custom smartphone application. The final format of the test replicates all mechanical, optical and electronic functions of a laboratory-based enzyme-linked immunosorbent assay in a single and multiple analytes formats. This assay was evaluated validated in Uganda using sera from nearly 130 Sudan Ebola Virus survivors and controls, demonstrating 96% sensitivity and 100% specificity.

In addition to the single analyte format, two configurations of the multiple analyte formats were constructed. The first configuration simultaneously detected the immune response to three viral antigens. In the second configuration, the point-of-care test was translated for Bundibugyo Ebola Virus species and used for the identification of immune cross reactivity between viral species.

The second system was targeted for earlier stage viral detection via direct detection of fragments of viral nucleic acids. This assay comprised spherical nucleic acids nanoparticles as the biosensing interface and Exonuclease III for target recycling amplification. Although the system is operated in a laboratory, it was addressed to function with the simplest form of laborious equipment to enable its used in resource-limited facilities.

These complementary systems have to potential to be utilised for patient management, surveillance during and post outbreaks and vaccine and therapeutic evaluation.

Declaration of originality

I, Polina Brangel, declare that the work reported within this thesis is my own, unless otherwise stated. Collaborations are acknowledged in the text.

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Table of Contents

Abstract.....	3
Declaration of originality.....	5
Acknowledgements.....	7
Chapter 1 - Introduction.....	21
1.1. Commentary.....	21
1.1.1. The outline of the thesis.....	22
1.2. Nanoparticles in biosensing and clinical diagnostics.....	24
1.2.1. Gold nanoparticles in medical diagnostics	25
1.2.2. Fluorescence.....	27
1.3. Point-of-Care diagnostic test.....	29
1.4. Lateral flow tests	32
1.5. Smartphone-based optical biosensors in medical diagnostics	34
1.6. Ebola virus	35
1.6.1. Viral hemorrhagic fever	35
1.6.2. Taxonomy and species.....	36
1.6.3. Ebola virus structure and viral proteins.....	37
1.6.4. EVD vaccine development.....	39
1.6.5. Ebola virus Diagnostics	40
Chapter 2 - The Development of an Ebola Virus Antibody Test Using Lateral Flow and Smartphone Technologies.....	46
2.1 Introduction	46
2.2. Material and Methods.....	48
2.2.1. Cloning, expression and purification of recombinant viral proteins	48
2.2.2. Colorimetric ELISA.....	50
2.2.3. Western blot	52
2.2.4. Characterisation of AuNPs conjugation to secondary antibody	53
2.2.5. Lateral flow immunochromatographic strip test preparation	55
2.2.6. Testing nonhuman antibodies via lateral flow test strip	56
2.2.7. Development of the smartphone application	56
2.2.8. Human positive and negative samples.....	57
2.3. Results and discussion	57
2.3.1. System design and operation.....	57
2.3.2. Characterisation of the recombinant viral proteins.....	58
2.3.3. Preparation of AuNPs conjugates with secondary antibodies	64
2.3.4. Strip design and preparation	70
2.3.5. Lateral flow assay assembly and optimisation	71
2.3.6. Development of smartphone application.....	74
2.3.7. Determination of the optimal size of the AuNPs	75
2.3.8. Evaluating the limit of detection of the developed assay	77
2.3.9. Assay optimisation for testing human samples	81
2.4. Conclusions.....	88
Chapter 3 - Serological Surveillance of Human Ebola Virus Survivors Using the Developed Smartphone-based Lateral Flow Test.....	93
3.1. Introduction	93

3.1.1. 2000-2001 Ebola virus outbreak in Uganda.....	94
3.1.2. Ebola clinical features.....	95
3.2. Material sand methods.....	96
3.2.1. Ethics statement	96
3.2.2. Samples collection.....	97
3.2.3. Cloning and expression and purification of recombinant viral proteins	97
3.2.4. Lateral flow immunochromatographic strip test.....	97
3.2.5. Testing nonhuman antibodies in spiked serum	98
3.2.6. Human sera analysis with lateral flow strip	99
3.2.7. Development of the next generation smartphone application	100
3.2.8. Human IgG ELISA	100
3.2.8.1. Chemiluminescence ELISA.....	100
3.2.9. Data analysis.....	101
3.2. Results and discussion	102
3.3.1. Presentation of the smartphone-based lateral flow for human samples	102
3.3.2. Adjusting the smartphone application for testing real samples under field conditions.....	105
3.3.3. Evaluation of SUDV monoplex assay with human sera	107
3.3.4. Evaluation of the SUDV multiplex assay with human sera.....	112
3.3.5. Species multiplex assay.....	117
3.4. Conclusions.....	119
Chapter 4 - Bundibugyo Ebola Virus Serological Screening and Viral Cross Reactivity.....	123
4.1 Introduction	123
4.1.1. Scope of the chapter	123
4.1.2. BDBV outbreaks review.....	124
4.1.3. Cross-reactivity between species.....	125
4.2. Materials and methods.....	126
4.2.1. Ethics statement	126
4.2.2. Samples collection.....	127
4.2.3. Cloning and expression and purification of recombinant GP ₁₋₆₄₉	127
4.2.4. Lateral flow immunochromatographic strip test.....	127
4.2.5. Human sera testing with lateral flow strip.....	128
4.2.6. Test strips analysis via smartphone application.....	128
4.2.7. Samples testing via chemiluminescence ELISA.....	128
4.2.8. Data analysis of lateral flow strips and ELISA.....	128
4.3. Results and discussion	129
4.3.1. Sample collection.....	129
4.3.2. Sera characterisation via chemiluminscense ELISA	130
4.3.3. BDBV Monoplex lateral flow assay	132
4.3.4. Multiplex analysis using EVD species multiplex assay	133
4.4. Conclusions	138
Chapter 5 - Detection of Ebola Virus Nucleic Acids using SNA and Enzymatic Target-Recycling Amplification.....	141
5.1 Introduction	141
5.1.1. Spherical Nucleic Acids	142

5.1.2. Fluorescence dye quenching as the analytical signal of the assay	143
5.1.3. Target-recycling with Exonuclease III.....	145
5.1.4. Using <i>In-silico</i> simulation for the design of the recognition oligonucleotides.....	145
5.2. Materials and methods.....	146
5.2.1. <i>In-silico</i> simulation of Ebola virus oligonucleotide probes	146
5.2.2 In-silico analysis of secondary structure of DNA oligonucleotides.....	147
5.2.3. Melt curve analysis of the designed oligonucleotides.....	148
5.2.4. Gold nano particles synthesis.....	148
5.2.5. SNA Synthesis	149
5.2.6. SNAs optical characterization.....	150
5.2.7. Dynamic Light Scattering	150
5.2.8. Transmission electron microscopy	150
5.2.9. Flare titer	151
5.2.10. Flare hybridisation onto SNA.....	151
5.2.11 Assay dose-response	151
5.2.12 Assay dose response with Exo III	152
5.3 Results and discussion	152
5.3.1. Scope of the assay.....	152
5.3.2 <i>In-silico</i> design of detection probes.....	153
5.3.3. Hybridisation test of the selected probes	160
5.3.4. Synthesis and characterisation of SNA	163
5.3.5. Assay development and assembly	168
5.3.6. Detection of short target.....	172
5.3.7. Detection of full 60 mer SUDV target.....	174
5.3.8. Calculation of the required clinical range for the assay	175
5.3.9. Addition of Exonuclease III to the assay.....	176
5.4. Conclusions	182
Chapter 6 - Conclusions and outlook.....	185
6.1. Conclusions	185
6.2. Outlook	188
Reference.....	191
Appendix A - Biosensing.....	205
Appendix B.....	207
Appendix C	208

Table of Figures

Figure 1.1. Illustration of the development process of NP-based biosensing system. Starting from the left, selection of NPs, surface modification with biosensing mechanism and the scaling up for clinical applications	26
Figure 1.2. Jablonski fluorescence diagram	28
Figure 1.3. Hypothetical absorption (green) and emission (red) spectra of a fluorophore	29
Figure 1.4. Analysis of clinical samples with the conventional diagnostic tools might take several days	30
Figure 1.5. Schematic presentation of the lateral flow test strip	33
Figure 1.6. Scanning electron microscope image of the structure of Ebola virus virion	37
Figure 1.7. Schematic illustration of Ebola virus (A) genomic RNA: NP-nucleoprotein gene; VP-viral protein gene; GP-glycoprotein gene; L-RNA-dependent RNA polymerase gene, and (B) virion	38
Figure 1.8. Collection of the operating BSL 4 facilities around the world as to 2010	41
Figure 2.1. Schematic overview of the developed assay for the detection of IgG response to EVD in human sera samples	60
Figure 2.2. Characterisation of GP ₁₋₆₄₉ – SUDV using colorimetric ELISA	61
Figure 2.3. Characterisation of GP ₁₋₆₄₉ – EBOV using colorimetric ELISA	62
Figure 2.4: Characterisation of GP ₁₋₆₄₉ – BDBV using colorimetric ELISA	63
Figure 2.5. Characterisation of VP40 and NP from SUDV.....	65
Figure 2.6. AuNPs ‘salt stress’ test	66
Figure 2.7. A. Absorbance spectra of labelled and bare 20 and 40 nm AuNPs	67
Figure 2.8. Size distribution by volume of the AuNPs upon conjugation to capture antibody, Z-average size	68
Figure 2.9. AuNPs morphology analysis via TEM. TEM images of 20 nm and 40 nm AuNPs	69
Figure 2.10. Setting for NC membrane spotting	71
Figure 2.11. Schematic illustration of the lateral flow test strip assembly	72
Figure 2.12. Determination of running buffer	73

Figure 2.13. Determination of blocking reagent in the running buffer	74
Figure 2.14. Smartphone application operation flow chart	76
Figure 2.15. Dependence of the colour intensity of the lateral flow test zone on the size of the AuNPs, quantification in RTLI of the control line and test line intensity of 20 and 40 nm AuNPs, conjugated to capture antibody without target analyte	77
Figure 2.16. A-C: 3C10, BDBV and 6D8 antibody titer dose-response curves, respectively	79
Figure 2.17. Species-multiplex lateral flow. Strip consists of three test lines spotted with GP ₁₋₆₄₉ from SUDV, BDBV and EBOV	80
Figure 2.18. Analysis of human plasma and serum	82
Figure 2.19. Comparison between different secondary antibodies against human IgG	84
Figure 2.20. Serum volume optimisation	85
Figure 2.21. Sequence of the multiplex test lines	87
Figure 3.1. Ebola virus outbreak in 2000-2001 in Uganda	95
Figure 3.2. Schematic graph representing EVD time frames of disease onset and development of disease signs and symptoms associated with severe EVD infections (bottom) and the corresponding diagnosis assays (top)	96
Figure 3.3. Host response, assay and smartphone app	104
Figure 3.4. Assay development	106
Figure 3.5. Monoplex SUDV-GP ₁₋₆₄₉ lateral flow test	111
Figure 3.6. Samples readout comparison by ELISA and mono-SUDV lateral flow	112
Figure 3.7. Venn diagram of the IgG immune-reactivity between SUDV recombinant proteins in human survivors detected via the developed SUDV-multiplex lateral flow test strip	115
Figure 3.8. SUDV-multiplex test strip with three test lines for simultaneous detection of IgG response to SUDV EBO against VP40, GP and NP in sera	117
Figure 3.9. Photo of the tested samples with the EVD species multiplex lateral flow test strips for identification of the immune response against a specific EVD species	118
Figure 4.1. Map of the Democratic Republic of the Congo and Uganda showing the locations of BDBV outbreaks, each red ellipse represents the location of the outbreak and the Number of recorded cases/deaths	124

Figure 4.2. Phylogenetic tree presenting full-length genomes of Ebola virus species	126
Figure 4.3. Venn diagram representation of positive immunoreactivity between Bundigugyo, Zaire and Sudan GP ₁₋₆₄₉ of screened BDBV survivors	132
Figure 4.4. Lateral flow and smartphone quantitative analysis of BDBV samples	134
Figure 4.5. Screening BDBV samples with EVD species multiplex assay	137-8
Figure 5.1. Schematic illustration of the synthesis method of SNA. Citrate coated AuNPs are incubated with DNA modified with thiol in its terminus to form low-density monolayer	144
Figure 5.2. Cy5 (A) chemical structure and (B) Optical properties. Absorbance spectrum is in blue and emission spectrum is in red	145
Figure 5.3. Schematic illustration of the assay	155
Figure 5.4. In-silico prediction of secondary structure of the selected BVDV DNA oligonucleotide at 25 ⁰ C and 50 mM NaCL. Predicted free energy of the secondary structure -6.37 kcal mol ⁻¹	158
Figure 5.5. In-silico prediction of secondary structure of the selected EBOV DNA oligonucleotide at 25 ⁰ C and 50 mM NaCL. Predicted free energy of the secondary structure -4.76 kcal mol ⁻¹	159
Figure 5.6. In-silico prediction of secondary structure of the selected SUDV DNA oligonucleotide at 25 ⁰ C and 50 mM NaCL. Predicted free energy of the secondary structure -6.52 kcal mol ⁻¹	160
Figure 5.7. Melt curves show the relation between fluorescence and temperature of free DNA oligonucleotides strands, thiolated-probe, flare and SUDV target.	
Figure 5.8. Derivatives melt curves show the relation between the negative first derivative of the melting curve and temperature of free DNA oligonucleotides strands, thiolated-probe, flare and SUDV target	162
Figure 5.9. T _m of sets DNA oligonucleotides obtained from melt curves represents the temperature of the dissociation of the 50% of the double stranded DNA	163
Figure 5.10. Image of SNA synthesised with thiolated oligonucleotide probe ..	165
Figure 5.11. Normalised absorption spectra of SNA synthesized with SUDV thiolated oligonucleotide (3) after each wash into water from the synthesis solution	167
Figure 5.12. Normalised absorption spectra of two populations of SNA synthesized with (3) and (7) and bare AuNPs used for the SNA synthesis	167

Figure 5.13. Particles average diameter measured by dynamic light scattering of bare AuNPs, SNA synthesized with SUDV thiolated oligonucleotide (3) and SNA synthesized with control thiolated oligonucleotide (7)	168
Figure 5.14. Dynamic light scattering intensity distribution curves of (A) Bare AuNPs, (B) SNA synthesized with SUDV thiolated oligonucleotide (3) and (C) SNA synthesized with control thiolated oligonucleotide (7)	169
Figure 5.15. TEM images of SNA synthesised with SUDV thiolated oligonucleotide, using four different magnifications	170
Figure 5.16. Normalised absorption (black) and fluorescence emission (blue) spectra of SNA (dashed line) and Cy5 flare (solid lines) in PBS	170
Figure 5.17. Absolute fluorescence emissions at 665 nm of different concentrations of Cy5 flare in PBS. Data obtained from three independent experimental repeats in triplicates	172
Figure 5.18. Absolute fluorescence emissions at 665 nm of different titer ratios of Cy5 flare and SNA (1 nM) with complementary oligonucleotide (light blue) and non-complementary oligonucleotide (dark blue)	172
Figure 5.19. Initial set point of the assay prior target oligonucleotide addition	174
Figure 5.20. Logarithmic dose response curve. Absolute fluorescence emission recorded at 665 nm upon 60 min incubation of the target SUDV oligonucleotide (1) with initially hybridised SNA + flare (square), SNA (circle) and free flare (triangle) in PBS at room temperature	174
Figure 5.21. Comparison of binding of short SUDV oligonucleotide target (1), blue and full length SUDV oligonucleotide target (2), black to SNA (synthesised with thiolated SUDV oligonucleotide (3)) previously hybridised to flare in PBS	175
Figure 5.22. Normalised absorbance spectra of SNA in different dilutions of CutSmart® Buffer in water	178
Figure 5.23. SNA aggregation kinetics over 50 min in the following dilutions of CutSmart® Buffer and water, from top line to bottom line: 1:1, 1:2, 1:3, 1:5, 1:8, 1:10, 1:0	178
Figure 5.24. Selectivity of the assay between three Ebola virus species. Dose response between the concentrations of subtype-specific 60 mer sequences and the relative fluorescence intensity	180
Figure 5.25: Normalised readout of SNA/flare/Exo III, SNA/flare and free flare at the set point (dash black) and after 10 h (dash blue) incubation at room temperature	181
Figure 5.26. Dose response curves of the assay with and without Exo III	182

Figure 5.27. Specificity of the assay: SNA/Flare/Exo III incubated with 1 nM SUDV (black), 10 µM BDBV (blue) and 10 µM EBOV (red) for 1 hr 183

Table of Tables

Table 1.1. Diagnostic assays used to detect EBOV infection in humans - Current diagnostics methods used for EVD detection in humans 42

Table 2.1. Summary of the available NC membranes for lateral flow test strips. 70

Table 3.1. Summary of serum screening results of SUDV-GP₁₋₆₄₉ monoplex lateral flow assay of SUDV survivors and non-infected controls 109

Table 3.2. Summary of serum screening results of SUDV multiplex and monoplex lateral assays and full antigen ELISA of survivors and non-infected controls .. 115

Table 3.3. ELISA serology screening of SUDV survivors** and controls** against multiple recombinant viral proteins, NP, GP₁₋₆₄₉ and VP40 116

Table 4.1. ELISA serology screening of BDBV survivors and controls against GP proteins of three viral species. Results are classified in a semi-qualitative approach for the presence of IgG antibodies against the tested protein 131

Table 5.1. BDBV top scored 60 mer 156

Table 5.2. EBOV top scored 60 mer 157

Table 5.3: SUDV top scored60 mer 158

Table 5.4. Probes for the SUDV nucleic acid assay. Purple colour corresponds to the hybridisation regions between the sequences (1), (2) to (4) 161

List of Abbreviations

%PP	Positive percentage
app	Application
APS	Ammonium persulfate
BDBV	Bundibugyo Ebola Virus
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BSL 2	Biological Safety Level 2
BSL 4	Biological Safety Level 4
C	Non-infected control
cDNA	Complementary DNA
Cy5	Cyanine 5
DLS	Dynamic light scattering
DRC	Democratic Republic of Congo
EBOV	Zaire Ebola Virus
ELISA	Enzyme Linked Immunosorbent Assay
EVD	Ebola virus disease
Exo III	Exonuclease III
FRET	Fluorescence Resonance Energy Transfer
GP	Glycoprotein
GP ₁₋₆₄₉	Recombinant glycoprotein
hr	Hour
HRP	Horseradish peroxidase
I	Intensity
IFA	Indirect immune-fluorescence assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
LB-Kan	LB agar containing Kanamycin
LOC	Lab-on-chip
MP	Mobile-phones
MW	Molecular weight
NaCl	Sodium chloride

NC	Nitrocellulose
NCBI	National Centre for Biotechnology Information
nm	Nanometres
NP	Nucleoprotein
NPs	Nanoparticles
OD	Optical density
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline, 0.01% w/v Tween-20
PCR	Polymerase chain reaction
POC	Point-of-Care
QDs	Quantum dots
RefSeq	Reference sequence
ROI	Region of interest
RT	Real time
RTLI	Relative test line intensity
S	Survivor
SNA	Spherical Nucleic Acids
SPR	Surface plasma resonance
SS	Stokes Shift
SUDV	Sudan Ebola Virus
TEM	Transmission electron microscopy
T _m	Disassociation temperature
UV	Ultraviolet
VHF	Viral hemorrhagic fevers
VP	Matrix protein

Chapter 1

Introduction

1.1.Commentary

Biosensing is an analytical method for the detection of analyte based on biological components. Biosensing has played a key role in evolution itself as all living systems interrogate and respond to their environment with sensing capacities. Individual biological systems have developed a wide range of complex solutions to sensory processes and their sensing receptors respond through highly specific biochemical cascades. Such biological and chemical receptors exhibit remarkable selectivity, sensitivity and specificity to their target groups within a relatively rapid response. Hence, mimicking these biological mechanisms is the fundamental principle of biosensing implementation in diagnostics, imaging, and targeted therapeutics. For instance, the first biosensor appeared in 1962 and utilised glucose oxidase enzyme for amperometric detection of oxygen during cardiac operations [1].

In the field of biomedical diagnostics, biosensing processes have been successfully developed for accurate and timely diagnosis of medical conditions and diseases [2]. Diagnosis through the detection of biomarkers tend to be less invasive [3] and is able to identify the disease in an earlier stage [4]. As a result, patient treatment is significantly improved and the impact of the disease is reduced. Furthermore, these diagnostics are usually scalable for a large number of samples and are especially well suited for infectious disease epidemics and pandemics.

Emergence and re-emergence of diseases of high risk to individuals, and communities, are of specific interest and concern to public health systems, whether in developed or developing countries. Ebola virus disease (EVD) represents a prime example of such re-emerging pathogens. Although rare, EVD outbreaks are unpredictable with a high fatality rate and increased frequency in

the last 15 years [5]. These facts, together with the lack of vaccines or therapeutics, explain why EVD has been categorised as top risk threat [6]. In contrast to other viruses affecting millions of people every year, EVD stands out from all other diseases and disclose an alarming gap in our immune system's ability to defend itself against it. However, although it appears that EVD outbreaks occur mainly in remote, rural areas and are quarantined effectively, Ebola virus is still considered a worldwide threat.

The 2014-15 West African EVD outbreak, 29,000 people were infected, with over 11,000 fatalities [7], presented a global threat to emerge from rural areas in Africa and spread rapidly worldwide. The portrayal of EVD in the media has created public fear of EVD [8] and EVD is commonly misperceived as an active agent transmissible through air, although it can be only transmitted through body liquids. Following five decades since its discovery, EVD is still considered as a major concern and threat in both endemic and non-endemic regions of the world. The potential threat of its use as a bio-weapon has also dramatically changed world perspective regarding its risks [9], and thus encouraged new research and resources in the fight against EVD diseases.

1.1.1. The outline of the thesis

This thesis is concerned with the development of biosensing mechanisms, which are based on properties of nanoscale materials. Using novel design principles to build assays for the detection of antibodies and nucleic acids related to Ebola virus. The motivation for this thesis was driven by the need for better diagnostics and prognostics technologies for infectious diseases.

In this thesis, two different assays were developed, a serological assay for the detection of EVD antibodies from human samples and a solution nanoparticles (NPs)-based assay for the detection of Ebola nucleic acids.

The Introduction chapter covers the principle of biosensing, the theoretical background, optical properties of NP and their use in biosensing. In addition, the concept of point of care tests and smartphone use is reviewed in context of the latest literature. To complete the introduction, a review of Ebola virus is established and new diagnostic systems are presented.

Chapter 2 describes the development of a lateral flow test strip for the detection of specific antibodies against the most immunogenic EVD viral proteins, including glycoprotein (GP), nucleoprotein (NP) and matrix protein 40 (VP40). These viral proteins were prepared in a recombinant form and were used as the recognition entities in the biosensing mechanism. The development of lateral flow strip also included functionalisation of gold nanoparticles (AuNPs) as the visual labelling of the target analytes. The assay was developed in two formats – single analyte (monoplex) and multiple analytes (multiplex) detection.

The monoplex format consisted of a single test line of GP and a control line. The multiplex format was designed in two configurations, in the first configuration three GP proteins from three viral species were presented as independent test lines. In the second configuration of the multiplex, the GP, NP and VP40 from SUDV species were integrated as three test lines. In addition, a complementary smartphone application was designed and developed. This application was used for quantitative analysis of the lateral flow test strips, for data storage and connectivity.

Chapter 3 demonstrates the evaluation and validation of the smartphone-based lateral flow assay. The evaluation and validation are performed by testing nearly 130 real human serum samples for the detection of immune response to EVD. The majority of these samples were obtained from previously infected and recovered patients from the EVD during the 2000-1 outbreak in Gulu district in Uganda. This chapter presents the correlation between the performances of the novel assay compared to the standard serological test, Enzyme Linked Immunosorbent Assay (ELISA). Both the monoplex and the multiplex formats are tested throughout Chapter 3. Additionally the developed assay was tested under field conditions during sample collection in 2015 in Uganda.

Chapter 4 presents the use of the developed smartphone-based lateral flow assay for the detection of immune cross-reactivity between EVD species. As such, human samples of survivors from the 2008 outbreak in Bundibugyo district, Uganda are screened via the monoplex and the multiplex assay formats.

Chapter 5 demonstrates another approach for the diagnosis of Ebola virus via the detection of the viral nucleic acids. This assay was designed with the use of fluorescence transduction mechanism through quenching of a fluorescent dye

in presence of the AuNPs, which is mediated by of viral complementary DNA (cDNA) fragment. This chapter also demonstrates the strength of an *in-silico* approach for the design of oligonucleotides probes sequences, which yields in especially high specificity.

In chapter 6, the main findings of the thesis will be reviewed, alongside suggestions for further research stemming from it.

In this thesis, biosensing assays have been designed, developed and characterised in terms of their sensitivity, ease of use, and general applicability. These two assays provide a complementary assessment for the detection of Ebola virus.

1.2.Nanoparticles in biosensing and clinical diagnostics

The implementation of nanotechnology in biosensing (For the sake of clarity and presentation, a detailed description of the three key components of the analytical biosensing systems is provided in the Appendix A) has emerged over the past decade. This growth has been driven by the development of novel nanomaterials, which are used as building blocks for nanomaterial-biomolecule hybrid systems. In such a short time, the nanomaterial landscape grew immensely using in particular colloidal nanoparticles (NPs), for analyte detection with improved detection limits, greater specificity and stability in complex matrices. This section covers the use of colloidal nanoparticles for biosensing systems and biosensor devices.

NPs exhibit unique properties, which are not present in the bulk form of their materials [10] and NPs respond differently to absorption of light. For instance, inorganic semiconductor NPs absorb a wide range of light wavelengths and emit specific wavelength that is proportional to their size. Their emission intensity can then be modulated as a response to the presence of analyte. Other metallic NPs display size-dependent localised surface plasma resonance (SPR) [11]. These examples present general applications for NPs as optical transducers in biosensing systems [12].

Figure 1.1 describes the steps in the development of NPs-based biosensing assays. The core composition of the NPs reflects on the physical and

optical properties are utilised for the signal transduction. Modification of the surface of these NPs is performed to enable the linkage between the NP and the biorecognition element. The biosensing mechanism is then designed and introduced to the surface of the construct. This mechanism responds to the presence of the analyte by changing the optical properties of the whole system. The final step is usually the most challenging, as it requires operation of the biosensing system in clinical relevant conditions (e.g. blood, urine). Hence, evaluation of the developed biosensing system requires stabilising the mechanism against non-specific interaction, increasing its stability over time as well as its sensitivity within the clinical range of the analyte [13]. The vast amount of developed NPs-based biosensing assays stay within a lab-based proof of concept. Therefore, the ability to scale up these novel technologies for large clinical studies remains a challenge.

1.2.1. Gold nanoparticles in medical diagnostics

Fluorescent quantum dots (QDs) [14], conjugated polymers [15], silica NPs [16] and noble metal NPs [12] are widely used in NP-based biosensors. As this thesis focuses on the use of AuNPs for the development of novel biosensing concepts, properties and biosensing applications of AuNPs are reviewed in this section.

Although the preparation of colloidal AuNPs has been known since Roman times [17]. The theoretical basis for the characteristic absorbance peak, coherent electron oscillations, referred as localised SPR, was first described in Lorenz–Mie solution in 1908 [18]. Their experimental work revealed that AuNPs exhibit a broad absorbance spectrum with a size tunable SPR peak.

Analyte-dependent aggregation/dispersion assays induce shifts in the SPR peak wavelength and thus have been employed as the basis for many biosensing systems. Sandwich-based assays utilising antibody-antigen specificity were initially described by Leuvening *et al.* in 1980 [19]. Antibodies specific to the target of interest were physically adsorbed to the surface of 60 nm AuNPs, and upon the introduction of analyte, the solution colour changed from red to blue, indicating immunorecognition. In fluorescence assays, AuNPs were used to

quench the fluorescence emission of either a fluorescent dye or particle [14]. Depending on the presence of analyte, the emission would change.

The relatively high stability of AuNPs in biological samples enabled development of ultrasensitive assays. In particular, the plasmonic ELISA enable naked eye detection of prostate specific antigen in whole serum at the ultralow concentration of attogram per milliliter [12].

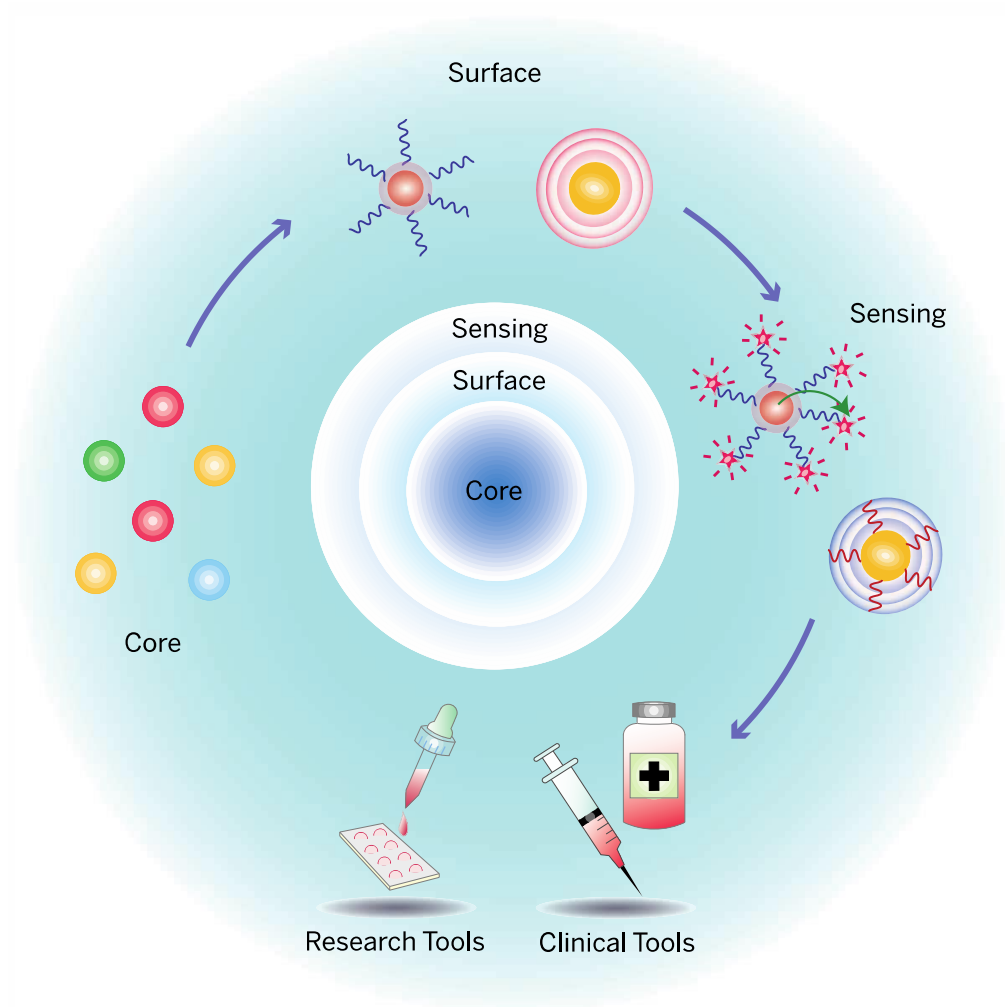


Figure 1.1. Illustration of the development process of NPs-based biosensing system. Starting from the left, selection of NPs, surface modification with biosensing mechanism and the scaling up for clinical applications. Red circles represent fluorescent NP and orange circles represent metallic NPs. Adapted from P. D. Howes et al. copyright 2014 [13].

1.2.2. Fluorescence

Fluorescence is used as an analytical readout signal in many biosensing assays. Fluorescence is a classification of the luminescence in which certain molecules emit light due to an electronically excited state. This usually occurs by chemical, mechanical or physical mechanisms that cause energy absorbance.

Photoluminescence is a process of excitation of a molecule with ultraviolet (UV) or visible light wavelengths. Depending on the configuration of electrons in a molecule and the excited state, either fluorescence or phosphorescence emission pathways are possible.

Fluorescence emission occurs as a fluorophore absorbs light of a specific wavelength (that can excite an electron from ground state to excited state) and re-emits photons of a higher wavelength. The brief interval between the two is known as the fluorescence lifetime and generally lasts few nano seconds.

Fluorescence can be separated into three main events that classify the energy levels of different excited states in molecular orbitals. These energy level classifications are often presented via a Jablonski energy diagram, see Figure 1.2 and explained in three steps. In the first step, fluorophore absorbance of a high-energy photon (blue) leads to system's electronic and vibration at excitation. In the second step, the excited electron vibrational relaxes (red). In the third step, the relaxation of the excited electron back to the ground state is accompanied by specific wavelength photon emission, corresponding to the energetic gap between the excited state and the ground state [20]. Although the emission and excitation cycle can be repeated hundreds of times, fluorescent dyes often reach a photobleached state, which results in destruction of their fluorescence emission properties.

The absorbance spectrum of a fluorophore usually presents a maximum peak. Excitation at the wavelength of this peak causes maximum excitation of the substance and results in fluorescence emission.

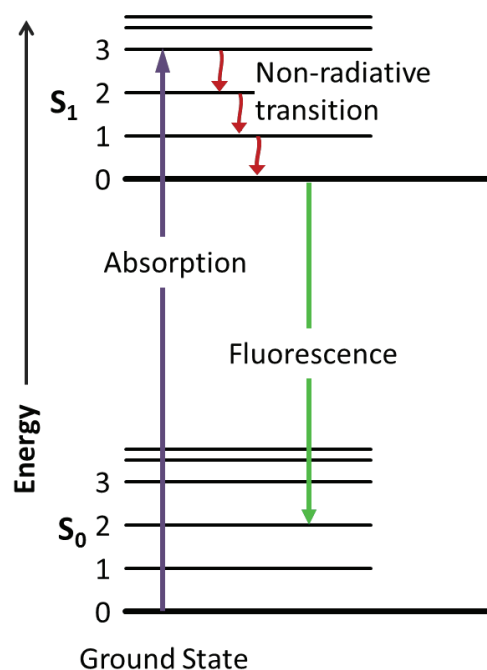


Figure 1.2. Jablonski fluorescence diagram. When sufficient energy is absorbed it excites an electron, followed by non-radiative relaxation, and then photon emission at a specific wavelength. Adapted and modified from Google images.

Figure 1.3 presents an illustration of absorbance and emission spectra of a hypothetical fluorophore. The peaks of the absorbance and the emission spectra are separated and this separation is known as Stokes Shift (SS). The SS occurs by rapid relaxation from vibrational state. For this reason, photon emission occurs most of the times when the vibrational energy state is lower than the excitation and therefore emits energy and at a higher wavelength. The smaller the SS then the closer the optimal excitation wavelength to the emission peak is. In this case, more complex sets of filters are required to separate the absorbance wavelength from the emission wavelength [20].

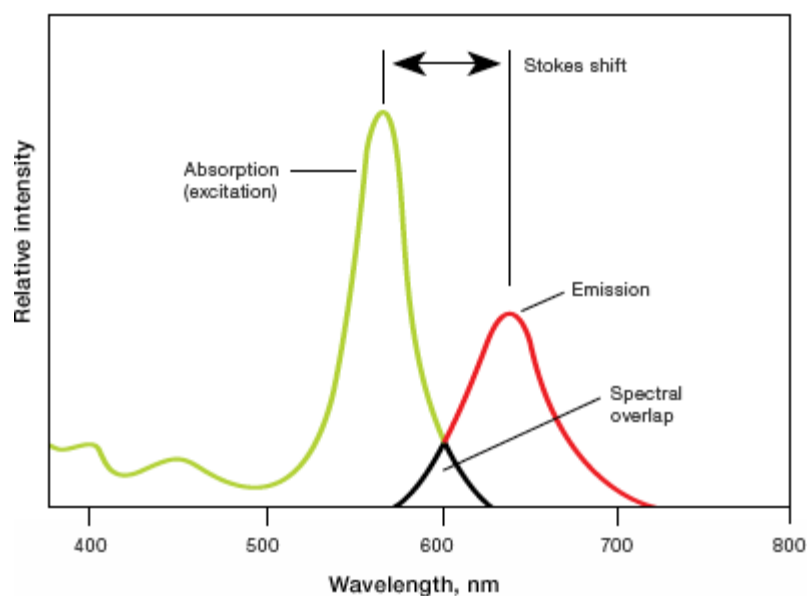


Figure 1.3. Hypothetical absorption (green) and emission (red) spectra of a fluorophore. Adapted and modified from Quantitative Fluorescence Microscopy text book [21].

1.3. Point-of-Care diagnostic test

In the last century, the approach of traditional medicine has been changing from medication to prevention by early stage diagnosis. Specific and accurate identification of the medical condition is the key for better patient management and targeted treatment. Indeed, molecular diagnostics provide such solutions through the detection of specific biomarkers to identify a condition or a disease, in various indication spaces such as cardiology, oncology, neurology and infectious diseases.

The molecular diagnostic techniques can be classified in two groups as presented in Figure 1.4. The first group consists of standard diagnostics tools. These operations normally take up to several days requiring specialised laboratory facilities and trained personal. Point of Care (POC) tests form the second group. These operations are considered faster, cheaper and simpler to perform.

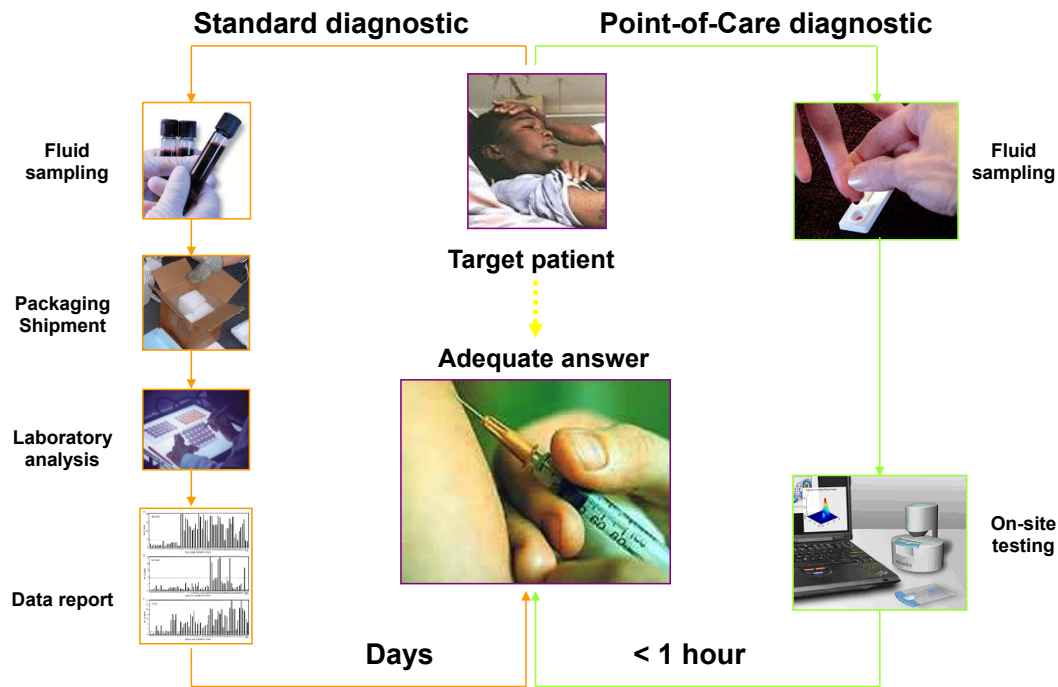


Figure 1.4. Analysis of clinical samples with the conventional diagnostic tools might take several days. The collected sample has to be transferred to the closest diagnosis center. The analytical work is then assembled to produce results converted into a readily accessible data. The concept of point of care diagnostics is to reduce the time for the sample analysis to less than an hour with a simple portable device. Adapted from P. Brangel [22].

In general, POC testing is defined as biochemical analysis performed at the site of the patient and at the time of need for medical care. This type of analysis provides immediate result regarding the condition of the tested individual and therefore facilitates treatment decision or further and more complex screening [23].

Ideally, the POC analysis can be operated at the bedside of the patient, at the office of a clinician or under field conditions at a secondary and tertiary care service. Hence, these devices must be rapid and simple for operation, and they must display initial results within minutes and be a simple procedure with up to three steps for the analysis of biological samples that are easily obtainable (e.g. whole blood, serum and urine) [24] [25].

Indeed, POC tests are especially useful for medical care in resource-poor settings and are crucial for diagnostics, monitoring and surveillance in low and

middle-income countries where the health care infrastructure is weak and the availability of sustainable medical care is limited [26].

A POC test is usually composed of a disposable cartridge or strip that produce qualitative or quantitative analysis of the sample and a complementary or built-in reader [27]. Furthermore, up to a year shelf life, components stability in room temperature and humidity are imperative performance requirements for such systems that normally employed outside of laboratory settings.

The technologies behind these disposable cartridge or strips mainly rely on capillarity flow in either microfluidic-based devices or paper-based strips also known as dipsticks [27], which will be further reviewed in section 1.4. The microfluidic devices normally uptake small volumes of fluid samples and enable their mixing while flowing along the device [28].

The concept of these microfluidic devices is used to form lab-on-chip (LOC) POC platforms that can be fabricated from polymers, paper, silicon, glass and ceramics [29]. The LOC produce a range of ancillary procedures within the device for sample preparation, separation and pre-concentration of the target analyte and its detection [30].

The POC tests have a wide range of applications in medical diagnosis. Such applications include detection of markers specific to respiratory diseases, oncology, women health, cardiovascular disorders and infectious diseases. Urinalysis is another major application of the POC platforms, which is used for the detection of pregnancy, ketones, proteins and drug abuse [27].

The target analyte can be classified by size, from small molecules to whole cell detection. The detection of small molecules is usually performed via electrochemical or optical transducers. For instance, Glucose-meters are the most commonly known POC tests. This meter uses the enzymatic activity of Glucose Oxidase to electrochemically quantify the level of glucose in a blood sample [31]. POC analyzer for continuous and real time screening of lipids profile and cholesterol has been recently presented by A.R. Dale *et al.* [32]. Another especially exciting configuration of POC sensor was integrated in contact lenses for analysis of electrolytes (Na^+ , K^+ , Ca^{2+} , Cl^-) composition [33].

Proteins are a rather large class of target analytes for POC tests, such as antibodies against HIV, Streptococcus and Influenza. Home screening POC tests

for cardiac injury biomarkers aim at predicting heart failure at an early stage by detecting cardiac troponins [34]. Moreover, whole cell POC for full blood cell counts is also available. This POC provide crucial information for the diagnosis, prognosis and monitoring of conditions such as HIV/AIDS by performing CD4+ T-lymphocyte count[35], which determines patients' eligibility for antiretroviral therapy.

Despite the great potential of POC technologies, their commercialisation faces a range of challenges [23]. The emerging POC systems often face lower reproducibility than the conventional diagnostic tools as most POC operate on a qualitative yes/no basis. In addition, sensitivity and specificity remain a practical challenge to fit the required clinical range of certain analyte, especially in infections with low viral loading [36]. In addition, documentation of data produced by POC test presents another challenge. As POC are targeted to make clinical decision, the documentation of the results of the analysis needs to fulfill accreditation requirements, which can often be problematic due to lack of connectivity of these devices to patient charts [36]. Nonetheless, this is currently being addressed by combining these tests with smartphones technologies and will be further discussed in section 1.5.

1.4. Lateral flow tests

Lateral flow immuno-chromatography is a technology that is widely applied in the field of medical diagnosis. It is used for qualitative or semi-quantitative detection of target analyte. For quantitative analysis, external readers are usually required [37]. This cost effective, specific and rapid method can be operated under field conditions, making it especially attractive for resource-limited or non-laborious conditions. Therefore, it is supplied in a dry format and can be stored at ambient temperature for a long period of time (up to 2 years).

This technology was invented in the early 80's and has been utilised in a wide range of applications, including detection of pathogens, drugs, hormones and metabolites [38].

The operation procedure of a standard format of lateral flow test strip is described in this section and described in Figure 1.5. It consists of porous Nitrocellulose (NC) membrane referred as the analytical zone of the test. The membrane includes spotted lines of recognition biomolecules, on the test line and the control line. The test line is used for the detection of the analyte and therefore presents recognition biomolecule with high affinity to the target. The control line exhibits another recognition biomolecules with high specificity against other (than analyte) component in the system. In addition, the lateral flow test strip includes colorimetric particles that utilise the recognition and tagging of the analyte. These are used for the visual labeling of test results [37].

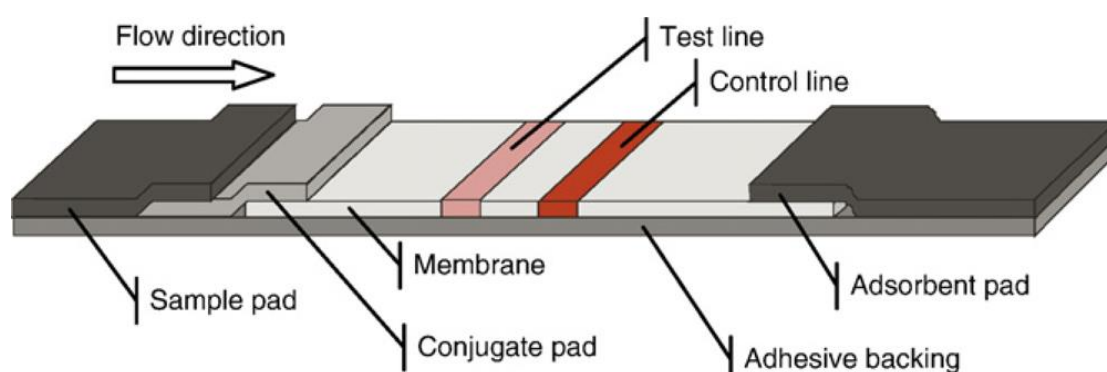


Figure 1.5. Schematic presentation of the lateral flow test strip. Adapted and modified from S. Assadollahi [39].

Liquid sample is applied onto the sample pad, located on the edge of the strip. The sample penetrates through the pad to the conjugate pad, which is located underneath the sample pad. Hydration of the conjugate pad releases the colorimetric particles, which are functionalised with another recognition biomolecule. Typically, these particles can be either colloidal AuNPs, QDs, magnetic particles or mono disperse latex particles [40].

Once the sample rehydrates the functionalised particles, the formation of the analyte-recognition complex occurs and both migrate into the analytical zone of the strip. Then the formed complex reaches the test line and a sandwich format is formed between the spotted recognition biomolecule and the complex.

Analyte-free particles are then recognised and captured in the control line. Excess reagents move past the analytical zone and are entrapped in the absorbent pad. Results are interpreted from the analytical zone by the presence or absence of visual lines, by either naked eye or a reader. Two lines stand for a positive readout and a single line represents a negative result.

Standard lateral flow technology is considered to be less sensitive than other analytical methods, such as ELISA. As such, novel approaches have been developed to increase its sensitivity through signal amplification. C. Parolo *et al.* demonstrated that functionalising AuNPs with horseradish peroxidase (HRP) can increase the sensitivity of such technology by up to one order of magnitude [41] and can be also utilised for detection of DNA oligonucleotides [42]. Nanoparticles with catalytic properties, also known as nanozymes, can introduce non-biological amplification to the lateral flow technology. D. Duan *et al.* developed a 100 times more sensitive lateral flow strip than a AuNPs strip for the detection Ebola virus antigen via Nanozyme-strip [43]. The sensitivity can be also improved by using a fluorescence transducer, as the readout intensity of fluorescence particles is significantly greater than from colorimetric ones when used as the labelling component of the lateral flow test [44]. Nonetheless, all amplified lateral flow strips require either additional equipment, such as a fluorescent reader, or operate in multiple steps.

Many multiplex lateral flow assays have been also developed. These assays are based on multiple test lines or other capture configurations [45]. Microarrays printed on the analytical zone, enable analysis of wide range of proteins [46], allergens [47] and nucleic acids [48].

1.5. Smartphone-based optical biosensors in medical diagnostics

The concept of using mobile-phones (MP) in bioanalytical science is becoming a fast growing trend. From using simple telemedicine concept with basic MP, introduced by G.M. Whitesides [49], to designing advanced smartphone-based LOC device [50]. Currently, there are up to 2.1 billion smartphone users around the world, meaning that nearly 24% of the world population owns a smartphone [51].

The increase in the availability of cost effective smartphones enhances their penetration to the market, introducing them to low and middle-income countries. In addition, to their computational abilities, their inherently user-friendly interfaces and digital connectivity make them prime candidates to be integrated with POC technologies [49] [50]. These properties allowed the smartphone usage for imaging of digital microscopy [54], cytometry [55], record of the readout of optical [56], electrochemical [57] and SPR [58] biosensors.

The high-resolution camera enables using smartphones as portable readers of optical POC tests. The lateral flow test strip is probably ideal platform to form POC test with smartphones, as their quantification normally requires a high-resolution colorimetric intensity count. D. J. You *et al.* developed a lateral flow and smartphone assay for semi-quantitative detection of thyroid stimulating hormone [59]. A smartphone application was developed for commercially available lateral flow test strips for *Botrytis cinerea* and provided a quantitative analysis platform to assess the stage of crop infection by the mold [60].

The rapid multiple analyte serological test for HIV and syphilis was developed and then evaluated by local Rwandan health care workers, exhibiting the simplicity in the operation of smartphone-based assays [53].

While smartphones have been rapidly integrated with POC tests for medical diagnosis. Their use in medical diagnosis should be extended beyond reading ability [61], by leveraging fast data processing, wireless data connectivity and Cloud capabilities storage.

1.6. Ebola virus

1.6.1. Viral hemorrhagic fever

Viral hemorrhagic fevers (VHFs) are caused by different RNA viruses that trigger viral infections in animals and humans with a high case fatality rates [62] [63]. The original definition for hemorrhagic fever involves a widespread disease with renal syndrome, elicited by members from the *Hantaviruses* family. This family includes a wide range of viruses and they are able to cause severe

hemorrhage, jaundice, multiple organ failure and encephalopathy. Among others, this list includes EVD [64]. Traditionally, VHFs have been considered as rare, exotic and high profile diseases that have been affecting humans. VHFs are typically transmitted to humans through direct contact with an infected human or animal, reservoir or arthropod vectors. The complete transmission pathway has yet to be proven for EVD, for which no natural reservoirs have been conclusively identified [65].

There are currently no fully specific and effective treatments for most of the VHFs. In addition, most of the pathogens that are causing VHFs are classified as the most dangerous hazard level, which require operation in laboratory of Biological Safety Level 4 (BSL 4) [65]. These are also identified as Category A bio-weapon agents due to their potential to cause a widespread illness with a major public health impact [66]. Moreover, the increase in global travel facilitated the emergence of VHFs and highlighted their importance in both endemic and non-endemic areas [67].

1.6.2. Taxonomy and species

VHFs are taxonomically classified with *Mononegavirales*, which is a family of enveloped, non-segmented negative-stranded RNA viruses [68]. The unique and characteristic members of the Filoviridae family include the Ebola viruses causing EVD, with five different species: Zaire Ebola Virus (EBOV), Sudan Ebola Virus (SUDV), Bundibugyo Ebola Virus (BDBV), Reston Ebola Virus and Ivory Coast Ebola Virus [69]. The case of fatality ratio of EVD ranges from roughly 25% for BDBV, 53% for SUDV, to 60–90% for EBOV [69].

In general, Ebola virus virion morphology is described as either a long filamentous shape, often with branches shaped as a short loop or with a circular configuration [69] as presented in Figure 1.6.

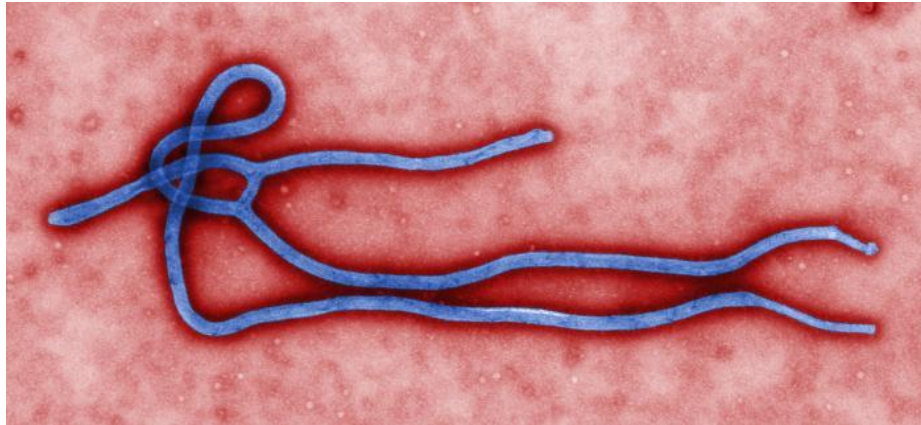


Figure 1.6. Scanning electron microscope image of the structure of Ebola virus virion. Adapted from CDC's Public Health Image [70], copyright 2005.

The first case of EVD outbreak was recorded in 1976 northern Zaire, now the Democratic Republic of Congo (DRC), and named Ebola virus after a small river located in northwestern of DRC [71]. The isolated viral species was named EBOV. During the same year, another outbreak occurred in South Sudan and was also isolated from patients and identified as another EVD species, which was named SUDV [72].

In 1994, an additional African Ebola virus species was identified in the single case of infected traveler, who worked in the Tai Forest in Cote d'Ivoire, and was named Ivory Coast Ebola virus [69]. The final African and human-pathogenic EVD species was reported in 2008 during an outbreak of EVD in the Bundibugyo district of Western Uganda [73], named BDBV and will be further reviewed in Chapter 4.

Finally, Reston Ebola virus was discovered in 1989 after infecting multiple laboratory employees in Reston, Virginia, USA who were working with *Cynomolgus* Monkeys imported from the Philippines [74].

1.6.3. Ebola virus structure and viral proteins

The virus genome consists of a negative sense single stranded RNA of about 19 thousand bases. It encodes seven structural proteins, including nucleoprotein (NP), polymerase cofactor, four matrix proteins (VP40, VP35,

VP30 and VP24), glycoprotein (GP), replication-transcription protein, minor matrix protein, and RNA polymerase (L) [69] (see Figure 1.7).

In this thesis, GP, NP and VP40 proteins are used as recognition entities for the developed biosensing systems and therefore are further revised in this section.

The GP is an *N*- and *O*-glycosylated glycoprotein expressed on the viral surface and is responsible for receptor binding and fusion of the viral envelope with host cell membranes as part of the Ebola virus infection [64]. Upon infection, GP (amongst other proteins) induces the primary immune protection mechanism in the host [75].

The sequence of GP differs substantially between viral species, sharing less than 60% identity across the three species (i.e. EBOV, SUDV and BDBV), and only 45% identity with Marburg virus [76]. Such distinction makes this viral protein especially useful for differentiating between the viral species. Other viral proteins of different species share relatively similar sequences.

In addition, GP is the most studied viral protein mainly due to its key role in the pathogenesis, antigenic and immunogenic, which makes it the most attractive for understanding the immunisation process against Ebola virus [77].

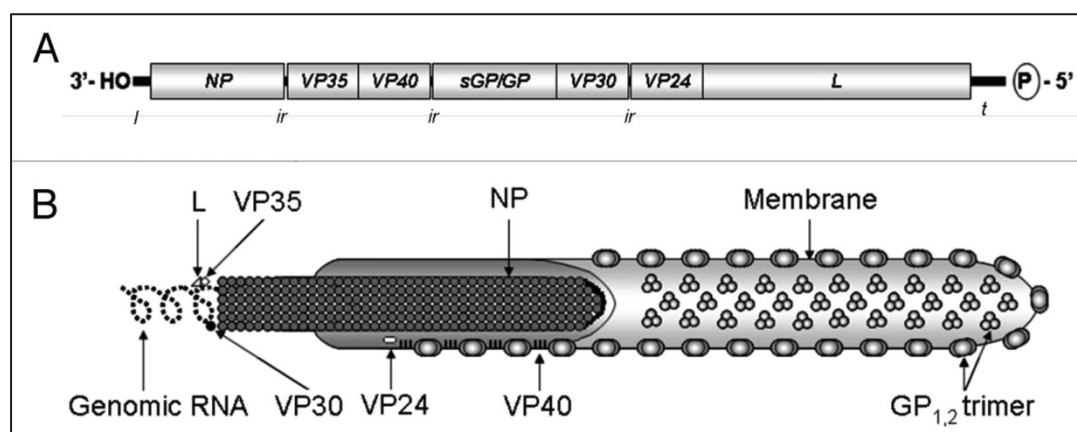


Figure 1.7. Schematic illustration of Ebola virus (A) genomic RNA: NP-nucleoprotein gene; VP-viral protein gene; GP-glycoprotein gene; L-RNA-dependent RNA polymerase gene, and (B) virion. Adapted and modified from J. Richardson et al. Copyright 2010 [78].

The NP consists of 739 amino acid residues with a molecular weight

(MW) of 115 kDa. NP was shown to be required for the assembly and budding of the nucleocapsid and for replication of the viral genome [79]. Both the N- and C-termini of the protein are important for NP–NP interactions and for the formation of viral filament-like structures. Viral matrix proteins take part in the assembly of the viral lipid-envelop by providing a link between the surrounding membrane and the NP structure [79].

The VP40 consists of 326 amino-acid residues with a MW of 43 kDa. VP40 is considered the most abundant matrix protein in the viral virion. VP40 is involved in viral and host RNA metabolism during the replication cycle as suggested by its capability of sequestering host RNA [80].

Together with GP, VP40 is presented on the surface of the viral particle and therefore was seen to be capable of eliciting protective immune responses in EVD patients [81].

Finally, it has been demonstrated that GP, NP and VP40 are the main proteins producing human immune response to the viral infection and therefore present specific antibodies in many infected patients[82].

1.6.4. EVD vaccine development

During the 2014-15 Western African Ebola virus outbreak, international health organisations expressed an urgent need for vaccine development. This incentive fast tracked the evaluation of two leading vaccine products, cAd3-EBOV, from Glaxo- Smith Kline and rVSVΔG-EBOV-GP, from NewLink Genetics [83]. Both vaccines are based on expression and immunisation against viral GP of the EBOV species[84].[85].

Vaccine efficacy is usually tested via immunogenicity demonstrated by antibody response, which is currently performed by ELISA and neutralisation assays. In addition, T-cell immune responses is also tested and measured by intracellular cytokine staining [83].

As these methods are laboratory-based, there is still an unmet need for simpler, portable and rapid methods to assess the efficacy of the potential vaccines.

1.6.5. Ebola virus Diagnostics

Despite the wide range of methods and capabilities of laboratory diagnostics, the initial EVD diagnosis is typically based on clinical assessment and known contact with suspected patients. In principle, EVD is confirmed by identification of the host specific immune response for Ebola virus or/and viral components, such as proteins or nucleic acids sequences. Confirmation is typically obtained by a positive finding in various methods, see Table 1.1, including virus isolation, electron microscopy, histological techniques, specific detection of nucleic acid, immune-fluorescence, and immunoassays for the detection of both antigen and antibodies [86] [87] [88] [89] [90].

Although the constant improvements of analytical methods, such as new reagents and equipment, have drastically reduced operation time and enhanced sensitivity, they are still not employed routinely and are not well established[64]. On average, EVD diagnostic results can be obtained within 24 to 48 hours, once samples are received at the appropriate laboratories. However, as most of the outbreaks occur in remote areas, the actual diagnosis time can take up to one week [7].

Serology, the diagnostic identification of antibodies in serum, is a necessary tool in the physiological assessment of infected, previously infected or at risk persons [91]. Typically serological testing is performed with ELISA. Serological ELISA is used reliably for screening large numbers of samples in laboratory environments, with high sensitivity and specificity, compared to neutralisation culture approaches.

Most commonly used serological assays for antibodies detection are direct Immunoglobulin G (IgG) and Immunoglobulin M (IgM) ELISAs and IgM capture ELISA [92]. These assays, aimed to detect antibodies against different viral species of EVD using the NP [93], VP40 [94], and GP [95] genes as target proteins. However, in the course of Ebola virus infection, most patients die before a significant antibody response can be tested.

Hence, serological diagnostics are more suitable for infected patients who are likely to survive than those who succumb to the virus [69].

High viremic titers are present in the blood and tissues of EVD patients even at early stages of the illness, indicating that direct detection of virus antigens is more suitable for early stage diagnosis. Assays directly intended for the detection of antigen or nucleic acids were developed and are based on polymerase chain reaction (PCR) [96] and antigen ELISA [90] [97].

The detection of antigens or antibodies has proven to be a useful and efficient diagnostic tool, both for the early and late stages of the disease. Overall, diagnosis of EHF remains a key element for curtailing the spread of the disease and providing more time for effective intervention as therapeutics are developed [64].

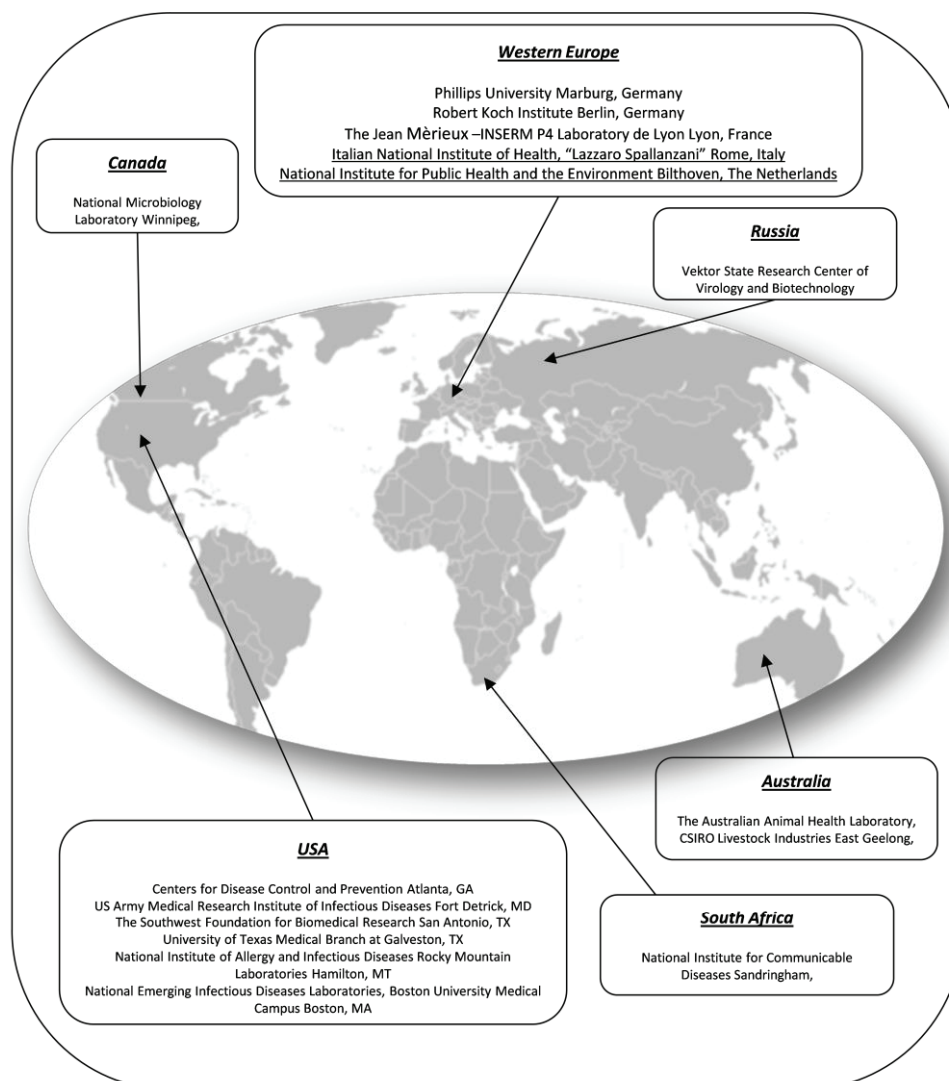


Figure 1.8. Collection of the operating BSL 4 facilities around the world as to 2010. The map was generated by using the list of facilities reviewed by Feldmann et al. [69].

Table 1.1. Diagnostic assays used to detect EBOV infection in humans - Current diagnostics methods used for EVD detection in humans.

Test	Target	Specimen	Advantages	Disadvantages	Could be useful in field laboratory
IFA*	Antiviral antibodies	Serum	Simple to operate	Nonspecific positives, subjective	N
ELISA**	Antiviral antibodies	Serum	Specific, sensitive, high throughout	Complex (multiple steps), slow (~ 5 hr)	Y
Immunoblots	Antiviral antibodies	Serum	Protein specific	Interpretation sometimes difficult, slow	N
Antigen detection ELISA	Viral antigen	Blood, serum, tissue, oral/nasal washes	Sensitive, high throughout	Complex (multiple steps), slow (~ 5 hr)	Y
PCR***	Viral nucleic acid	Blood, serum, tissue, oral/nasal washes	Sensitive, can distinguish between viral species	Requires specialised equipment	Y
Immunohistochemistry	Viral antigen	Tissue	Shows histologic location of the viral antigens	Very slow (~2 days), requires formalin for sample fixation	N
Electron microscope	Viral particles	Blood, tissue	Distinguish unique morphologies	Requires specialised equipment	N
Viral isolation	Viral particles	Blood, tissue		Very slow (~1 week)	N

*IFA: indirect immune-fluorescence assay; **ELISA: enzyme-linked immune-sorbent assay; ***PCR: polymerase chain reactio

1.6.5.1. The importance of a point of care test for Ebola

The severe threat of infectious disease was exemplified by the 2014-5 outbreak of Ebola virus in West Africa with nearly 29,000 infected people and an extreme case fatality rate of over 11,000 fatalities [7]. Local populations were severely affected, both by the disease and its devastating social and economic ramifications. There were also many fatalities amongst those who travelled to infected areas on humanitarian grounds, including health workers on the front line [69], [98].

During this outbreak, the most commonly used techniques for diagnostics of an acute Ebola infection were based on real time (RT)-PCR technology, antigen capture ELISA, and antibody detection assays. EVD diagnosis with these standard technologies is restricted to be operated in BSL 4 facilities.

Figure 1.8 schematically represents the distribution of BSL 4 facilities around the world and emphasises on the limited availability of such facilities in Africa. As the EVD epidemics usually occur in rural areas, the transportation of samples for diagnosis becomes almost impossible. This may explain the fact that during the 2014-5 outbreak, less than 60% of cases were actually diagnosed [99].

There is no doubt that the availability of accurate and cost-effective POC testing for active and past infection would be a significant progress. International health organisations have encouraged the development of such POC test for Ebola [100].

New protocols and methods in nucleic acid detection [101] and viral antigen assays [102] have been introduced and tested under field conditions to provide a simple and rapid solution. A recent example of such rapid antigen assay was developed by *C. Yen et al.* [103]. This assay is based on a multiplex lateral flow strip for the detection of Ebola virus GP along with dengue, yellow fever detection. Another less sophisticated serological method was developed by *Lunchet et al* [104].

An assay based on magnetic lateral flow test strip for the detection of Ebola virus antigens was introduced [43]. In addition, high sensitivity was achieved by combining synthetic biology with isothermal amplification in a

paper-based POC test for detection of viral nucleic material [105].

A. Lucht *et al.* demonstrated for the first time the applicability of the immuno-filtration system to detect viral antigen and its usefulness under field conditions. Although this assay was less sensitive than other tests but with a rapidity far less prone to technical complications [104].

Finally, *ReBOV Antigen Rapid Test* kit based portable PCR technology was brought for evaluation during the latest outbreak but was proven not efficient as the sample preparation was too complex [101].

Nowadays in the post outbreak period, there is a great number of previously infected survivors that require follow up and monitoring [106]. This population has yet to undergo much needed assessment both for patient management and epidemiological understanding of the disease. Unfortunately, tools suitable for on-site, rapid, effective and economic analysis do not yet exist [107].

Chapter 2

The Development of an Ebola Virus Antibody Test Using Lateral Flow and Smartphone Technologies

2.1 Introduction

Accurate, portable and cost-effective medical diagnostic tests for EVD can empower developing countries to take action against the spread of emerging diseases in terms of individual patient diagnostics, prognostics and care. Standard medical diagnostic tests are too expensive and inflexible to be deployed on a sufficient scale for effective surveillance in remote and rural areas. Certainly, POC tests offer ideal solutions to such need as they can be employed under field conditions or in mobile hospitals and provide a logistical and financially sustainable solution for epidemic control and prevention [100].

This scenario was reported during the 2014-15 West African Ebola virus outbreak. Local and international health authorities joined forces to encourage the development of simple to use, cheap and portable diagnostic tests for Ebola virus [108] [102]. Indeed, rapid testing was essential for both clinical detection and prevention from further disease spread. As a result, during 2015-16 great effort on behalf of the medical and scientific communities for healthcare diagnostics was invested in working on POC tests for EVD [107].

These developed tests can be classified into two categories, direct antigen tests and nucleic acids diagnostic assays. The direct antigen tests were designed to detect whole Ebola virion [109] or specific viral protein such as VP 40 and NP using mainly paper-based qualitative tests [103] [107].

The nucleic acids diagnosis comprised PCR-based Ebola kits or simplified assays based on isothermal amplification of nucleic acids [101] [97]. All these new assays were made for the detection of targets of the viremic stage of Ebola infection, which is relatively short (up to 7 days from onset). Hence, serological POC tests for the detection of patient's immune response to EVD were not addressed [107].

Immune response can report on the exposure to a viral infection and is crucial for epidemiology and surveillance studies. Furthermore, it can predict the survival probability of an infected patient, as reviewed in section 1.6.3.

IgG and IgM antibodies against viral proteins are detectable 12 days after infection onset and is currently diagnosed using a standard ELISA method [110]. The analysis of these samples with ELISA must be performed in BSL 4 facilities, especially as ELISA may be using whole viral antigen as the recognition element.

Here, the use of recombinant viral proteins as the recognition elements has a great advantage. Generally, recombinant proteins are relatively cheap and robust to manufacture and can be handled in BSL 2 facilities [77]. Nonetheless, the availability of recombinant viral proteins for Ebola Virus remains limited, especially for the SUDV species.

The key motivation for Chapters 2, 3 and 4 was to address the need for a specific, rapid, and cost-effective point-of care platform for the detection of antibodies against Ebola virus.

This chapter describes the development of a diagnostic test for the humoral response to Ebola virus. The developed system uses in-house made recombinant viral protein as recognition elements. This POC is test comprised two components, the lateral flow test strip and a smartphone application (app). Sample analysis is performed with the strip and the complementary app produces quantitative analysis of the tested sample.

The lateral flow test strip was developed in both single (monoplex) and multiple (multiplex) formats. The monoplex format includes recombinant glycoprotein (GP₁₋₆₄₉) from the SUDV and designed to single population of SUDV antibodies.

The multiplex format was developed in two configurations enabling simultaneous detection of multiple IgG antibodies against several SUDV viral

proteins (VP40, NP and GP₁₋₆₄₉) or for the detection of different viral species including SUDV, EBOV and BDBV.

The complementary app was designed to provide quantitative results of the test strip alongside data storage and geographical mapping of the tested samples. In addition, this chapter demonstrates the selection and characterisation of custom made Ebola virus recombinant proteins and their incorporation in a lateral flow test strip. AuNPs were functionalised with secondary antibodies and used for visual labeling of the test result.

Finally, analytical parameters of the smartphone-based lateral flow assay for Ebola Virus antibodies were evaluated, including optimal conditions for operation, running time, shelf life and limit of detection.

2.2. Material and Methods

2.2.1. Cloning, expression and purification of recombinant viral proteins

Purified recombinant His-tagged (His₆) glycoproteins (GP₁₋₆₄₉) viral polypeptide (lacking the transmembrane domain) for SUDV, EBOV and BDBV, were prepared as previously described by L. Lobel *et al.* [111].

The cDNA constructs coding for SUDV nucleoprotein (NP) and viral protein 40 (VP40) genes (GenBank AY729654) were synthesised and cloned commercially (DNA2.0) using optimised codon frequencies for *E. coli*. A His₆ was added to the C or N- terminus of NP and VP40 protein, respectively.

The cDNA constructs were transformed into chemically competent *E. coli* BL21 (DE3) (Stratagene, Catalog #200131) and plated onto LB agar containing 25 µg/ml of Kanamycin (LB-Kan). A single colony of the transformant was inoculated with 10 ml of LB-Kan broth and cultured at 37 °C with vigorous shaking overnight at 225 rpm. The overnight culture was diluted 1:50 into LB-Kan and grown at 37 °C with vigorous shaking until the optical density (OD) at 600 nm reached 0.6–0.8. Then, isopropyl-beta-*D*-thiogalactopyranoside (IPTG) was added into the culture which was further incubated at 37 °C for 4 h. Cells were harvested by centrifugation at 6000 rpm for 20 minutes and frozen until

use at -80 °C. The pellet was resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 5 mM b-mercaptoethanol (b-Me) and 10 mM imidazole, pH 8.0). Next, 1 mg/ml lysozyme, 3 U/ml benzonase nuclease, and protease inhibitor cocktail (1:100) (P-8465; Sigma-Aldrich) were added to the lysis buffer and incubated for 30 minutes on ice. Cell membranes were disrupted by three cycles of ultrasonication for 1 minute at 100%. The whole process was carried out on ice. The lysed cells were centrifuged at 10,000 g for 60 minutes at 4 °C to separate the soluble and insoluble proteins.

VP40 was then purified from the soluble fraction: via addition of the supernatant containing His-tagged VP40 to a column containing 1 ml Ni-NTA agarose resin (Qiagen) and incubated with the resin mixture for 1 h on a rocking platform. The column was washed with lysis buffer and the protein was eluted with a buffer consisting of 50 mM NaH₂PO₄, 300 mM NaCl, 5 mM b-Me and 250 mM imidazole, pH 8.0. Using a Centricon 10K cutoff spin filter (Millipore), the eluted protein was concentrated and buffer was exchanged with 50 mM Tris-HCl, 150 mM NaCl, 5mM b-Me, pH 8.0.

NP protein was purified from the insoluble fraction or inclusion bodies. Protein expression, cell lysis, disruption and centrifugation were carried out as described above, except that insoluble material was collected. The insoluble material was washed once with lysis buffer containing 1% v/v Triton X-100 followed by two washes with lysis buffer without Triton X-100. The insoluble material was dissolved in the denaturing solubilisation buffer (50 mM Tris, 500 mM NaCl, 8 M urea, 10 mM imidazole, pH 8.0) and mixed on a platform shaker for ca. 1 h at room temperature. The mixture was sonicated again as described above and centrifuged at 10,000 g for 30 minutes at 4°C. The supernatant containing solubilised NP was collected and loaded onto 1 ml of Ni-NTA resin (Qiagen), which was pre-equilibrated with solubilisation buffer. The protein was eluted from the column with solubilisation buffer containing 500 mM imidazole.

Refolding of NP protein was carried out by dilution to a uniform concentration of 0.1 mg/ml and dialyzing against refolding buffer containing 50 mM Tris-HCl, 500 mM NaCl 0.5 M L-Arginin, 50 µM ZnCl₂, 10% v/v glycerol, 0.5 mM DTT, 50 mM imidazole and 3 M urea, pH 8.0, at 4 °C for 4 h. Afterwards, another dialysis step was performed against a buffer containing 50 mM Tris-HCl,

500 mM NaCl 0.5 M L-Arginine, 50 μ M ZnCl₂, 10% v/v glycerol, 3 mM reduced glutathione, 0.3 mM oxidised glutathione, 50 mM imidazole and 1.5 M urea, pH 8.0, at 4 °C overnight. A third dialysis step was performed against a buffer containing 50 mM Tris-HCl, 500 mM NaCl 0.5 M L-Arginine, 50 μ M ZnCl₂, 10% v/v glycerol, 1.5 mM reduced glutathione, 0.15 mM oxidised glutathione, 50 mM imidazole and 0.75 M urea, pH 8.0, at 4 °C for 4 h. A final dialysis step was performed against buffer containing 50 mM Tris-HCl, 300 mM NaCl, 0.2 M L-Arginine, 50 μ M ZnCl₂, 10% v/v glycerol, 0.3 mM DTT and 0 M urea, pH 8, at 4 °C for 4 h. Dialyzed protein was concentrated using a Centricon 30 K cutoff spin filter (Millipore).

Endotoxins were removed from the purified proteins using a ToxinEraser™ endotoxin removal kit (L00338, GenScript, USA) according to the manufacturer's instructions. Quantification of endotoxins was performed using a ToxinSensor™ gel clot endotoxin assay kit (L00402, GenScript, USA) according to the manufacturer's instructions. The purity of the protein samples was estimated by SDS-PAGE staining with Coomassie brilliant blue R-250. The total protein concentration was determined using a Bradford™ protein assay kit. Endotoxin content of the purified proteins was less than 1 EU/mg for all recombinant proteins used in the assay.

2.2.2. Colorimetric ELISA

2.2.2.1. Reagents and solutions

Monoclonal IgG antibodies against GP₁₋₆₄₉ for SUDV, referred to as 3C10, and GP₁₋₆₄₉ for EBOV, referred to as 6D8, were produced in mice as previously described by J. Dye et. al.[112]. Rabbit polyclonal IgG antibodies against GP₁₋₆₄₉ for BDBV, referred as pol-BDBV, were purchased from Ibt Bioservices (0304-001). Additional antibodies, including Monoclonal anti-poly Histidine (Sigma Aldrich), Bovine Serum Albumin (Sigma Aldrich) (BSA), HRP conjugated Anti-Rabbit IgG (Fc specific) antibody produced in goat (abcam) and HRP conjugated Anti-Mouse IgG (Fc specific) antibody produced in goat (abcam) were used as

secondary antibodies. The following reagents were used in the assay, including One step TMB (biorad) and H₂SO₄ (Fluka), PBS tablets (Sigma), Tween-20 (Sigma).

2.2.2.2. ELISA procedure

The recombinant GP₁₋₆₄₉ proteins were analyzed using a standard ELISA protocol, following established protocols, with minor adjustments. A final concentration of 2 µg/ml in a volume of 100 µl/well of the recombinant and purified viral protein was pipetted into each well of a 96-well microtiter plate (MaxiSorp, Nunc). The plate was covered and incubated overnight at 4°C. The plates were then washed with 200 µl of PBST (PBS (Sigma P4417) with 0.05% (w/v) Tween-20 (Sigma P7949), pH 7.4. Next, 200 µl/well of blocking solution 10% (w/v) skimmed milk in PBST buffer, was added and incubated for 1 hour at 37 °C. Following incubation, plates were washed using PBST. Next, 3C10, 6D8 and pol-BDBV were diluted into a range of concentrations: 0.01, 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000 ng/ml in PBST with 2% skimmed milk. Subsequently 100 µl of each diluted antibody was added to each well and incubated for 1 hour at 37 °C along with 1:1000 dilution of monoclonal anti-poly Histidine for the positive control. Plates were then washed with PBST, and 100 µl/well of secondary (mouse and rabbit) antibody conjugated to HRP (diluted 1:5000) was added and incubated for 1 hour at 37 °C. Plates were washed with PBST prior to the reading step. Then a total of 50 µl/well of one step TMB was added and incubated for 10 minutes in room temperature in the dark. Then 10% H₂SO₄ was prepared and 50 µl/well added to stop the reaction. Plates were read using a standard absorbance measurement at 650 nm using a Perkin-Elmer Envision plate reader.

2.2.3. Western blot

2.2.3.1. *Reagents and Solution*

SDS-PAGE gel: [trisaminomethane (Tris)-Cl 1.5M pH 8.8, Tris-Cl 0.5M pH 6.8, deionised water, 10% SDS, 30% Acrylamide bis solution, 10% Ammonium persulfate (APS) and Temed. Protein Electrophoresis: 50 mM Tris-HCl (pH 7.5), 1% NP-40, 1mM EGTA, 1mM sodium vanadate, 1mM NaF, 10mM sodium β -glycerophosphate, 150mM NaCl, 0.25% sodium deoxycholate, 5mM sodium pyrophosphate], sample buffer [0.25M Tris-HCl pH 6.8, 8% SDS, 30% Glycerol, 0.02% Bromophenol Blue and 0.3M DTT] and SDS Running Buffer [Trizma base, Glycine, SDS]. Transfer buffer [25mM Tris pH8.5, 0.2M Glycine, 10% Methanol]. Protein Labeling: Ponceau S solution [0.1% ponceau, 5% acetic acid] and TTBS [Trizma base, NaCl, Tween20 0.05%].

2.2.3.2. *Western blot procedure*

The purity of the protein was analyzed by SDS-PAGE electrophoresis using standard methodology [Upper (5%) and bottom (10%) Gels: DDW, 30% Bis- Acrylamide (Sigma), Tris buffer, sodium dodecyl sulfate (SDS; Sigma), ammonium peresulfate (Sigma), Tetramethylethylenediamine (TEMED; Sigma)], and using Mini- protein tetra Electrophoresis system (Bio Rad Laboratories). Sample buffer containing Tris buffer, glycerol, SDS and bromophenol blue was added to each of the samples. The gel was run for 1.5 hours with a 150mV potential in running buffer purchased from Sigma (T1165). Next, the gel was stained with Coomassie Brilliant Blue stain (Coomassie Brilliant Blue (sigma 83003) dissolved in 90ml methanol and acetic acid (1:1)) for 2 hours and washed with DDW, Methanol and acetic acid (1:4:4) for an additional 4 hours. The gel was then visualised using gel scanner and analyzed with Dolphin-1D software (WEALTEC) operation for SDS-PAGE gel.

Proteins are transferred from the SDS-PAG gel to a 0.45 μ m nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) using transfer buffer (10%

methanol) using standard methodology. The proteins were transferred for 5 hr at a voltage of 100 V. Once transferred, proteins were visualised using Ponceau S solution and washed with Tween-20 (Sigma) 0.1% (v/v)-TBS buffer (TTBS). WB immuno-assay: Once blotted, non-specific binding was blocked with 4% (w/v) BSA in TTBS for 1 hour and then the membranes were directly incubated with an anti-His₆ tag murine monoclonal antibody diluted 1:3000 or human polyclonal sera samples diluted 1:400 at RT for an additional hour. Following incubation, two washes of 5 minutes each with TTBS were performed, and goat anti-murine IgG conjugated with horseradish peroxidase (HRP) at a dilution of 1:10,000 was added for 1 hour at RT. Finally, the membranes were washed twice with TTBS. Following the final membrane washing, the recombinant viral proteins were detected using WB luminol commercial reagents (Santa Cruz Biotechnology). Briefly, 500µl of reagents A and B, mixed in a ratio 1:1, were incubated on the blotted membrane for a period of 15-30 seconds. The membrane was then gently dried, and a Fuji X-ray film was exposed to the membranes for 30-60 seconds. Immediately after, exposure films were developed and visualised. The analysis of viral recombinant protein migration was done by comparing the migration to a broad-range protein marker (Fermentas), which was present on each blot.

2.2.4. Characterisation of AuNPs conjugation to secondary antibody

2.2.4.1. Optimisation of the AuNPs conjugation to secondary antibodies

A total of 120 µl of citrate coated AuNPs were mixed with a wide range of concentrations of secondary antibodies in water in a low binding clear 96 well plate and incubated for 1 hour at room temperature and shaken at 400 RPM. Then 10 µl of 10% w/v of NaCl in water was added to each well. A change of color from red to blue corresponded to aggregation of the AuNPs.

2.2.4.2. *AuNPs optical characterization*

The solution of AuNPs conjugated to secondary antibody was diluted 1:15 in 700 μ l of PSB in low volume disposable cuvettes. UV-Visible absorption spectra were recorded from 350-700 nm using a UV-vis spectrophotometer. The absorbance intensity of the spectrum was normalised to the maximum absorbance peak of the Surface Plasmon Resonance (SPR) and presented as normalised absorbance.

2.2.4.3. *Dynamic Light Scattering*

The size of the labeled AuNP was determined by DLS using a ZetaSizer Nano ZS (Malvern Instruments). Size measurements of the functionalised nanoparticles were performed in disposable low volume clear cuvette. The particles were diluted to less than 1 OD at the SPR peak. Highly concentrated samples (>5 OD) lead to inaccurate sizing due to viscosity effects and can cause multiple scattering (i.e. scattering from one particle is re-scattered by another particle), potentially lead to an artificially low particle size. The measurements were carried out at 25°C with an equilibration time of 5 minutes, with 3 measurements for each sample and 3 runs of 1 minute for each measurement. The data was presented in size distribution by volume and intensity.

2.2.4.4. *Transmission electron microscopy*

A 20 nm and a 40 nm AuNPs conjugated to anti-human IgG (Fc specific) antibody were diluted 1:25 from stock in water. Samples were prepared by depositing 2 μ L labeled AuNPs on the top of carbon-coated copper grids and dried in a falcon tube, to prevent accumulation of dust, for approximately 5 hours. Transmission electron microscopy (TEM) images were produced using a JEOL 2010 transmission electron microscope operated at an accelerating voltage in the range of 50 to 150 mV. Analysis of size of the AuNPs was performed by measuring AuNPs diameters directly from the images.

2.2.5. Lateral flow immunochromatographic strip test preparation

2.2.5.1. Gold nanoparticles conjugation

Citrate coated AuNPs of 40 nm and 20 nm diameters were purchased from BBI Solutions, UK. Different AuNPs conjugates were prepared following an identical protocol with different secondary antibodies, including anti-human IgG (FC/Fab and whole IgG specific), anti-rabbit IgG (FC specific) and anti-mouse IgG (FC specific) (Sigma Aldrich). Then 100 µl of 100 µg/ml secondary antibodies in water were added to 1200 µl of AuNPs solution, and incubated at room temperature (shaking/rotating at 900 rpm) for 20 minutes. Subsequently 100 µl of 1 mg/ml of bovine serum albumin (BSA) was added as a blocking agent and incubated for an additional 20 minutes at room temperature. The particles were centrifuged for 10 minutes at 6000 g (the supernatant was discarded) and the pellet was resuspended in 120 µl of PBS pH 7.4. The concentration was adjusted to 7 OD at a wavelength of 525 nm and 540 nm for 20 nm and 40 nm, respectively. The stock solution of the conjugated particles was then stored in low binding 1.5 ml eppendorf tubes at 4 °C.

2.2.5.2. Lateral flow strip assembly

All materials for the lateral flow test strip were obtained from Millipore (Billerica, USA). Specifically, sample and absorbent pads (CFSP001700), conjugation pads (GFCP00080000), detection pad nitrocellulose membrane (SHF2400425) and backing card (HF000MC100) were used. Test and control lines were spotted using a microspotter (Microdrop, autodrop system AD-P-800). All spotted viral proteins were diluted in water to a final concentration of 1 mg/ml. The initial concentration of VP40 and NP, 0.2 and 0.4 mg/ml, respectively, was adjusted to 1 mg/ml with BSA. The control lines of the strips used for human sample testing were spotted with 1 mg/ml (in water) of human IgG antibodies from human serum, purchased from Sigma. The control lines of the non-human strips were spotted with a 1:1 mix of rabbit and mouse IgG antibodies at a final concentration of 1 mg/ml. The minimum distance between

spotted lines was set to be 2 mm using the 'inflight mode' of the microspotter. Finally, 3 mm single lateral flow strips were cut with a paper guillotine.

2.2.6. Testing nonhuman antibodies via lateral flow test strip

3C10, 6D8 and pol-BDBV were used as target antibodies for GP₁₋₆₄₉ for SUDV, EBOV and BDBV, respectively. Monoclonal anti-poly Histidine antibody produced in mice was purchased from Sigma Aldrich and used as a positive control for all the recombinant viral proteins (dilution 1:1000). A range of concentrations of 3C10, 6D8 and pol-BDBV were tested using the following procedure. Each antibody was diluted to 100-50,000 dilution range in running buffer consisting of PBS pH 7.2, 0.1% Tween-20 and 5% w/v BSA, 10 µl of the antibody solution was mixed with 10 µl functionalised AuNPs and added onto the sample pad of the lateral flow. After 5 minutes, the strips were washed with 10 µl running buffer. Then, the absorbance pad was removed to prevent an increase in background noise. The readout of the test line was quantified in relative test line intensity (RTL_I) units using the developed smartphone application (described in section 2.2.7.). The limit of detection of the lateral flow test strip for each GP₁₋₆₄₉ subtype was determined as the RTL_I signal above a cut off, set as the average blank plus three-times the standard deviation. For the multiplex test strip mixtures of 3C10, 6D8 and pol-BDBV antibodies diluted to 1:1000 in running buffer were applied in the same procedure onto the strips. Images were then captured via the smartphone camera.

2.2.7. Development of the smartphone application

The first generation of the app was used (without the geographical tagging). The smartphone application used the camera to capture a photograph of the lateral flow strip, and to extract the intensity of the test line. The app was developed for the Android operating system using the Eclipse Integrated Development Environment 4.5.0 with the Android Development Toolkit 23.0.7.2120684. Once the patient details are filled out, the app enables access to the camera, launches the OpenCV 2.4.9 library, supposing software, which is an

open source application provided by Android. The camera resolution class was based on the OpenCV Camera Control Example, using JavaCameraView, with red lines overlaid to aid strip alignment. A 4x digital zoom was used to further aid user alignment. The automatic continuous picture focus mode was used. When aligned, the user taps the screen to capture the image, which is then cropped to the region of interest (ROI): 3 mm of the analytical zone of the lateral flow test strip. The intensity of the green channel of the photo was averaged across the width of the strip to reduce noise, producing an intensity line profile along the length of the strip. The result includes the score of the test and control line in relative intensity, and the values of the intensity plot, which are saved in a .csv format. The files are accessed once the phone is connected to a computer through the open source library, open.csv, which was used to read the .csv files.

2.2.8. Human positive and negative samples

This study was approved by the Helsinki committees to collect these samples and conduct this study in Uganda Virus Research Institute and Soroka Hospital, Beer-Sheva, Israel (Appendix B). Four human samples were obtained from obtained from SUDV survivors and non-infected controls, characterised to the presence of IgG antibodies against GP₁₋₆₄₉ of SUDV and referred to as positive samples. Control samples were obtained from healthy volunteers. Samples were stored at -80°C and thawed prior analysis.

2.3. Results and discussion

2.3.1. System design and operation

This section describes the design of the new smartphone-based lateral flow assay targeted for the detection of Ebola virus antibodies.

The proposed assay consists of two components; a lateral flow test strip and a complementary smartphone application. The lateral flow test strip was

developed in two formats to detect single (monoplex) and multiple (multiplex) target analytes as schematically presented in Figure 1A.

The monoplex format comprised single test line spotted with GP₁₋₆₄₉, purified recombinant protein containing 649 terminal amino acids of GP without the trans-membrane domain.

The multiplex format included two configurations; the first comprised three test lines with different recombinant viral proteins from the SUDV species, i.e. NP, VP40, and GP₁₋₆₄₉. The second configuration of the multiplex format consisted three test lines of GP₁₋₆₄₉ from different EVD species, i.e. SUDV, EBOV and BDBV.

The assay was designed to utilise of AuNPs conjugated to a secondary antibody as the visual labelling of the analytical zones of the test strip, the test and control lines. Ebola virus viral proteins were spotted on the test line to directly bind IgG antibodies against the virus.

The application of this assay was targeted as a serological tool to assess previously infected patients. Hence, it was developed to analyse human body fluids that present a humoral response (e.g. serum).

In practice, the operation of the assay begins with application of the sample to the test line. Then, the target IgG antibodies are labelled with AuNPs through the secondary antibodies to form an immunocomplex, which then binds to the test line/s and control line. As such, visual appearance of more than a single line corresponded to positive test readout. Quantitative analysis is perfumed with the app that was also engineered to provide store and share the produced results and geographically tag the location of the tested sample.

2.3.2. Characterisation of the recombinant viral proteins

2.3.2.1. Characterisation of Ebola viral glycoproteins

Recombinant GP₁₋₆₄₉ from SUDV, EBOV and BDBV were produced in collaboration with the laboratory of Virology and Immunology at Ben Gurion University, Beer-Sheve, Israel. In addition, Virology Division at the US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick,

Maryland, provided murine monoclonal antibodies, 3C10 and 6D8, against SUDV – GP₁₋₆₄₉^S (GP^S) and EBOV – GP₁₋₆₄₉^Z (GP^Z), respectively. Rabbit Polyclonal antibody against BDBV-GP₁₋₆₄₉^B (GP^B) was also used. These antibodies were used to characterise the immune reactivity of the provided recombinant GP₁₋₆₄₉ proteins via standard ELISA.

Figures 2.2, 2.3 and 2.4 present the dose response curve of 3C10 6D8 and pol-BDBV antibody tested against GP₁₋₆₄₉^S, GP₁₋₆₄₉^Z and GP₁₋₆₄₉^B, respectively. A wide range of concentrations of antibodies were tested, 0.01, 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000 ng/ml.

The lowest antibody concentration that was detected with GP₁₋₆₄₉^S, GP₁₋₆₄₉^Z and GP₁₋₆₄₉^B was determined to be 500 pg/ml, 1 ng/ml and 1 ng/ml, respectively, demonstrating strong affinity of the recombinant protein and the target antibodies.

In addition, the selectivity against non-orthogonal antibodies against the other EVD species of each GP₁₋₆₄₉ was tested. GP₁₋₆₄₉^S exhibited high selectivity to the orthogonal antibody (3C10) demonstrating a significantly higher binding affinity to 5 ng/ml of 3C10 than 500 ng/ml of both 6D8 and pol-BDBV (Figure 2.2B).

Similarly, GP₁₋₆₄₉^Z and GP₁₋₆₄₉^B demonstrated high selectivity to their orthogonal antibody and low binding to the non-orthogonal antibodies (Figure 2.3B, 2.4B). Finally, these enabled confirmation that the three provided recombinant glycoproteins have high affinity and selectivity to be able to detect IgG response to SUDV, BDBV and EBOV.

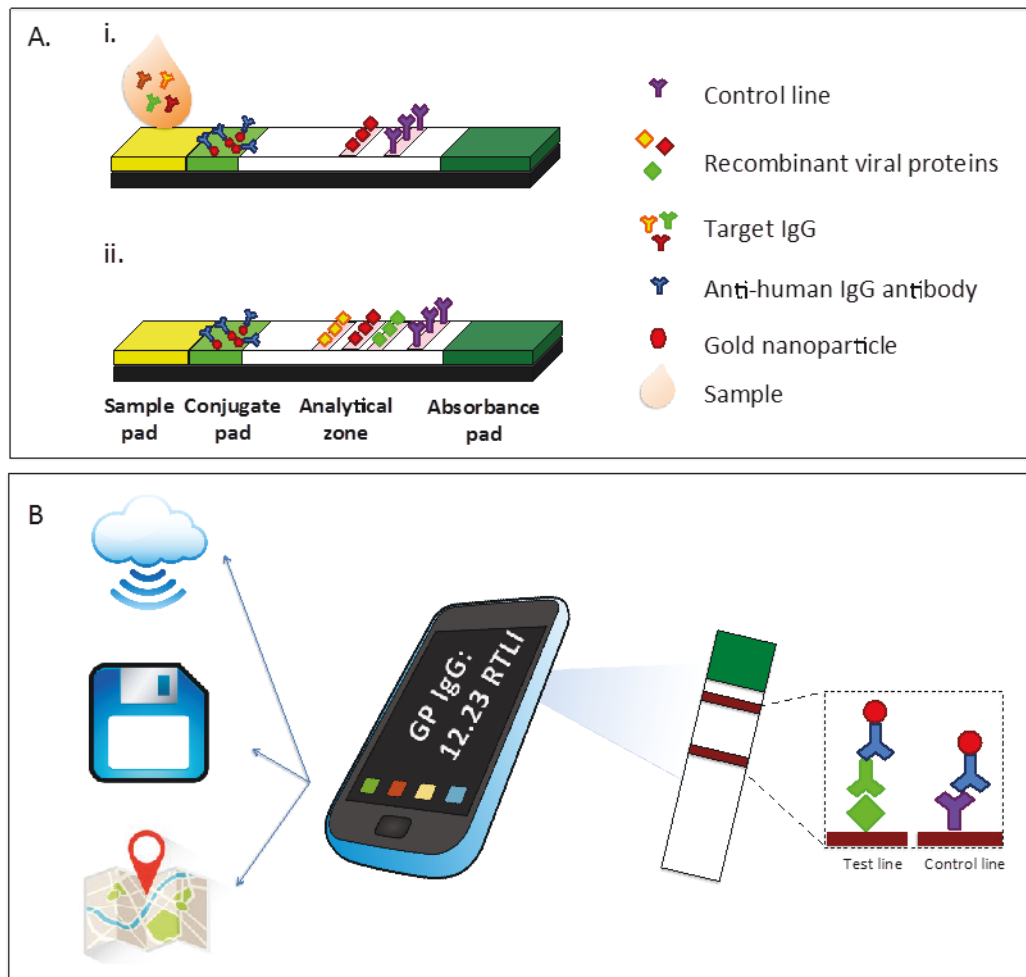


Figure 2.1. Schematic overview of the developed assay for the detection of IgG response to EVD in human sera samples. A. A sample is applied to the sample pad and migrates through the analytical area forming a complex between the labelled AuNP and the target IgG antibodies. Specific target IgG antibodies against single (i) or multiple (ii) Ebola viral proteins bind to pre-printed test lines to form a visual red-purple signature. An additional control line for assay performance is used for detection of total IgG antibodies. Visualisation of assay results is obtained after 15 minutes. B. Visualised results are quantified using a smartphone accessory to facilitate data storage, share and map the result within its geographical location.

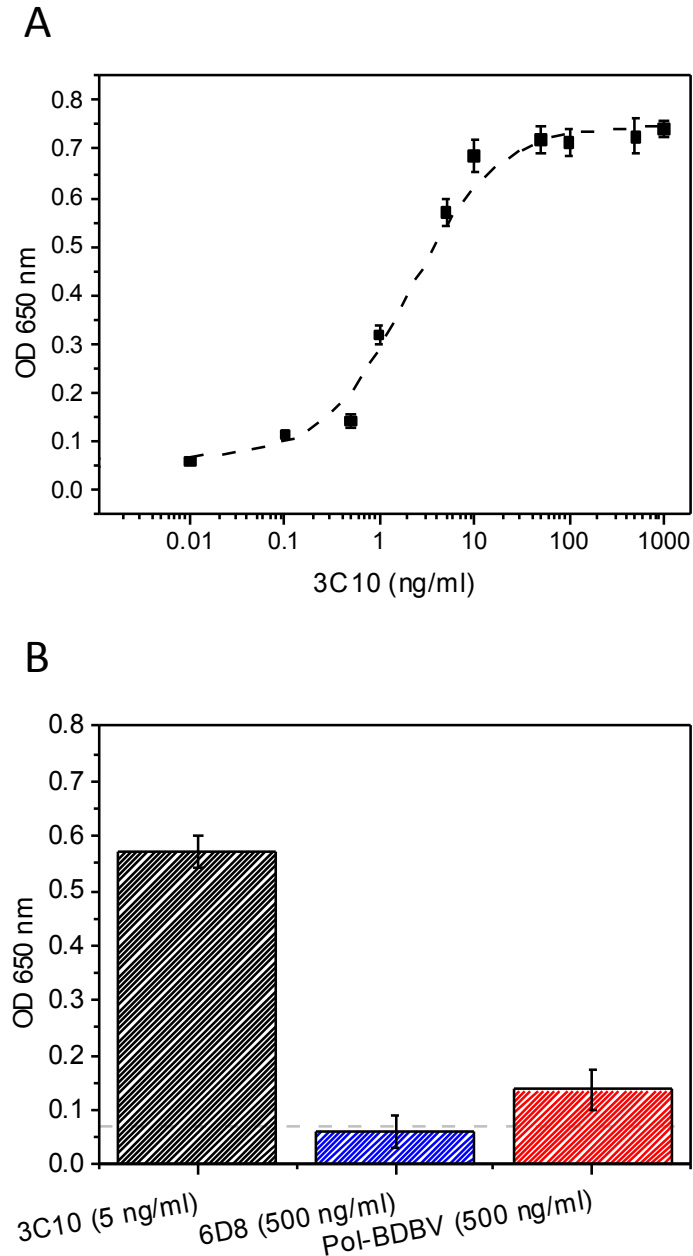


Figure 2.2. Characterisation of GP₁₋₆₄₉ – SUDV using colorimetric ELISA. A. GP₁₋₆₄₉ immune reactivity was tested with monoclonal antibody (3C10) using an indirect ELISA assay. 3C10 antibody titer dose-response curves, concentration ranges from 1 µg/ml to 0.01 pg/ml. The series of results was plotted and fitted to a sigmoidal curve ($r^2 = 0.982$). Bars indicate means $\pm$ standard deviations ($n=3$). B. Selectivity of the GP₁₋₆₄₉ – SUDV testing 3C10 (black) 5 ng/ml, 6D8 (blue) 500 ng/ml) and pol-BDBV 500 ng/ml antibodies against GP SUDV. Bars indicate means $\pm$ standard deviations ($n=3$).

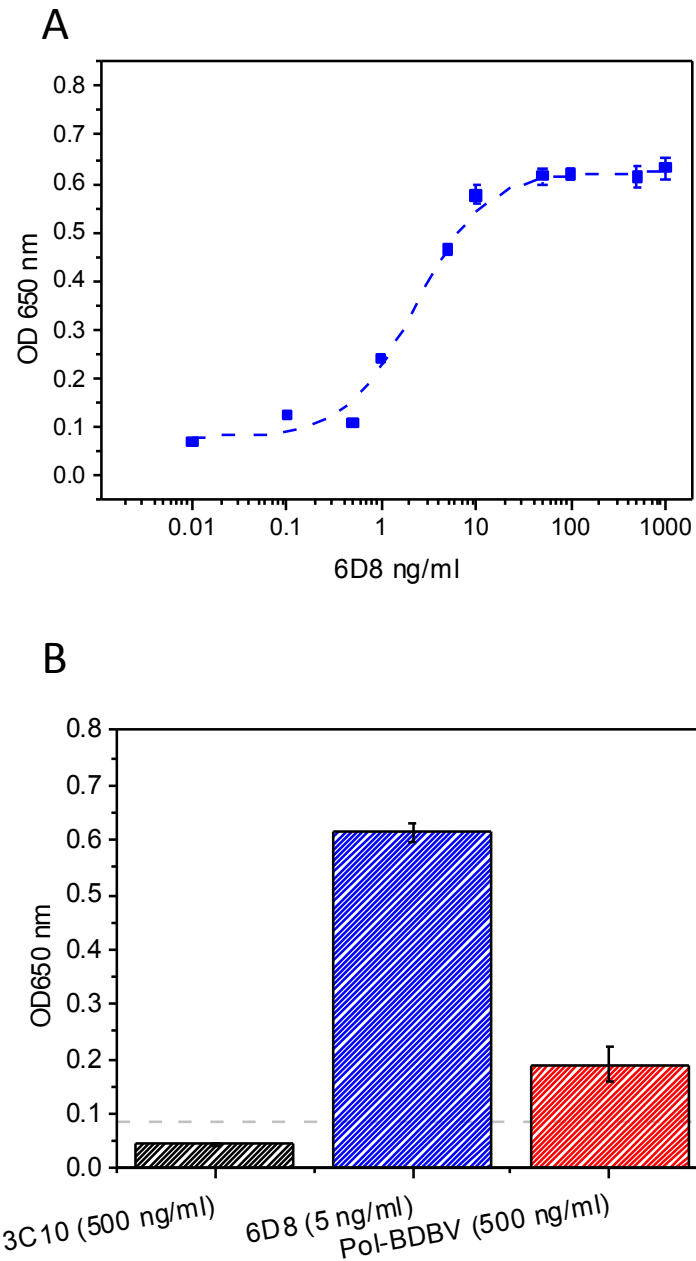


Figure 2.3. Characterisation of GP₁₋₆₄₉ – EBOV using colorimetric ELISA. A. Zaire GP₁₋₆₄₉ immune reactivity was tested with monoclonal antibody (6D8) using an indirect ELISA assay. 6D8 antibody titer dose-response curves, concentration ranges from 1 μ g/ml to 0.01 pg/ml. The series of results was plotted and fitted to a sigmoidal curve ($r^2= 0.989$). Bars indicate means $\pm$ standard deviations ($n=3$). B. Selectivity of the GP₁₋₆₄₉ – SUDV testing 3C10 (black) 500 ng/ml, 6D8 (blue) 5 ng/ml) and pol-BDBV 500 ng/ml antibodies against GP EBOV. Bars indicate means $\pm$ standard deviations ($n=3$).

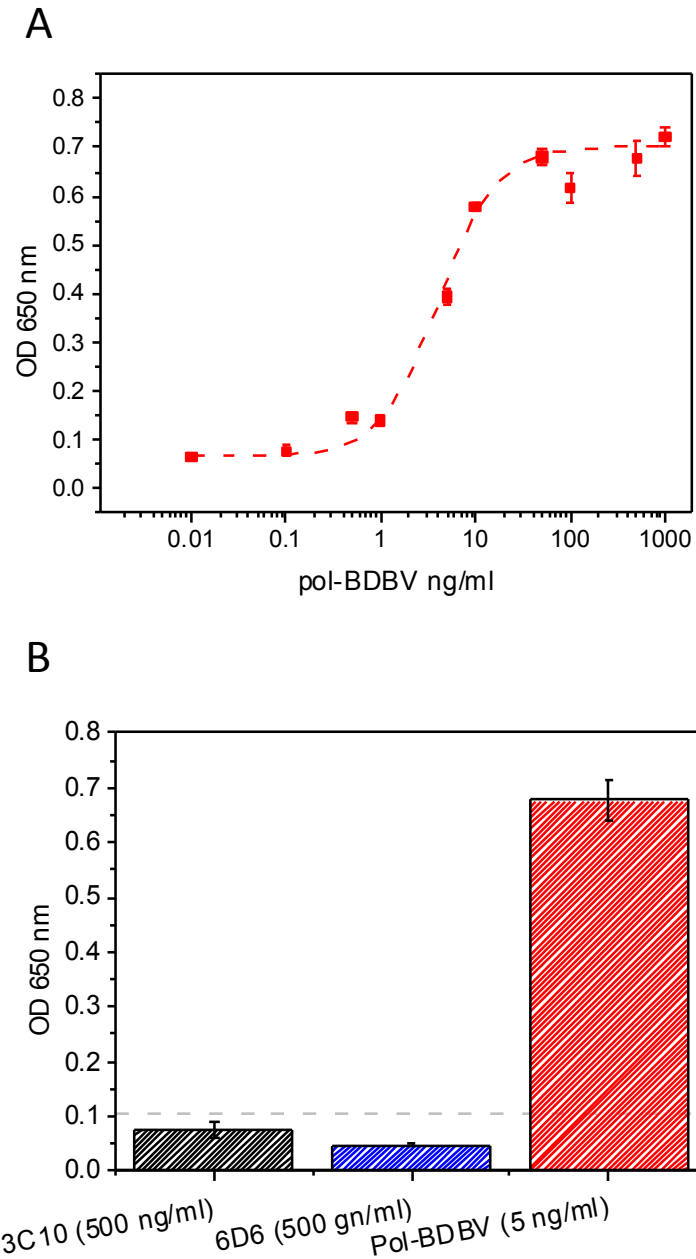


Figure 2.4: Characterisation of GP₁₋₆₄₉ – BDBV using colorimetric ELISA. A. Bundibugyo GP₁₋₆₄₉ immune reactivity was tested with polyclonal antibody (pol-BDBV) using an indirect ELISA assay. Pol-BDBV antibody titer dose-response curves, concentration ranges from 1 μ g/ml to 0.01 μ g/ml. The series of results was plotted and fitted to a sigmoidal curve ($r^2 = 0.996$). Bars indicate means $\pm$ standard deviations ($n=3$). B. Selectivity of the GP₁₋₆₄₉ – BDBV testing 3C10 (black) 500 ng/ml, 6D8 (blue) 500 ng/ml) and pol-BDBV 5 ng/ml antibodies against GP BDBV. Bars indicate means $\pm$ standard deviations ($n=3$).

2.3.2.2. *Characterisation of other viral proteins from SUDV species*

Viral proteins, VP40 and NP, were cloned from the SUDV species genome and produced in collaboration with the laboratory of Virology and Immunology led by L. Lobel at Ben Gurion University, Beer-Sheve, Israel. These proteins were produced with a His₆ tag.

These unique proteins had no available specific antibodies for affinity characterisation. As such, NP and VP40 were characterised via SDS-PAGE electrophoresis and western blot, presented in Figure 2.5.

The SDS PAGE gel (Figure 2.5A) exhibited single bands suggesting high degree of purity and presence of a single protein for both NP and VP40. However, some level of degradation of VP40 occurred.

The molecular weight was evaluated based on the SDS PAGE migration exhibiting 116 kDa for NP and almost 44 kDa for VP40, as expected [113]. In addition, BSA (0.8, 0.4 and 0.2 mg/ml) was used as a control and for calibration and quantification of the concentration of the tested protein, 0.2 and 0.4 mg/ml for NP and VP40, respectively.

Western blots demonstrated that these proteins were indeed labelled with His₆ tag and were a single (high purity level) product of the cloning process.

2.3.3. Preparation of AuNPs conjugates with secondary antibodies

2.3.3.1. *Optimisation of AuNPs conjugation to secondary antibody*

The assay was developed using 20 nm and 40 nm AuNPs. Conjugation of sufficient amount of proteins onto the surface is expected to prevent AuNPs aggregation in high salt concentrations [114].

Conjugation process is performed in pH conditions at the isoelectric point (IP) of the conjugated protein. Because at the IP, the protein has no charge enabling more efficient physical adsorption onto the surface of AuNPs is enabled [37]. IP of goat IgG antibodies that were used as secondary antibodies, is between pH 6.8 to 7.6 [115], and therefore PBS pH 7.2 was used for the conjugation process.

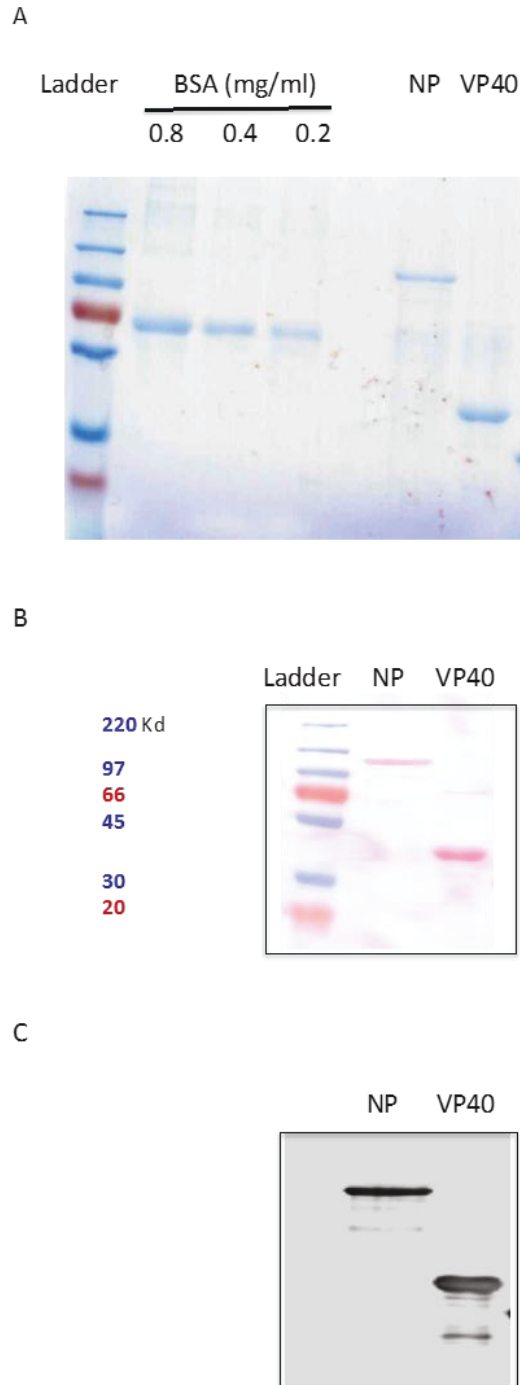


Figure 2.5. Characterisation of VP40 and NP from SUDV. A. SDS-PAGE electrophoresis Coomassie staining with protein ladder, BSA as a control and for protein quantification 0.8, 0.4 and 0.2 mg/ml, NP and VP40. Ab. Molecular weights of 65 kd, 116 kd and 44 kd were observed for BSA viral proteins NP, and VP40 respectively. B. Nitrocellulose membrane blotted with A stained with Ponceau S. Lane order;-ladder, NP and VP40. C. Western blot, the detection of two of the recombinant viral proteins of SUDV was done using murine monoclonal anti His₆ tag.

The optimal concentration of the secondary antibody to be functionalised on AuNPs was determined using the 'salt stress' test. During this study, a range of concentrations of secondary antibodies, 200, 150, 100, 50, 20, 10 and 1 $\mu\text{g ml}^{-1}$, was conjugated to the surface of the AuNPs.

Upon conjugation completion, saline solution that resembled the salt molarity of physiological blood was added. Colour change from red to blue of the AuNPs mixture related aggregation of the particles occurred due to an insufficient amount of conjugated antibody (Figure 2.6A). This resulted from low colloidal stability of the particles with a red-shift of the SPR peak, as recorded in Figure 2.6. A 100 $\mu\text{g/ml}$ was determined as the optimal concentration of the secondary antibody to be used for the conjugation of 20 nm and 40 nm AuNPs.

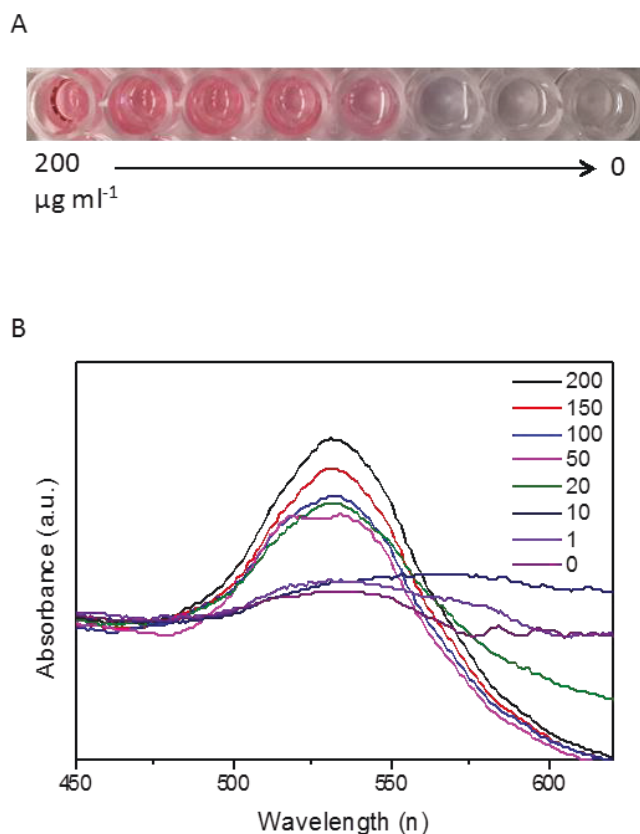


Figure 2.6. AuNPs 'salt stress' test. A. Colour of the AuNPs solution upon saline addition to 40 nm AuNPs conjugated to a range of antibody concentrations (from left) 200, 150, 100, 50, 20, 10, 1 $\mu\text{g/ml}$. B. Absorbance spectra of the AuNPs, above 50 $\mu\text{g/ml}$ the AuNPs retained their colloidal stability, with full particle aggregation below 50 $\mu\text{g/ml}$.

2.3.3.2. Characterisation of conjugated AuNPs

AuNPs conjugated to secondary antibodies were characterised to determine their optical properties, colloidal stability and size. 40 nm and 20 nm AuNPs present SPR peak in the range of 520-550 nm [116].

The SPR peaks of bare and conjugated AuNPs was compared via absorbance spectra and presented in Figure 2.7. The maximum absorbance wavelength of bare 20 nm and 40 nm was recorded 525 and 540, respectively. As expected, upon antibody conjugation a shift of 4 nm was recorded in the SPR peak of 20 nm and 40 nm particles, confirming the functionalisation of the antibody onto their surface.

In addition, the narrow and uniform spectrum of the functionalised particles demonstrated colloidal stability and that the morphology of the particles remained similar to the bare particles confirming the success of the antibody labelling process.

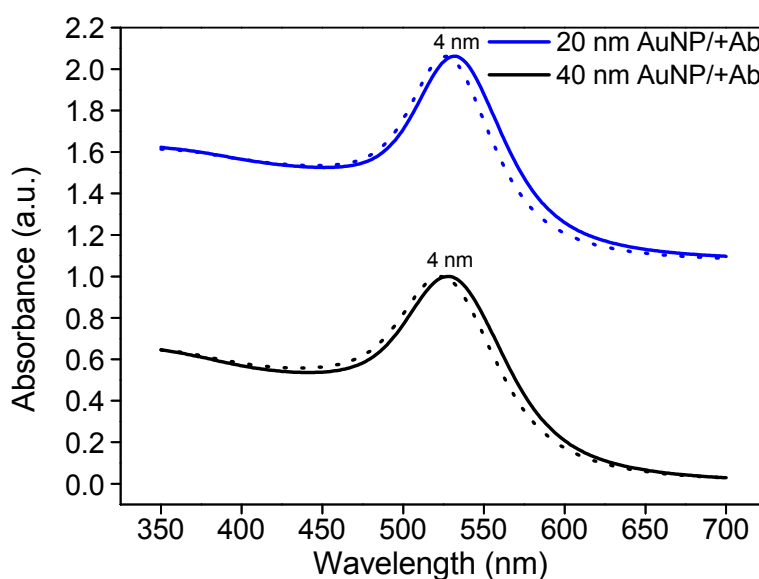


Figure 2.7. Absorbance spectra of labelled and bare 20 and 40 nm AuNPs. Size dependent UV-Vis spectra of 20 nm (blue) and 40 nm (black) AuNPs. The shift (4 nm) in the plasmonic resonance peak between bare AuNPs (dashed) and conjugated capture antibody provides confirmation of the conjugation. 20 nm AuNPs presented with an offset of 1 a.u.

The size of the functionalised nanoparticles was examined via dynamic light scattering (DLS) and presented by volume in Figure 2.8. The size of the conjugated particles can provide further information regarding the functionalisation of the antibody to the surface of the particles. The 20 nm and 40 nm functionalised AuNPs exhibited a size of 44 and 55 nm, respectively. The average size of an antibody is about 7-8 nm [117], the conjugated secondary antibody forms a monolayer onto the surface of the 40 nm particles as their size is increased by about 15 nm (single antibody on each size). However, on the surface of the 20 nm, there is more than a single layer of antibodies as the total size increases by over 20 nm. In addition, the DLS shows a single population of particle size for both AuNPs, which suggests that no aggregates of either antibodies or AuNPs occurred upon the functionalisation process.

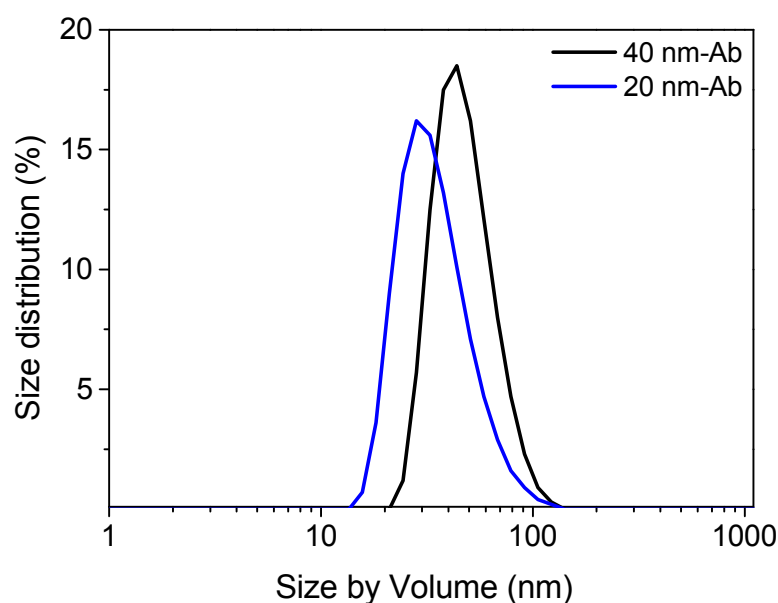


Figure 2.8. *Size distribution by volume of the AuNPs upon conjugation to capture antibody, Z-average size correspond to 44.51 ± 0.14 nm, 55.39 ± 0.11 nm for 20 and 40 nm, respectively.*

The morphology of the functionalised 20 nm and 40 nm AuNPs were analysed using TEM and presented in Figure 2.9.

The image of the 20 nm particles demonstrates highly disperse particles with mainly spherical morphology without any aggregates. Their size was evaluated as around 40 nm, as expected. The 40 nm particles had a higher

density. The thin light film around each particle demonstrated that these AuNPs were dispersed in solution.

The gathering of 40 nm AuNPs normally occurs during the sample preparation that requires drying at room temperature. Most particles retain their spherical morphology although several triangles and elliptical particles were recorded. The size of the particles based on the analysis was around 52 nm, as expected.

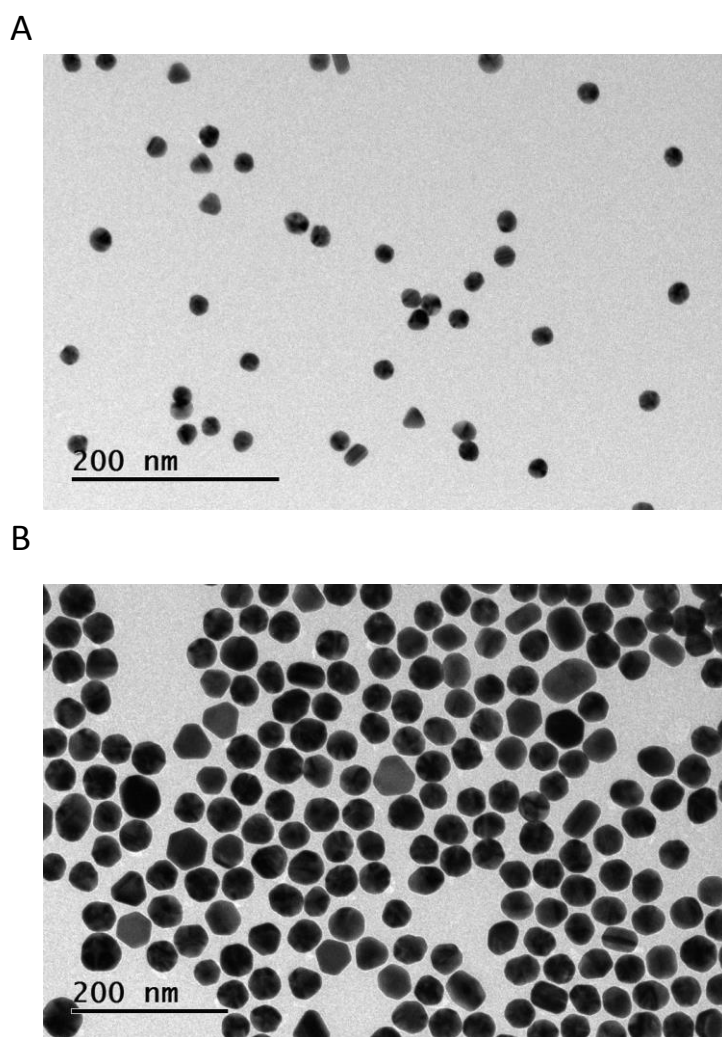


Figure 2.9. AuNPs morphology analysis via TEM. TEM images of 20 nm (A) and 40 nm (B) AuNPs labelled with secondary antibody. Both particles retain spherical shape, no aggregates were recorded. TEM imaging performed by Dr Michael Thomas.

2.3.4. Strip design and preparation

The engineering of the lateral flow test strip is a key step in the process of the assay development. This process includes selection of the optimal crosslinking density in the NC membrane. In particular, the higher the amount of the crosslinking component of the NC causes, the smaller porosity of the NC, leads to a slower flow rate and therefore enables more time for the formation of the desirable immunocomplex (Table 2.1).

However, too slow flow rate may often induce lower specificity and higher non-specific interactions. As such, following the guidance of Millipore [118], HF180 was selected as the optimal NC configuration for the detection of IgG antibodies in serum samples with nanometric AuNPs.

Table 2.1. Summary of the available NC membranes for lateral flow test strips

Membrane HF ¹ # ²	Flow rate	Sensitivity
HF240	Slowest	Most sensitive
HF180		
HF135		
HF120		
HF090		
HF075	Fastest	Least sensitive

¹ High Flow rate, suitable mainly for lateral flow test strips

²The amount of cross linker

The NC membrane was spotted with test line/s and control line using the equipment setting presented in Figure 2.10.

This setting was calibrated to accurately deposit these lines by spraying the desired protein at a flow rate of 0.8 μ l per 1 mm of membrane.

Each test line was spotted with different recombinant viral proteins, as described earlier. The pipette of the machine was especially sensitive to blockage be caused by either salt crystallisation or protein agglomeration.

As such, the concentration of the proteins was set to be 1 mg ml⁻¹ based on a consultation with a lateral flow company (Mologic) [119].

Once the NC membrane dried, the strip was assembled as presented in Figure 2.11. The NC membrane was placed first onto the plastic backing. The Absorbance pad was placed with a 2 mm overlap with the membrane. The shelf life of the whole strips was evaluated to be up 16 weeks.

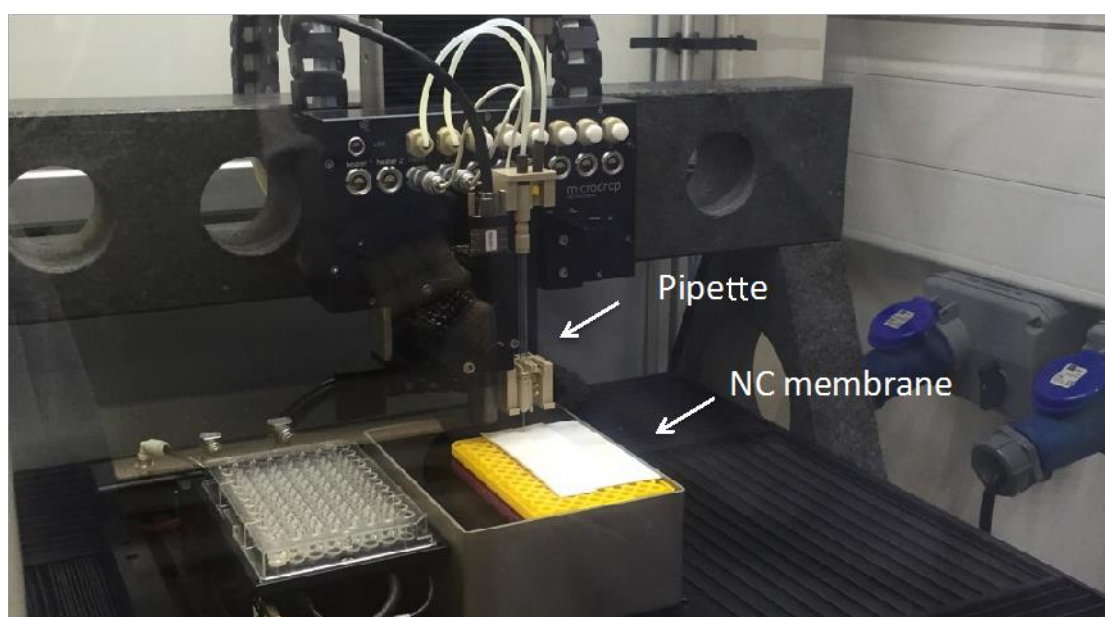


Figure 2.10. Setting for NC membrane spotting. The 96 well plate contains the solution with the viral proteins from which the pipette (white arrow) collects the sample. The pipette sprays the membrane in continuous 'flight mode'.

2.3.5. Lateral flow assay assembly and optimisation

This section describes the determination of the operation setting of the lateral flow assay to enable the testing of human body fluids that were reported to exhibit a humeral response to EVD (e.g. serum, plasma). Optimising these operation settings was crucial for the stability of the components of the assay, including AuNPs and the spotted proteins within the analytical line (test/control lines).

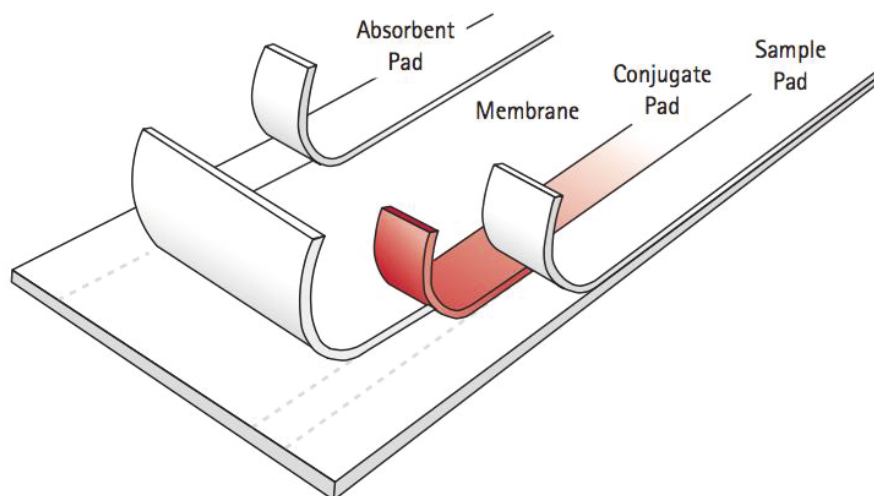


Figure 2.11. Schematic illustration of the lateral flow test strip assembly. Adapted and modified from Millipore hi-flow plus membranes text guide [118].

2.3.5.1. Determination of running buffer

The buffer, in which the assay is operated and referred to as the running buffer, has a key role in the function of the assay. An optimal running buffer should maintain the colloidal stability of the labelled AuNPs to enable their uniform migration through the NC membrane while supporting the formation of specific immune interactions of the detection components. Detergents are commonly used to stabilise NPs [120] and prevent non-specific interactions of biomolecules [121].

As such, PBS buffer with and without Tween-20 was tested. In Figure 2.12, AuNPs conjugated to a secondary antibody (anti-human IgG) in PBS (1) and PBS/Tween-20 (2) were applied to the lateral flow test strip with single test (GP₁₋₆₄₉^S) and control lines (human IgG) and recorded after 5 minutes. The absence of Tween-20, in the running buffer, caused aggregation of the AuNPs, which prevented their migration through the test strip therefore, an absence of the control line. Adding Tween-20 enabled rapid migration of the AuNPs and formation of the specific immunocomplex, labelling only the control line.

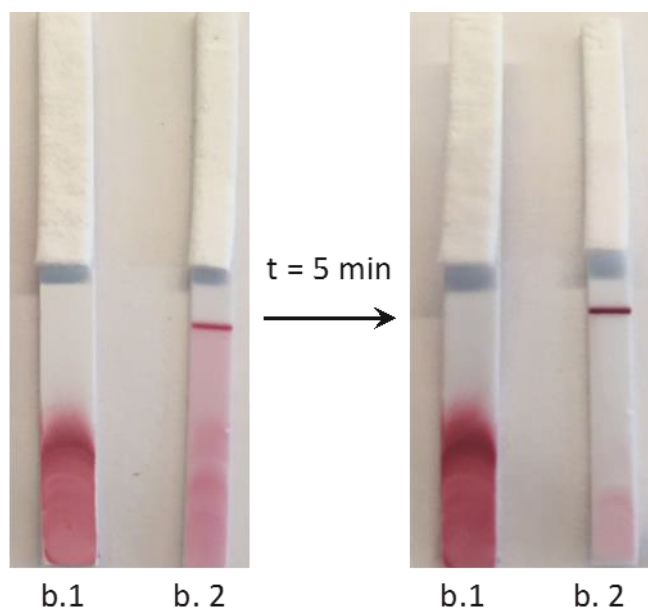


Figure 2.12. Determination of running buffer. Strips with control line spotted with mouse IgG were loaded with 40 nm AuNPs labelled with anti-mouse IgG antibodies in PBS pH 7.4 (b.1) and PBS pH 7.4, BSA 0.05% (w/v) Tween-20 (b.2). Upon 5 minutes the particles in b.1 aggregated preventing migration along the strip. Particles in b.2 migrated along the strip labelling the control line.

2.3.5.2. Determination of blocking reagents

The blocking reagent has an imperative function in prevention of non-specific interactions while enhancing the stability of the components of the assay, e.i. particles [122].

Four blocking conditions were examined onto manually spotted strips. These strips were prepared by depositing a spot with mouse IgG for the control line and GP₁₋₆₄₉^S for the test line as presented in the schematic illustration in Figure 2.13.

The analysed sample consisted AuNPs labelled with secondary antibody against mouse IgG antibodies and target solution. The target solution consisted of 3C10 and the running buffer with the following blocking components: 5% (w/v) skimmed milk (Figure 2.13.1), 1% (w/v) skimmed milk (Figure 2.13.2), 2% (w/v) Bovine Serum albumin (BSA) (Figure 2.13.3) and 5% (w/v) BSA (Figure 2.13.4).

Two clear spots were formed in the assay that had 5% (w/v) BSA, corresponded to detection and presence of the target IgG antibodies against GP₁₋₆₄₉^S. Although skimmed milk was used as the optimal blocker for the previously described ELISA assay, skimmed milk prevented the binding between the target antibody on NC, the test line of the system and the conjugated AuNPs. Higher concentrations of skimmed milk might have screened between the assay components that prevented them from interacting.

A 2% (w/v) of BSA was not sufficient for blocking the membrane of the strip and therefore it caused migration of the test line spot. As such, 5% (w/v) was determined as the optimal blocking condition for testing serum.

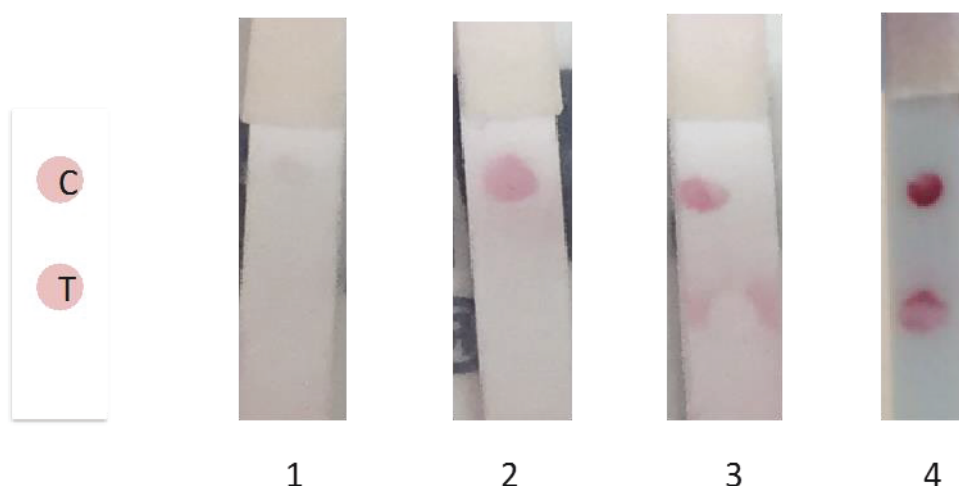


Figure 2.13. Determination of blocking reagent in the running buffer. NC strip test manually spotted with GP₁₋₆₄₉ (T) and mouse IgG (C). Dilution of 1:1000 of 3C10 was mixed with 40 nm AuNP labelled with anti-mouse IgG antibodies in different buffers (1) PBS pH 7.4, 0.05% (w/v) Tween-20, 5% (w/v) skimmed milk, (2) PBS pH 7.4, 0.05% (w/v) Tween-20, 1% (w/v) skimmed milk, (3) PBS pH 7.4, 0.05% (w/v) Tween-20, 2% (w/v) BSA, (4) PBS pH 7.4, 0.05% (w/v) Tween-20, 5% (w/v) BSA. Visual appearance of the red spots represented formation of immunocomplexes on the strip.

2.3.6. Development of smartphone application

An app was developed with the assistance of Ben Miller from University College London and was used for the analysis of the lateral flow test strip for the detection of Ebola virus antibodies.

The complementary app was designed to provide three features: i. provide quantitative analysis of the lateral flow test strips, ii. enable saving the results in a digital format that can be easily shared between other devices and iii. provide geographical tagging of the tested sample.

Figure 2.14 presents the interface of the application. In the first operation step personal details of the patients were recorded, including patient id, age, gender and the time since the recovery from EVD. In addition, the application automatically detected the location (latitude and altitude). Then, the lateral flow test strip was analysed via the camera window and the Red Cross is used for alignment and separation between the test and control lines. Tapping on the screen instantly provided the relative intensity of the test line and the control line.

The whole analysis of the strip was engineered as following; the app produced an image of each scanned strip and then generated an intensity plot corresponding to the colour intensity of the analytical zone of the strip (see Figure 2.14.4.ii). The change in the intensity peak corresponded to the amount of the target analyte tested, providing quantitative analysis. The baseline of the intensity plot was taken as the background signal (i.e. a non-analytical zone), to which the intensity of the test line was normalised. Finally, the result can be displayed on a geographical map.

2.3.7. Determination of the optimal size of the AuNPs

The AuNPs were used as the colorimetric labelling of the target antibody within the assay. The functionalisation of secondary antibody onto the surface of the particle enabled formation of immune-complex between the visual label (AuNPs) and the test line in the analytical zone of the assay.

In addition, the secondary antibodies had a significant role in controlling the assay function by directly binding to the spotted detection antibodies in the control line of the test strip. The 20 nm and 40 nm AuNPs conjugated to secondary antibodies were tested to determine the magnitude of intensity of the assay readout. Quantitative analysis was performed with the developed smartphone app and is presented in Figure 2.15. In this test no target analyte

was added to the system and the relative intensity of the test and control line was quantified using labelled and bare 20 and 40 nm AuNPs.

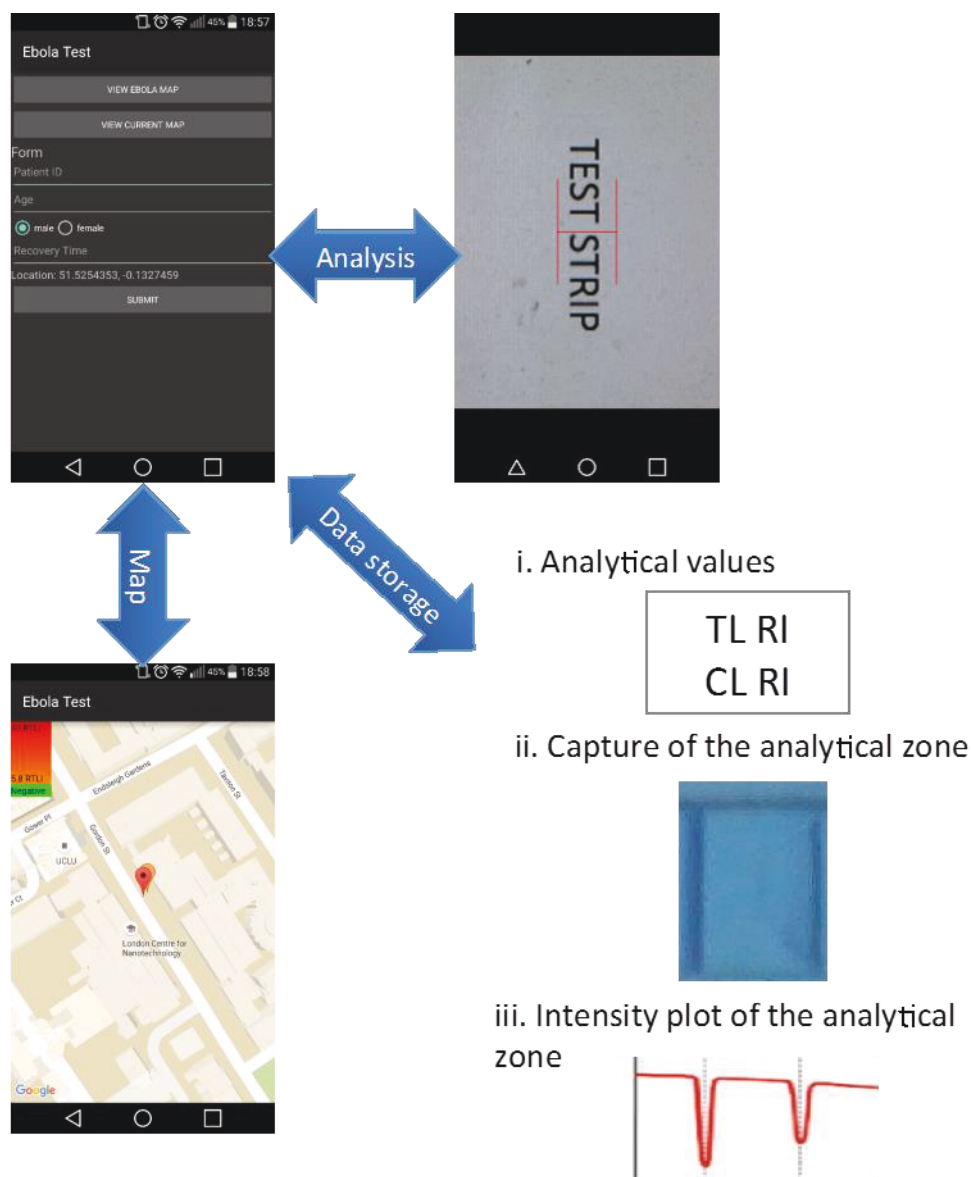


Figure 2.14. Smartphone application operation flow chart. Screen shots of the developed application, including details of log in window (top left), strip capture (top right), resulting geographical map (bottom left) and the output generated via the application (bottom right).

The result shown the readout of the assay with bare AuNPs was within background noise. This implied, that the binding of the AuNPs onto the control line is specific to the immune recognition between the antibodies on the surface

of the particles. In addition, this result confirmed that the functionalised secondary antibodies remained on the surface of AuNPs. The readout of the test lines demonstrated no non-specific binding between the functionalised AuNPs and the GP₁₋₆₄₉, as it was expected in absence of analyte.

Although the concentration of the 40 and 20 nm particles was identical, the 40 nm AuNPs produced a significantly higher intensity readout compare to the 20 nm AuNPs. This may be explained as the colour intensity of the AuNPs is normally correlated to the magnitude of their SPR and up to the size of 60 nm the OD at the SPR is proportional to the size of the particles. Hence, the colour intensity of 40 nm AuNPs is greater than the 20 nm ones.

Using 40 nm AuNPs in the final format of the assay may produce higher readouts, which improves the sensitivity of the assay while showing no non-specific interactions with the analytical zone and the test line.

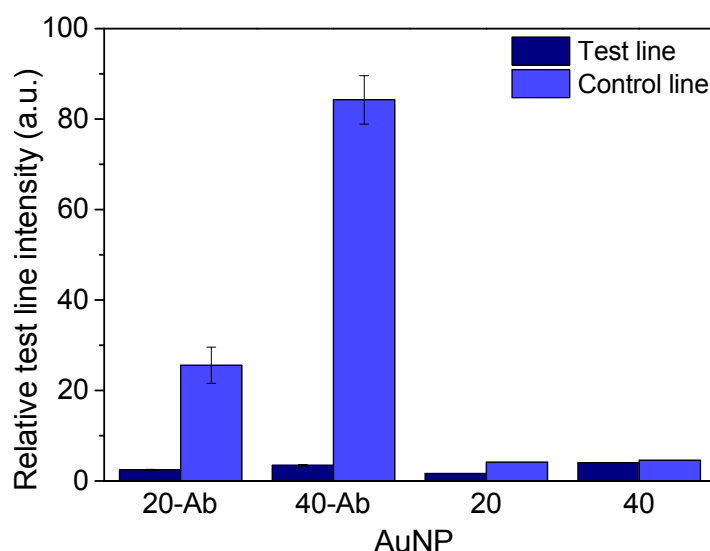


Figure 2.15. Dependence of the colour intensity of the lateral flow test zone on the size of the AuNPs, quantification in RTLI of the control line and test line intensity of 20 and 40 nm AuNPs, conjugated to capture antibody without target analyte. Bars indicate mean $\pm$ standard deviation ($n=3$). 40 nm AuNPs were selected as optimal to produce the highest signal readout.

2.3.8. Evaluating the limit of detection of the developed assay

The smartphone-based lateral flow assay for the detection of Ebola Virus antibodies was developed in two formats. The first format was engineered to

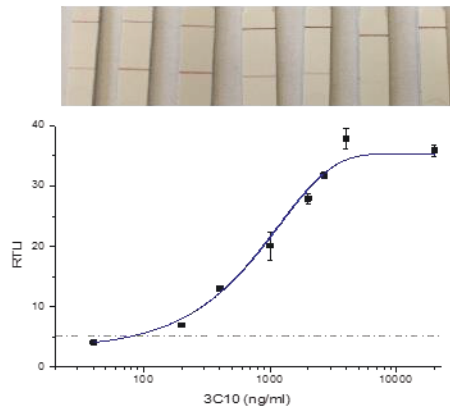
detect a single target IgG antibody and therefore consisted of a single test line spotted with recombinant GP₁₋₆₄₉ and is referred as the monoplex. The second format was designed to detect multiple target IgG antibodies against three recombinant GP₁₋₆₄₉ from three viral species which were spotted onto three independent test lines and was referred as the multiplex. The performances of these formats, monoplex and multiplex, are described in this section using specific IgG antibodies.

2.3.8.1. Monoplex GP₁₋₆₄₉ assay

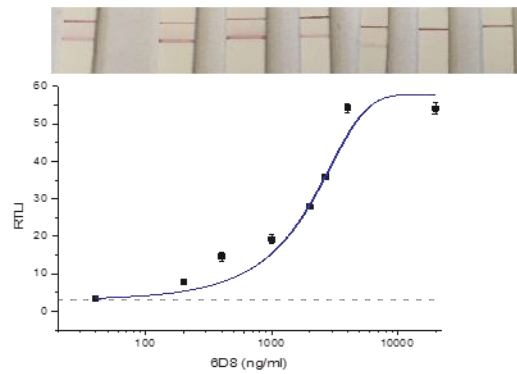
Specific IgG antibodies 3C10, 6D8 and pol-BVBD against GP₁₋₆₄₉^S, GP₁₋₆₄₉^Z and GP₁₋₆₄₉^B, respectively, were described earlier and were used for the evaluation of the monoplex assay format. Two of these antibodies were produced in mice, 3C10 and 6D8, whereas pol-BDBV was produced in rabbit. As such, lateral flow test strips were prepared with a mix of mouse and rabbit IgG antibodies spotted on the control line and a mix of AuNPs conjugated to anti-mouse and anti-rabbit IgG antibodies. Viral GP₁₋₆₄₉ proteins from different viral species were spotted on the test line resulting in a collection of three different monoplex assays. Dose response curves were produced for each specific IgG antibody by using the complementary test strip and presented in Figure 2.16. For instance, 3C10 was applied in a range of concentrations between 20 µg/ml to 40 ng/ml onto the strip with GP₁₋₆₄₉^S and quantified using the developed smartphone application. The cut-off value was evaluated and used to determine the lowest concentration of the target antibody detectable via the assay. The limit of detection of the SUDV, EBOV and BDBV assays was 200, 200 and 500 ng/ml, respectively.

The sensitivity of the developed assay was about 100-500 times below the sensitivity of the previously evaluated ELISA. This is expected as the ELISA has a signal amplification step via the HRP enzyme that improves its sensitivity. As such, the amount of the real sample analysed via the developed assay should be at about 500 times greater compare to the volume used in a standard ELISA. Previous serological analysis of human Ebola Virus survivors performed with a standard ELISA used diluted sera samples by 1000 fold [111]. Hence, to analyse real samples via the developed assay, a range of 5-15 µl of sample is required.

A



B



C

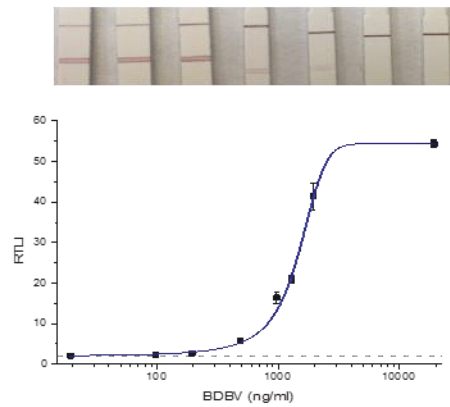


Figure 2.16. A-C: 3C10, BDBV and 6D8 antibody titer dose-response curves and images of randomly selected strips, respectively. Concentration range from 20 $\mu\text{g/ml}$ to 40 ng/ml, the series of results was plotted and fitted to a sigmoidal curve ($r^2 = 0.985$, 0.985 and 0.995 , respectively). Bars indicate means $\pm$ standard deviations ($n=3$).

2.3.8.2. EVD species multiplex assay

The identification of the species of the EVD is an imperative surveillance-monitoring tool for patient management and to understand the pattern of the disease distribution. In this section demonstrates simultaneous detection of specific IgG antibodies against different viral species including, EBOV, BDBV and SUDV via the lateral flow multiplex test strip.

Each test line of the test strip was spotted with a different viral GP₁₋₆₄₉ for each of the above viral species. The concept of the test was demonstrated using 3C10, 6D8 and pol-BDBV antibodies labelled as S, Z and B in Figure 2.17, respectively. In this case, the control line was spotted with a mix of rabbit and mouse IgG antibodies. The AuNPs used were a mix of particles conjugated to anti-mouse IgG and anti-rabbit IgG. The tested antibodies were mixed to form a final dilution titer of 1:1000.

The low background colour of the membrane represents the low non-specific interactions and the high specificity of the developed assay. Moreover, the appearance of a single test line when a single antibody is tested suggests the high specificity of the recognition components, the GP₁₋₆₄₉ proteins.

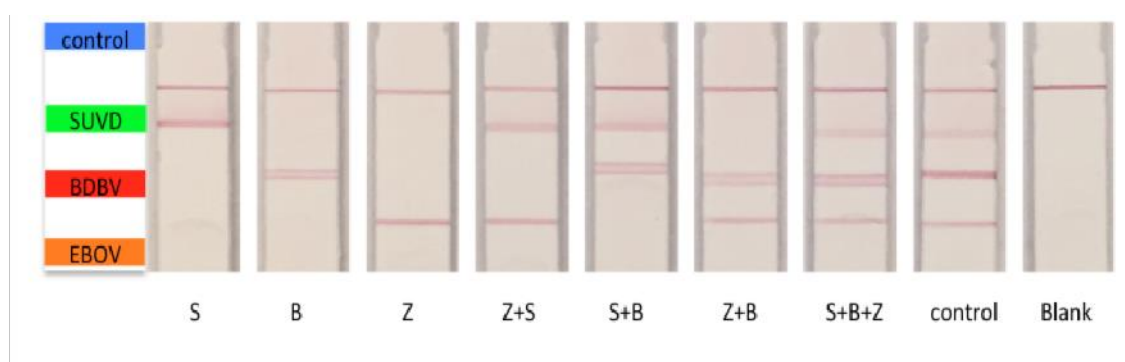


Figure 2.17. Species-multiplex lateral flow. Strip consists of three test lines spotted with GP₁₋₆₄₉ from SUDV, BDBV and EBOV. A. Simultaneous detection of monoclonal IgG antibodies against SUDV (S), EBOV (Z) and polyclonal BDBV (B), control lines were spotted with mouse and rabbit IgG and AuNPs conjugated to anti-mouse and rabbit antibodies. All antibodies were diluted 1:1000.

2.3.9. Assay optimisation for testing human samples

In this section, the transfer process of the developed assay for testing human samples is described. A total of 3 positive samples were obtained from human patients that were previously infected and recovered from SUDV, referred to as survivors, and demonstrated the presence of IgG antibodies against GP₁₋₆₄₉ for SUDV via ELISA. A single negative sample was obtained from a healthy non-infected volunteer and tested negative for IgG antibodies against GP₁₋₆₄₉ for SUDV.

The human lateral flow test strip comprised a control line spotted with human IgG antibodies and 40 nm AuNPs conjugated to secondary anti human antibodies.

2.3.9.1. *Plasma versus serum as assay sample*

High levels of the immune response to EVD are normally present in blood, serum or plasma. In addition, these body fluids are relatively simple to obtain and therefore ideal for point-of-care systems.

The developed lateral flow test strip was designed for cell-free samples and therefore plasma and serum were tested to determine the ideal form of specimen. Fresh positive (labelled as 1 in Figure 2.18) and negative (labelled as 2 in Figure 2.18) plasma (P) and serum (N) samples were tested via the lateral flow test strip. The resulting strips (in duplicates) are presented in Figure 2.18.

The sera samples migrated throughout the membrane presenting a visual test line for S1 that correlated to a strong IgG response to GP₁₋₆₄₉^S. The negative serum sample exhibited relatively low background colour around the test line region that might have been formed due to non-specific interactions. Nonetheless, the difference between the intensity of the test line of S1 and S2 was significant. In addition, these samples were characterised via ELISA confirming low levels of non-specific interaction of S2 and GP₁₋₆₄₉^S.

The analysis of the plasma samples demonstrated a high background noise throughout the whole analytical region of the test strip with no visual test line. This background colour, normally caused due to aggregation of the AuNPs,

might have been induced by coagulation factors in human plasma. As such, serum was determined as a more suitable specimen to be tested via the developed assay.

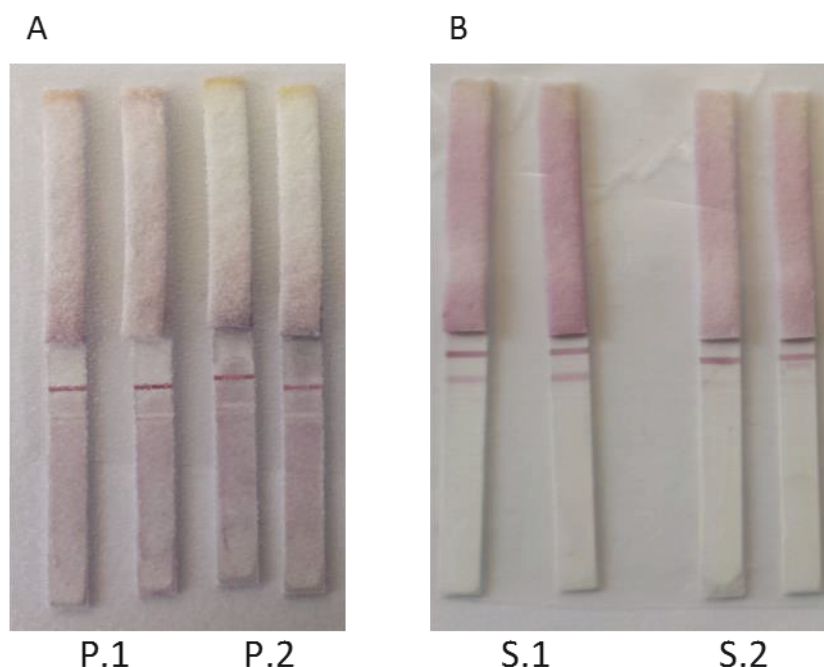


Figure 2.18. Analysis of human plasma and serum. A. Lateral flow test strips upon testing of plasma samples obtained from SUDV survivor (P1) and non-infected control (P2). B. Lateral flow test strips upon testing of serum samples obtained from SUDV survivor (S1) and non-infected control (S2). Each sample was analysed and presented in duplicate.

2.3.9.2. Selection of secondary anti human antibody

The secondary antibody conjugated to the surface of the AuNPs is a key component for the operation of the lateral flow immunoassay.

The detection target IgG antibody in human serum was designed as an indirect sandwich format between the antigen on the test line (GP₁₋₆₄₉) and the AuNP conjugated antibody. As such, the optimal secondary antibody must present an affinity to a different region of the target antibody to enable optimal conditions for the target to bind to both sandwich components.

Three positive and single negative samples were quantitatively analysed using the developed assay for human samples. These samples were analysed

with different anti-human secondary antibodies. Each antibody has affinity against different region in human IgG antibody. As such, all other elements were identical between the tested secondary antibodies, including the production origin (goat) and the conjugation process to the surface of 40 nm AuNPs.

The anti Fc antibody interacts with the fragment crystallisable region (Fc region), which is the tail region of an antibody, see Figure 2.19A.

The anti Fab antibody binds to the fragment and the anti-whole IgG binds non-specifically to various regions within the human IgG antibody. These antibodies conjugated to AuNPs were used to test three positive sera samples and a single negative sample, which were previously characterised via ELISA demonstrating that sample 3 had the highest levels of the target antibody, followed by 2 and then 1. The anti Fc antibody produced the highest readout intensity of the assay in all the three positive samples, corresponding to the expected relative levels, see Figure. 2.19. The anti-whole antibody exhibited the lowest readout especially in S.1 (Figure 2.19).

This suggests that certain interference occurred with the GP antigen and therefore reduced the efficiency of the assay. Anti Fab antibody was the worse secondary antibody demonstrating the lowest binding affinity to the target immunocomplex formed between the target antibody and GP antigen. Nonetheless, the levels of the readout with anti Fab antibody were comparable to the negative control for all three positive samples suggesting the target antibody indeed binds to the GP₁₋₆₄₉ via the Fab region. As such anti Fc antibody was determined as the optimal secondary antibody for the assay operation.

2.3.9.3. *Serum volume in the applied sample*

The structure and dimensions of the developed lateral flow test strip can take up to 20 µl of sample volume. This applied sample must include running buffer in a final volume of 20 µl. The composition of the running buffer is crucial for the stabilisation of the conjugated AuNPs and to block the membrane from non-specific binding, as demonstrated in Section 2.3.5.

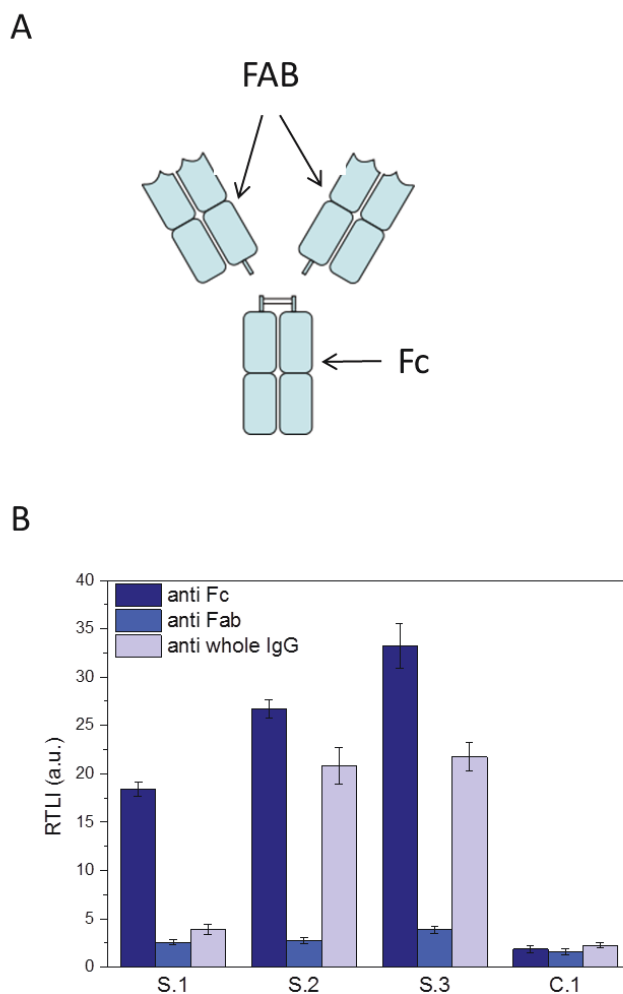


Figure 2.19. Comparison between different secondary antibodies against human IgG. A. Schematic illustration of the structure of IgG antibody including, the antigen-binding fragment (Fab), a region on the antibody that binds to antigens and the fragment crystallisable (Fc) region. B. Quantitative analysis of human sera positive samples (S1-3) and a negative control with different secondary antibodies conjugated to AuNPs. The RTLI readout of the tested survivors differs depending on the used antibody, from high to low signal was of Anti Fc (royal blue), anti-whole IgG (light blue) and anti Fab (medium blue). The RTLI of the non-infected control was within the background noise for all three tested antibodies. Bars indicate means $\pm$ standard deviations ($n=2$).

This section determined the partial volume of serum in the applied sample. Figure 2.20 presents the analysis of different sera volumes tested with the monoplex strip. The tested sera included three samples obtained from human SUDV survivors from different years (explanation about the sample database will be presented in chapter 3) and were referred as positive sera.

These positive sera samples were tested by mixing 5, 10, 12 and 15 μ l of serum with running buffer to a total sample volume of 20 μ l. Then these samples were analysed by using the developed human lateral flow test strip with a GP₁₋₆₄₉ on the test line. The volume range of the applied samples was estimated in section 2.3.8. All samples were originally characterised via ELISA and exhibited detectable levels of target IgG antibodies against GP₁₋₆₄₉.

Figure 2.20 demonstrated that using below 10 μ l of serum volume provided a relatively low readout, within the level of a background signal (blank sample). A total of 15 μ l of serum produced comparable quantitative result to the ELISA analysis and therefore was determined as the optimal serum volume for the sample preparation. In addition, 15 μ l serum produced comparable readout intensity to the positive control, which consisted of nonhuman test strips and using 3C10 antibody.

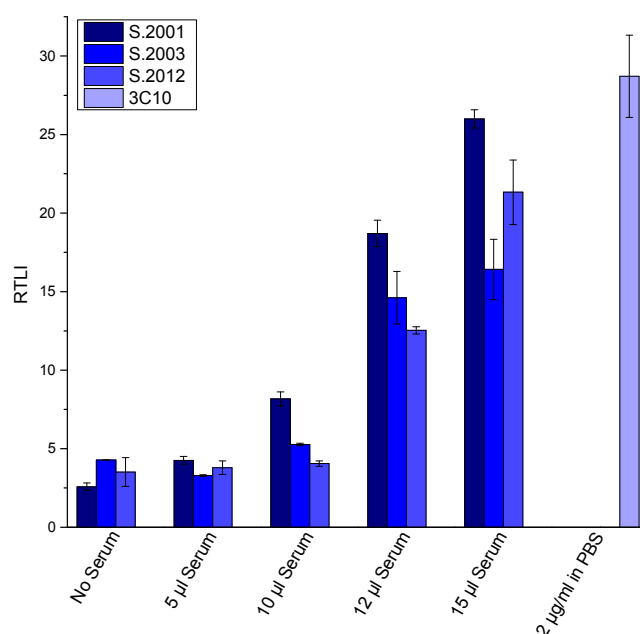


Figure 2.20. Serum volume optimisation. Different sera volumes obtained from survivors from three collections (2001, 2003 and 2012) were applied to the SUDV-GP₁₋₆₄₉ and quantified. Each sera volume was diluted to the optimal operation volume, 20 μ l. 15 μ l sera provided sufficient signal readout and with sufficient volume of running buffer for ensuring optimal sample migration onto the strip. Bars indicate mean $\pm$ standard deviation ($n=2$).

2.3.9.4. *The sequence of the test lines in the multiplex assay*

Multiplex test strips were prepared with GP₁₋₆₄₉ from different species spotted onto the test lines. The sequence of the spotted test lines was investigated by preparing three configurations of lateral flow test strips, as schematically presented in Figure 2.21A. The GPs were spotted in three random orders from the control line; (1): EBOV, BDBV and SUDV, 2: BDBV, SUDV and EBOV, 3: SUDV, EBOV and BDBV.

A single positive serum sample (dark blue) and a negative serum sample (light blue) were tested over these lateral flow test strips and quantified using the complementary smartphone app (Figure 2.21C). The tested positive sample exhibited a specific immune reaction against the SUDV species. The quantitative analysis of the positive sample demonstrated that the readout signal remained within the range of 24-28 RTLI regardless of the position of the test line. The negative sample exhibited no immune reaction, as expected.

Representative lateral flow test strips are presented in Figure 2.21B, demonstrating that the specificity in the detection of the orthogonal IgG target was independent of the sequence of the spotted test lines. To visualise the position of the spotted test lines, anti-his antibody was tested via configuration 1 and 3 and using AuNPs labelled with anti-goat IgG. In addition, the lack of the control line demonstrated the specificity of the assay for human specimens, as the anti-his was obtained from goat origin.

The reduction of the colour intensity of the test lines, from bottom to top, also demonstrated a non-sufficient amount of the target antibody that had similar affinity to each test line.

Finally, the third test line sequence was selected for the multiplex configuration mainly because no significant difference in the intensity of the readout was noticed.

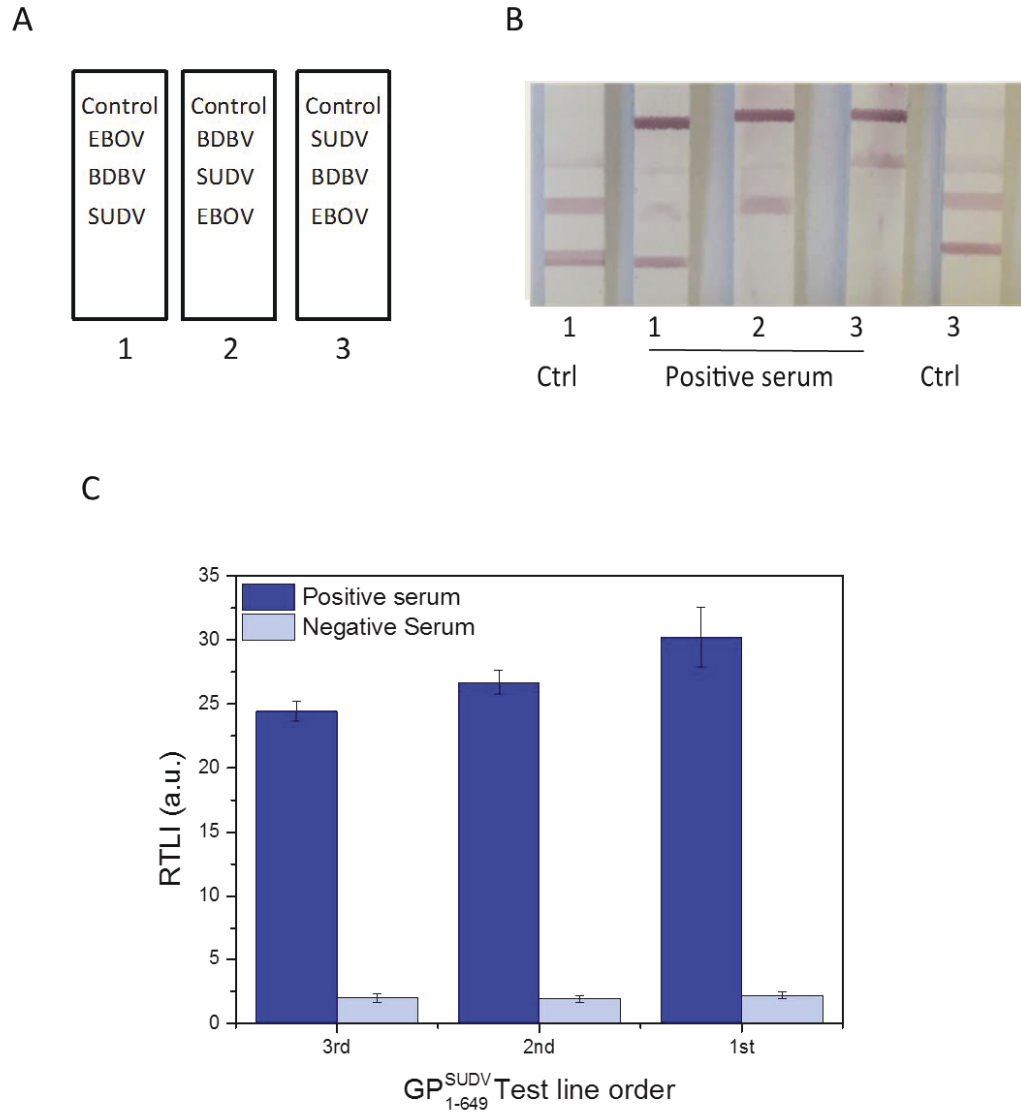


Figure 2.21. Sequence of the multiplex test lines. A. Three configurations of multiplex GP₁₋₆₄₉ format were prepared by spotting different sequences of the test lines with GP₁₋₆₄₉ from Ebola Virus species, including, SUDV, BDBV and EBOV. B. Analysis of human positive serum for SUDV with three configurations of lateral flow test strip 1,2 and 3. Visual red test line at the position of GP₁₋₆₄₉ from SUDV in each of the 1,2 and 3 strips. Visualisation of the test line positions of configuration 1 and 3 with control target, anti-his antibodies and AuNP labelled with anti-goat IgG. C. Quantitative analysis by using the smartphone app for positive and negative human samples to GP₁₋₆₄₉ SUDV over the three lateral flow configurations. Bars indicate mean $\pm$ standard deviation (n=2).

2.4. Conclusions

This thesis reviews two biosensing concepts for the detection of Ebola virus related targets. The first assay, presented in this chapter, utilises affinity recognition elements in a 'dry' paper-based format to detect antibodies. The second assay, presented in chapter 5, employs multiple nucleic acid recognition elements in a solution-based assay to identify target oligonucleotides. As reviewed in the Introduction in section 1.6.5, the diagnosis of Ebola virus requires detection of host's immunity and direct detection of viral components (e.g. antigen and nucleic acids). Hence, the overall aim in this thesis was to design alternative diagnostic tools that provide complementary analysis for the viral diagnosis.

This chapter described the development of a novel point of care assay for Ebola virus, which comprised a lateral flow test strip and complementary smartphone application. The developed assay was designed to detect specific antibodies against Ebola Virus viral proteins, which include glycoproteins from the SUDV, BDBV and EBOV viral species. The lateral flow test strip was developed in two formats, the monoplex format with a single test line and the multiplex format with three test lines.

Until the 2014-15 West African outbreak Ebola virus was addressed as a rare pathogen, with a relatively humbled research community. The low interest in this pathogen limited the amount and diversity of commercially available viral proteins and antibodies. In particular, viral proteins for SUDV still cannot be purchased via strand suppliers (e.g. Abcam, Sigma).

Hence, the work presented in this thesis relied on self-made recombinant viral proteins and monoclonal antibodies that were produced in collaboration with L. Lobel *et al* at the laboratory of Virology and Immunology at Ben Gurion University, Beer-Sheve, Israel and J. Dye *et al* at the Virology Division at the US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland (USAMRIID).

A range of viral recombinant proteins with His₆, including GP₁₋₆₄₉, NP and VP40 from SUDV and GP_{1-649S} from BDBV and EBOV, were prepared and characterised in this chapter. The binding between the recombinant viral

glycoproteins, GP₁₋₆₄₉, for three viral species, and specific antibodies were characterised with an indirect calorimetric ELISA test. The obtained GP₁₋₆₄₉ proteins demonstrated high selectivity in binding of the orthogonal antibodies with a limit of detection of 0.5, 1 and 1 ng ml⁻¹ for GP₁₋₆₄₉ from SUDV, EBOV and BDBV, respectively.

Additional recombinant SUDV viral proteins were provided, VP40 and NP. These proteins had no specific antibodies to be characterised via ELISA. As such, the specific molecular weight of these recombinant proteins was determined to be 116 and 44 kDa by using SDS-PAGE electrophoresis and matched with the literature values. In addition, a western blot was performed with anti-His₆ antibodies to confirm the purity of the provided proteins. These viral proteins will be included in a multiplex SUDV assay, which will be described in chapter 3. As such, all three recombinant proteins were confirmed to serve as the detection components of the developed assay.

AuNPs are considered cheap, stable in room temperature and simple for functionalisation [40] [114]. For these reasons AuNPs were selected as the labelling component of the lateral flow test strip and were functionalised with secondary antibodies.

The conjugation was optimised to retain the optical properties and the colloidal stability of AuNPs. This included conjugating 100 µg ml⁻¹ secondary antibody and blocking with BSA. In addition, the investigation of different secondary antibodies demonstrated that anti-Fc antibodies produced optimal detection abilities and therefore were used for the human lateral flow test strip configuration.

A 40 nm AuNPs produced a higher readout intensity of the test and therefore selected as the optimal particle size for the developed assay. The 40 nm AuNPs exhibited a stronger light scattering than 20 nm AuNPs and therefore produced a higher colour intensity on the test line [123]. Moreover, optimal operation conditions for the assay were evaluated and included the use of 5% and 0.05% w/v of BSA and Tween-20, respectively in the running buffer recipe.

The nitrocellulose membrane was spotted with the recombinant GP₁₋₆₄₉ proteins onto the test line. The control line was spotted with either human or a mix of rabbit and mouse IgG antibodies for human and non-human, respectively

lateral flow test strips. According to the dimension of the strip, a total of 20 μl was determined as the ideal sample volume to be applied to the strip.

The use of recombinant viral proteins was a key advantage as many of the serological tests use whole Ebola antigen [124]. As such, recombinant protein-based tests can be operated in basic biological safety laboratories, which is an important advantage due to a limited access to BSL-4 facilities that can operate Ebola virus samples in the affected areas of the outbreaks [125].

A complementary app was developed alongside the lateral flow strip. The use of a smartphone enabled portable operation of the assay. The application was designed to obtain information of the tested patient, provided quantitative analysis of the lateral flow strip, stored the result and geographically tagged the location. The use of smartphones in medicine is one of the growing trends [126] and previous studies demonstrated the use of smartphones as a lateral flow reader [53] [52]. Furthermore, in the Ebola virus assay developed herein, the smartphone was also built to provide storage and sharing of the collected data with the exact geographical tag. As such, this novel approach of using the smartphone not only as a test reader may enable the use of this test for surveillance for epidemiological studies.

The smartphone-based lateral flow test with was developed in monoplex and multiplex formats for three Ebola Virus species that mostly affect humans[69], including, SUDV, BDBV and EBOV. The test strips were spotted with recombinant GPs of these viral species and calibrated using specific nonhuman antibodies. The limit of detection was evaluated to be 200, 200 and 500 ng ml^{-1} for SUDV, EBOV and BDBV, respectively. Moreover, a proof of concept of the multiplex format demonstrated specific and simultaneous detection of these antibodies on the orthogonal test line.

At this stage the material-based development of the lateral flow test strip was achieved. This assay was further optimised and adjusted to analyse real clinical samples from human.

The immune response of individuals infected with Ebola virus is usually detected in blood [127], plasma [128] or serum [111]. As the developed assay configuration was not compatible for testing whole blood, plasma and serum of previously infected patients, each were tested via the developed lateral flow test

strip. The use of plasma caused uncontrolled aggregation of the AuNPs onto the membrane. The coagulation factors may reduce the colloidal stability of the AuNPs causing their aggregation [129], which prevent their migration along the analytical zone of the strip. As such, serum was determined as the optimal body fluid for the configuration of the developed assay. Nonetheless, analysing finger blood can significantly simplify the operation of such assay and is addressed in the future work.

The volume of the serum was determined. A total of 15 μ l of serum was required to obtain strong and specific test readout. The shelf life of the developed assay was over 16 weeks with operation time of 15 minutes. The sequence of the spotted test lines of the human multiplex format demonstrated no significant difference between the order of the printed GP₁₋₆₄₉ proteins from three viral species and the result of the test. As such, the test lines of the GP₁₋₆₄₉ proteins were prepared with the following sequence of species: EBOV, BDBV and SUDV followed by a control line.

Finally, data regarding host immune response to Ebola virus may provide information regarding the suitable treatment and maintenance of the infected individual. Surveillance screening for humoral response to Ebola Virus is crucial for epidemiological studies to understand the patterns of disease spread and in rapidly identifying its source. These serological tests are currently performed via standard ELISA, which require trained personnel, laboratory-based facilities and take up to several hours.

In this chapter, the critical need for faster, simpler and more portable serology tools for Ebola virus was addressed. Our new simple and portable smartphone-based system has demonstrated the ability to detect and classify specific IgG antibodies. Validation and evaluation of the developed assay is required. This can be performed by screening of a large cohort group of patients that were exposed to Ebola virus.

It is clear that the development and validation of such systems is essential and has been starkly emphasised in the wake of the 2014-15 West African outbreak.

Chapter 3

Serological Surveillance of Human Ebola Virus Survivors Using the Developed Smartphone-based Lateral Flow Test

3.1. Introduction

In this chapter, a small-scale evaluation of a developed smartphone-based lateral flow test for the detection of EVD IgG antibodies is presented. This system was evaluated on SUDV species in Uganda. SUDV was selected as a model species for the evaluation of the test. The evaluation was performed by testing a large cohort group of human serum samples obtained from previously infected and recovered (survivors) patients from the 2000-1 SUDV outbreak in Gulu district, Uganda.

The final formats of the assay combined lateral flow test strip with a complementarily smartphone app. In this chapter the smartphone app was used for quantitative analysis of the strips, data management and geographical tagging of the tested samples. Two independent platforms, monoplex and multiplex, of the assay were evaluated with human samples. In the monoplex format, host IgG antibodies against GP₁₋₆₄₉ from SUDV were targeted. In the multiplex format, the assay simultaneously detected IgG antibodies against three recombinant proteins from SUDV (VP40, NP and GP₁₋₆₄₉). Additionally, a proof of concept for viral species identification was demonstrated by using an additional multiplex text configuration and survivors from different EVD strains were analysed. In this multiplex, IgG antibodies against GP₁₋₆₄₉ from three EVD strains, including SUDV, EBOV and BDBV, were detected. This developed system produced a fast serological analysis and therefore might be highly suitable for patient management during and at a post-acute infection stage. Such a test can be highly beneficial for epidemiological studies and serological surveillance.

3.1.1. 2000-2001 Ebola virus outbreak in Uganda

From October 2000 to January 2001, the largest known epidemic of SUDV occurred in Uganda [130]. The causative agent was closely related to the EVD species of the Sudan 1976 and 1979 outbreaks with a fatality rate of 53% was in line with the mortality associated with this SUDV species [131].

Situated at the geographical heart of the African continent, Uganda lies in a region where many viral disease epidemics, leading to numerous deaths, have emerged over the years. In the first days of October 2000, an outbreak of an unusual febrile illness with occasional hemorrhage and significant mortality was reported to the Ministry of Health in Kampala. Clinical samples and contacts were forwarded several days later by the World Health Organisation (WHO) country office to a collaborating laboratory in Johannesburg, South Africa and the suspicion of EVD outbreak was confirmed [72].

In the following three months since the viral outbreak, more than 2000 human samples were collected and tested. Sample collections were performed throughout the different districts of the country, although confirmed with EVD cases were recorded from only three districts in Uganda: 393 (93%) from Gulu in Northern Uganda, 27 (6%) from Masindi in Western Uganda and five (1%) from Mbarara[132], as presented in Figure 3.1.

The effective testing and documentation of the infected patients provided great opportunities for clinical investigation of the SUDV and its pathogenesis [77] [107]. In total, during this outbreak, 425 namely cases, that met the clinical case definition of EVD, were identified and confirmed (with ELISA and PCR). This outbreak resulted in a total of 224 deaths.

The survived patients were classified as survivors and have been routinely screened since their recovery [132].



Figure 3.1. Ebola virus outbreak in 2000-2001 in Uganda. Adapted from C. H. Calisher et al. copyright 2008 [64].

3.1.2. Ebola clinical features

Based on close observations of acute human cases, EVD infections are one of the most severe type of VHF [124]. In general, the onset of infection is insidious, as the initial symptoms may resemble a cold or an infection by influenza virus. It then rapidly deteriorates into hemorrhaging from multiple body orifices and usually leads to death of the infected individual [133]. Figure 3.2 describes the course of the EVD infection in human. In the beginning the incubation period takes up to 21 days, with the majority of cases becoming symptomatic within 5 to 12 days following this incubation period, and death of

the patient usually occurring 7 to 14 days, after the onset of symptoms [134] [135].

In nonfatal cases, infected patients have fever for about 5 to 9 days and improvement typically occurs after 7 to 11 days, at about the same time the humoral antibody response becomes detectable [69]. Fatal cases develop EVD signs and symptoms relatively early during the infection, and death typically occurs between days 6 and 16, as a result of hemorrhage and hypovolemic shock [69].

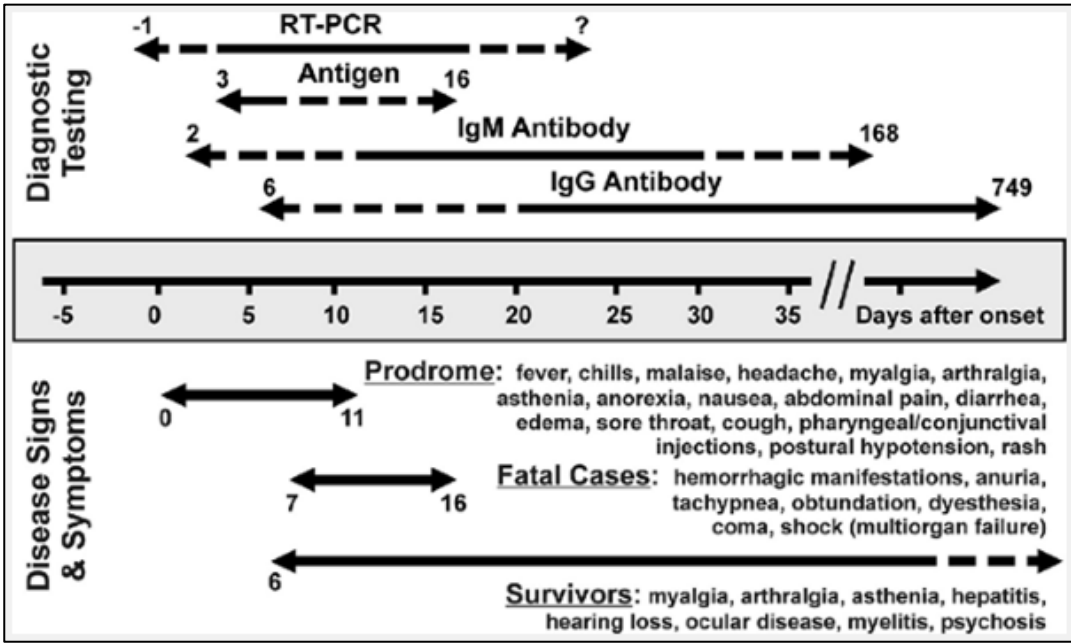


Figure 3.2. Schematic graph representing EVD time frames of disease onset and development of disease signs and symptoms associated with severe EVD infections (bottom) and the corresponding diagnosis assays (top). Dashed lines indicate approximate times where clinical features of disease or assay targets can be negative/absent or difficult to detect. Adapted from J. H. Kuhn et al. [64] copyright 2008.

3.2. Material and Methods

3.2.1. Ethics statement

The study was approved by the Helsinki committees of the Uganda Virus Research Institute in Entebbe, Uganda (reference number GC/127/13/01/15),

Soroka Hospital, Beer-sheva, Israel (protocol number 0263-13-SOR), presented in Appendix B, and the Ugandan National Council for Science and Technology (UNCST) (registration number HS1332). Written informed consent as well as a personal health questionnaire was completed for each subject.

3.2.2. Samples collection

A total of 128 tested serum samples, obtained from survivors of the 2000-2001, 2007 and 2014 EVD outbreaks in Uganda and West Africa. Subjects included confirmed survivors, according to patients PCR and ELISA results, and non-infected healthy local community members that were not infected[132]. The collected samples were divided into two groups; survivors, who were infected with the virus during the outbreak, and controls obtained healthy volunteers who were not infected with the virus. All samples were gamma irradiated.

3.2.3. Cloning and expression and purification of recombinant viral proteins

Purified recombinant His-tagged (His₆) GP₁₋₆₄₉ viral polypeptide (lacking the transmembrane domain) of SUDV, EBOV and BDBV, was prepared as previously described[111]. Other viral proteins, VP40 and NP were produced as previously described in section 2.2.1.

3.2.4. Lateral flow immunochromatographic strip test

3.2.4.1. Gold nanoparticles conjugation

Citrate coated AuNPs of 40 nm diameters were purchased from BBI Solutions, UK. Different AuNPs conjugates were prepared with secondary antibodies, including anti-human IgG (FC specific), anti-rabbit IgG (FC specific) and anti-mouse IgG (FC specific) (Sigma Aldrich). The AuNPs conjugation to the

secondary antibodies was performed as described in section 2.2.5.1. The functional AuNPs were used at a concentration that corresponded to 7 OD at a wavelength of 540 nm.

3.2.4.2. Strip assembly

All materials for the lateral flow assays were obtained from Millipore (Billerica, USA). Specifically, sample and absorbent pads (CFSP001700), conjugation pads (GFCP00080000), detection pad nitrocellulose membrane (SHF2400425) and backing card (HF000MC100) were used. Test and control lines were spotted using a microspotter (Microdrop, autodrop system AD-P-800). All spotted viral GP₁₋₆₄₉ proteins were diluted with water to final concentration of 1 mg/ml. The initial concentration of VP40 and NP, 0.2 and 0.4 mg/ml, respectively, was adjusted to 1 mg/ml with BSA. The control lines of the strips used for human samples testing were spotted with 1 mg/ml (in water) human IgG antibodies from human serum, purchased from Sigma. The control lines of the nonhuman strips were spotted with a 1:1 mix of rabbit and mouse IgG antibodies were at a final concentration of 1 mg/ml. The minimum distance between spotted lines was set to be 2 mm using the 'inflight mode' of the microspotter. Finally, 3 mm single lateral flow strips were cut with paper guillotine.

3.2.5. Testing nonhuman antibodies in spiked serum

Monoclonal IgG antibodies anti-SUDV-GP₁₋₆₄₉, EBOV-GP₁₋₆₄, 3C10 and 6D8 respectively, were produced in mice as previously described [43]. Rabbit polyclonal IgG antibodies against BDBV-GP₁₋₆₄₉ were purchased from Ibt Bioservices (0304-001). Monoclonal anti-poly Histidine antibody produced in mice was purchased from Sigma Aldrich and used as positive control (dilution 1:1000). A range of concentrations of each antibody was tested in the following way. Each antibody was diluted to a range of 100 and 50,000 dilution range in either running buffer or whole mouse serum purchased from Sigma. The running buffer was prepared in PBS pH 7.2, with 0.1% Tween-20 and 5% w/v

BSA. A 10 μ l of the antibody solution in either running buffer or serum was mixed with 10 μ l functionalised AuNPs and added onto the sample pad of the lateral flow. After 5 minutes, the strips were washed with 10 μ l running buffer. Then, the absorbance pad was removed, to prevent an increase in the background noise and the strips were quantified in RTLI units using the smartphone.

3.2.6. Human sera analysis with lateral flow strip

Two groups of samples were tested via lateral flow test strips: 1) samples that were previously collected and characterised, and 2) newly collected and tested fresh within 24 h upon collection. Samples from the first group were stored at -80 °C and fully thawed prior to use. In the second group, blood samples were collected into serum-separating tubes. The tubes were centrifuged at 2500 g for 10 minutes at room temperature.

The serum was collected into cryovials. A total sera volume of 15 μ l was mixed with 5 μ l of running buffer consisting of PBS pH 7.2, 0.1% v/v Tween-20 and 5% w/v BSA. The sample mixture was added at the end of the nitrocellulose membrane. 10 μ l of running buffer was added after 2 minutes. Then, 10 μ l of AuNPs labeled with anti-human antibodies were added to the strip, and the mixture migrated along the strip membrane to the test line, which bound and immobilised the gold conjugated capture antibodies. After 5 minutes, an additional wash was performed for 5 minutes. The results were quantified using the smartphone application within 15 minutes. A positive result, following the accumulation of complex (human specific IgG bound to AuNPs conjugated to secondary antibody), was demonstrated by the appearance of a red/purple color at the test and control lines. Coloring of the control line only signified a negative test result. To test the signal readout between the different collections by years between frozen and fresh samples, a one-way ANOVA was conducted using the Bonferroni means comparison test and showed no significant difference between the years at a p -value < 0.05.

3.2.7. Development of the next generation smartphone application

The developed smartphone application used the camera to capture a photograph of the lateral flow strip and to extract the intensity of the test-line. The application (app) was written for the Android operating system using the Eclipse Integrated Development Environment 4.5.0 with Android Development Toolkit 23.0.7.2120684. Following the patient details fill out, the app opens a camera activity, which uses the OpenCV 2.4.9 library. The camera class was based on the OpenCV Camera Control Example, using JavaCameraView, with red lines overlaid to aid strip alignment. A 4x digital zoom was used to further aid user alignment. The automatic continuous picture focus mode was used. When aligned, the user taps the screen to capture the image, which is then cropped to the region of interest (ROI): the analytical zone of the lateral flow test strip. The green channel of the ROI was averaged across the width of the strip to reduce noise, producing an intensity line profile along the length of the strip. The open source library opencv was used to read .csv files.

3.2.8. Human IgG ELISA

Two formats of ELISA were utilised for the analysis of the sera samples obtained from survivors and controls. Chemiluminescence ELISA was used to test samples collected at 2001, 2003 and 2012. Colorimetric ELISA was used for the analysis of the samples collected in 2015.

3.2.8.1. Chemiluminescence ELISA

The serum samples were analyzed using a standard ELISA protocol, following literature protocols [111], with minor adjustments. A final concentration of 2 µg/ml in PBS pH 7.4 in a volume of 100 µl/well of the recombinant and purified viral protein was pipetted into each well of a 96-well microtiter plate (MaxiSorp, Nunc). The plate was covered and incubated overnight at 4°C. The plates were then washed with 200 µl of PBST (PBS (Sigma P4417) with 0.05% (w/v) Tween-20 (Sigma P7949), pH 7.4. Next, 200 µl/well of blocking solution of 10% (w/v) skimmed milk was dissolved in PBST buffer,

added incubated for 1 h at 37 °C. Incubation plates were washed three times using PBST. Next, 100 µl of diluted 1:400 sera was added and incubated for 1 hour at 37 °C along with 1:1000 dilution of monoclonal antibody for the positive control. Next, plates were washed three times with PBST and 100 µl/well of secondary antibody conjugated to HRP (diluted 1:5000) was added. After a 1 hour incubation at 37 °C, plates were washed three times with PBST prior to the reading step. Oxidising reagent and enhanced Luminol reagent solutions were inserted into the wells in 1:1 ratio to reach a total volume of 80 µl/well. Plates were read using a standard luminometer (Thermolabsystems-Luminoskac Ascent).

3.2.8.2. Colorimetric ELISA

The procedure of the colorimetric ELISA is similar to section 2.2.8.1 with a minor modification in the final step in which a total of 50 µl/well of one step TMB was added and incubated for 10 minutes in room temperature in the dark. Then 10% H₂SO₄ was prepared and 50 µl/well added to stop the reaction. Plates were read using a standard absorbance measurement at 650 nm using Labtec Anthos Microplate reader.

3.2.9. Data analysis

The developed application was operated using Samsung Galaxy S-4 smartphone model to quantify the lateral flow test line. The resulting relative test line intensity was obtained by subtracting the average intensity of the test line from the average intensity of the bare membrane. The serum ELISA result is presented as a positive percentage (%PP), obtained as the normalised raw result divided by the positive control that contained antibodies against GP₁₋₆₄₉, 3C10, 6D8 and anti-BDBV. For both lateral flow and ELISA, the cut-off was established based on the average of the 27 tested non-infected controls with addition of three times the standard deviation. Based on this cut-off, the tested survivors samples were categorised as positive and negative. To test the signal readout between the different collections by years between frozen and fresh samples, a one-way ANOVA was conducted using the Bonferroni means comparison test

and showed no significant difference between the years at a p -value < 0.05 .

3.2. Results and discussion

3.3.1. Presentation of the smartphone-based lateral flow for human samples

The recently developed lateral flow assays for EVD detect either the whole viral antigen or specific viral proteins. Hence, in these assays antibodies are presented on the test lines [40]. In contrast, the lateral flow strips developed in this thesis and described in this section have recombinant viral proteins printed on the test lines of the strip, schematically presented in Figure 3.3B. Targeting this assay for a specific assessment of immune response of survivors to these viral proteins.

In Figure 3.3A, the general EVD viral infection course in human is presented. The whole cycle normally lasts up to 21 days [64], and the acute stage is symptomatic and corresponds to the viral replication period (Viremia). Within 6-7 days of the offset, the host's immune response is expected to produce specific IgG and IgM antibodies against the viral infection. The developed assay herein was targeted for the antibody response period and the post-acute stage.

The lateral flow strip test was developed in single (monoplex) and multiple (multiplex) analyte formats (Figure 3.3B) using purified recombinant viral proteins as recognition entities on the test lines. Using such recombinant proteins is an advantage for diagnostics assays as they can be produced in bulk with high purity, and remain antigenic even after several freeze-thaw cycles, as characterised in Chapter 2. Utilising these viral recombinant proteins enabled mimicking the virus immunogenicity without the safety concerns of working with highly pathogenic agents. The monoplex strip consisted of a single test line spotted with SUDV-GP₁₋₆₄₉ while the multiplex assay was developed in two configurations: for detection of multiple proteins of a single species, and a multi-species 'multiplex' assay to differentiate between several antibodies against different EVD GP₁₋₆₄₉ species (i.e. SUDV, EBOV and BDBV) by immobilising the glycoprotein GP₁₋₆₄₉ of each species onto the strip in separate test lines. Lateral flow multiplex assays configuration using different sequence combinations of

viral proteins of species showed no significant differences in assay performance (data not shown).

Antibody-functionalised AuNPs were used to provide visual colour change of the test lines on successful capture of the target antibodies. These AuNP were chosen due to their ready visual detection, cost-effectiveness, stability, robustness, and simple conjugation to secondary antibodies [13]. The optimisation of the lateral flow strip was described in Chapter 2, included testing different sized AuNPs, secondary antibodies and membrane configurations, as well as antigen and antibody calibration. A 40 nm AuNPs, anti-human FC secondary antibody as well as BSA blocking agent were utilised to assembly the optimised strip for a this clinical study. In addition, the prepared strips were compatible for use up to 16 weeks postproduction, when kept in low humidity and at room temperature, without showing degradation in assay performance. The operation time of the test required a total of 15 minutes.

All tested samples were analysed with the smartphone app, which performance described in section 3.3.2. In this chapter, the interface of the smartphone app was improved and presented in Figure 3.3C. This interface included a more intuitive log-in page that recorded patient's details, identification code, age, gender, number of years since recovered from EVD and selection option for the test protein (GP₁₋₆₄₉, VP40 and NP). The location was automatically synchronised with the GPS signal of the phone. In addition, the properties of the focus were also improved to enable faster and more accurate capture of the strip. Finally, cut off for the test were evaluated (described later in this chapter) and were included in the app to provide quantitative analysis within the detectable scale, as shown in the bottom of Figure 3.3C.

The strip quantification method of the app is described in this section. Briefly, each image of the test zone was converted into an intensity plot corresponding to the colour intensity of the test and control lines, as presented in Figure 3.4A. The average value of the intensity across the test line was proportional to the amount of analyte in the sample. The baseline of the intensity plot was taken as the background signal (i.e. area with no test/control line), to which the value of intensity of the test line was normalised. Therefore, the resulting value was reported in in relative test line intensity (RTL_I) units.

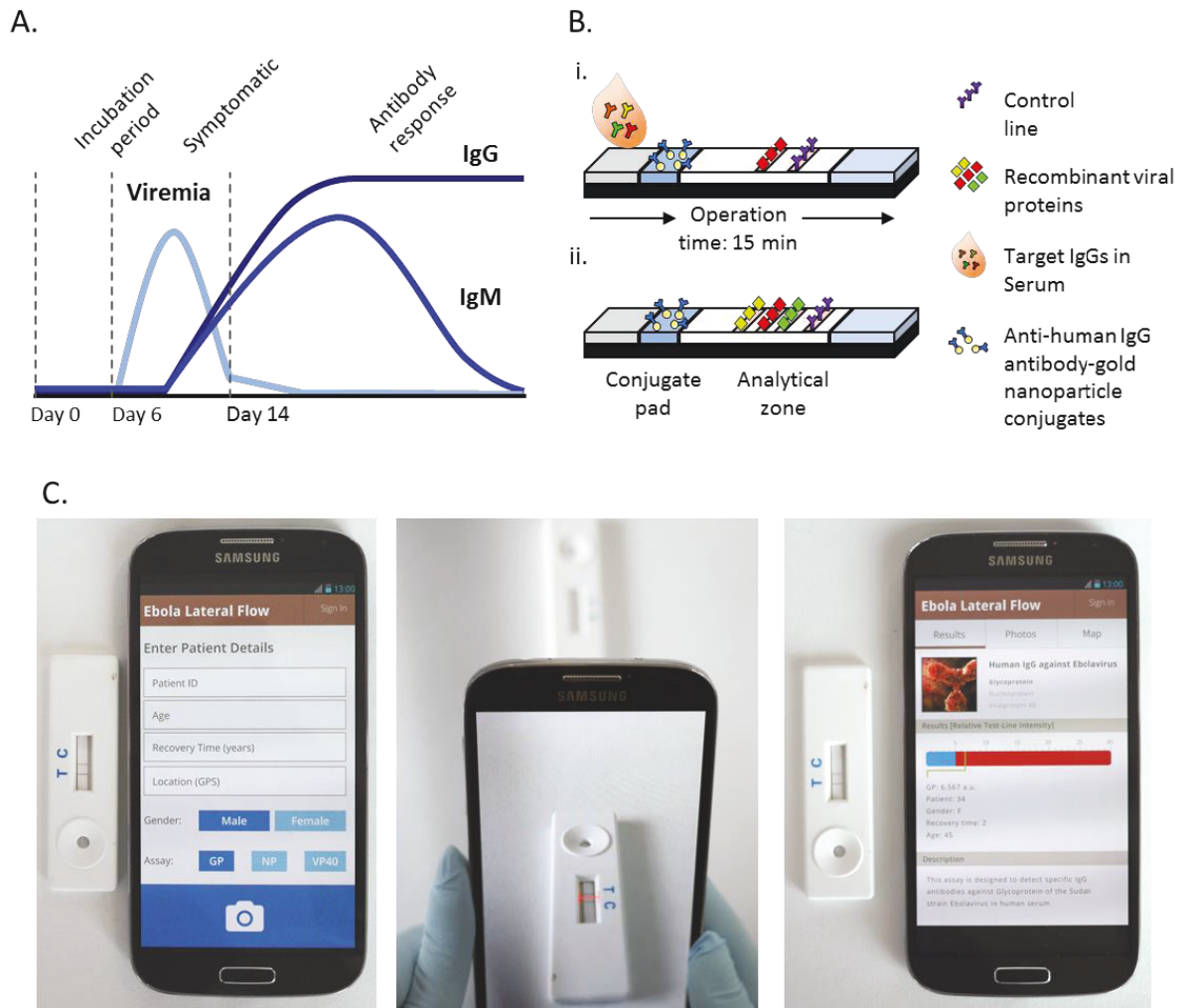


Figure 3.3. Host response, assay and smartphone app. A. illustration of a survival immune response to EVD, indicating IgG, IgM and viral levels during and post infection. B. Lateral flow strip illustration: serum is applied onto the sample pad, migrating through the analytical area and forming complexes between the labelled AuNPs and the target analytes. Targeted IgG serum antibodies against single or multiple recombinant Ebola viral proteins bind to pre-printed test lines, forming a visual red-purple line. A control line to validate assay function is used for the detection of anti-human antibody-AuNP conjugates. Visualisation of assay results is obtained after 15 minutes. C. Schematic smartphone application interface, from left to right; Login window to record patient details, including Patient ID, Age, Recovery time (years), Location, Gender and assay selection (GP, NP, VP40). Analysis window, once red box is aligned between the test and control lines, a tap on the screen provides strip analysis. Result summary window, which presents the RTLI and classifies the sample as positive or negative on a range scale and cut-off depending on the assay. The window also provides a summary of patient details, ID, gender, recovery time and age, and a description of the test taken (e.g. this assay is designed to detect IgG against GP SUDV).

Additionally to Chapter 2, the performance of the assay was compared between pure buffers and spiked serum as presented in Figure 3.4B. A high similarity between dose-response curves was obtained in both buffer and serum, validating that the assay can be operated in serum conditions. Furthermore, the specificity of the assay was investigated using monoclonal specific antibodies against the different GP₁₋₆₄₉ Ebola virus species and demonstrated high selectivity performance. As shown in Figure 3.4C, the non-specific affinity of 6D8, a monoclonal antibody against EBOV GP₁₋₆₄₉, to SUDV GP₁₋₆₄₉ is significantly lower than 3C10, a monoclonal antibody against SUDV GP₁₋₆₄₉, suggesting high assay specificity. The assay limit of detection was determined in Chapter 2 to be in a range of 200-500 ng/ml for the three different GP₁₋₆₄₉. Also in Chapter 2 different volumes of sera were applied to assess the visual appearance of the test line and a total of 15 µl of serum was required to produce a sufficient RTLI signal within a 15 minutes operation time.

3.3.2. Adjusting the smartphone application for testing real samples under field conditions

The promising potential of smartphone use in healthcare has been recognised by research, especially as readers of optical biosensors, replacing expensive and highly specialised systems [126]. In this chapter, quantitative analysis of the lateral flow strips was performed using a specially developed smartphone app. Compared to a standard lateral flow reader, the developed smartphone app provides additional functionalities such as real-time geological tagging of the tested samples, produces a surveillance map, and provides a ready means of data sharing.

The login page of the final version of the developed app included a form for entering patient details.

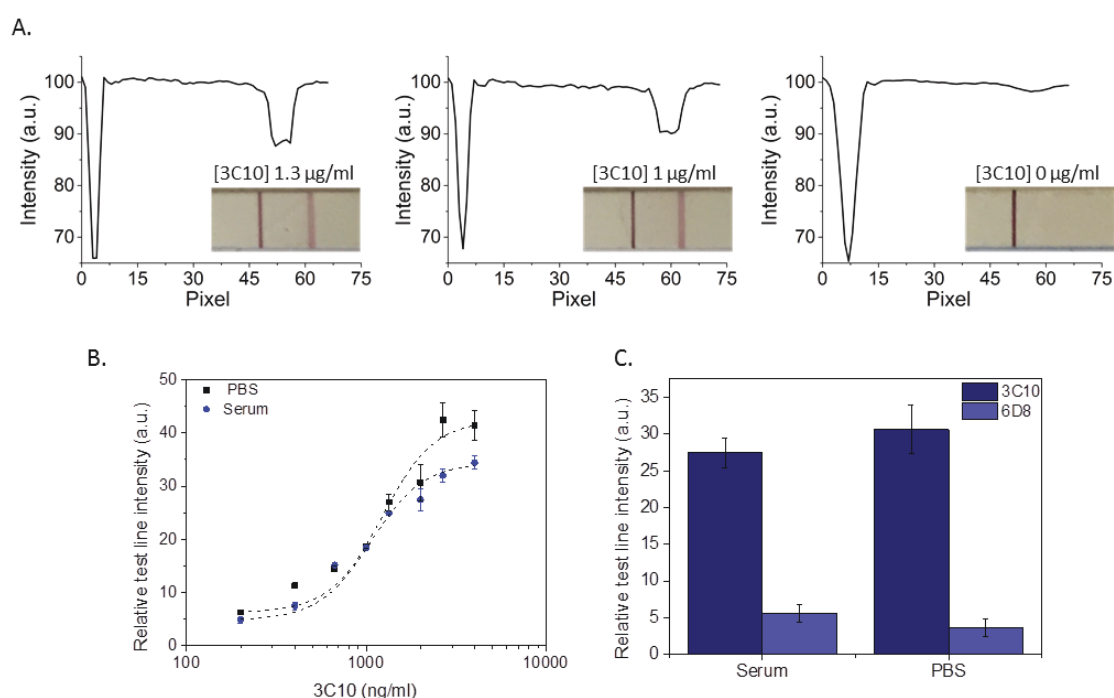


Figure 3.4. Assay development. A. Assay strips and corresponding raw intensity plots quantified by the mobile phone app. B. Dose-response curves for 3C10 obtained in phosphate buffer and serum quantified and normalised by the smartphone application. The series of results was plotted and fitted to a sigmoidal curve ($r^2= 0.960, 0.967$). Bars indicate mean $\pm$ standard deviation ($n=2$). C. Selectivity of SUDV-GP lateral flow, testing 3C10 and 6D8 ($2 \mu\text{g/ml}$) in spiked serum and in phosphate buffer ($n=2$).

The app used the Google Play Services Location API (application program interface) to ask for the most recent location and begin location updates automatically every 10 second with high accuracy. Once patient details are submitted the camera is activated with a red cross to aid with the strip alignment. The user (preferably a front line health worker) taps the screen to capture the image, which is then cropped to the region of interest and an intensity profile is produced. This profile is divided in half for the control line and test line areas, and the size of the peaks in each area quantified in RTLI units. The line profile, peak sizes, user data input and location are stored in a .csv file of size 2.6 KB. This patient information can then be read and displayed on a map using the Google Maps Android API, as presented in Figure 2.14 and Figure

3.5. The produced surveillance map correlates between the result of the test and the geographical location from where it was obtained. The testing can be performed on several devices in real-time screening and in different locations.

All serum samples screened using our lateral flow tests were quantified in RTLI units, directly proportional to IgG antibody levels and relative to the intensity-visualised signal. The strip analysis process included normalisation of the analytical zone against the background of the strip and therefore making the RTLI readout independent from the intensity and the lighting ambient.

3.3.3. Evaluation of SUDV monoplex assay with human sera

The development of the monoplex lateral flow assay was performed for SUDV as a viral subtype model. The key reason for selecting SUDV viral species and the 2000-1 Gulu outbreak in Uganda was its relatively lower mortality rate of around 50% compare to the 90% of case fatality from the EBOV [69]. As such, the 2000-1 Gulu outbreak yielded a relatively large survivors population, which have been monitored and studied over the years [111], [112].

Out of the seven structural viral proteins of the Ebola virus, the viral GP was selected as the monoplex recognition element. Previous research shown that the GP is responsible for the majority of human IgG responses against the virus, as well as the basis for most currently developed vaccines [83].

The SUDV monoplex assay was validated by testing 123 serum samples out of which 91 were obtained from SUDV survivors of the 2000-1 Gulu outbreak in Uganda and 32 from non-infected controls collected in 2001, 2003, 2012 and 2015.

Table 3.1 summarises the tested samples classified by their year of collection. Sera screening validation was performed using two sets of groups, referred to as *known* and *unknown* sample groups.

The *known* sample group comprised samples collected in 2001, 2003 and 2012, which have been previously analysed and published by A. Sobarzo *et al* [137].

The *unknown* group of samples were collected at 2015 and were tested fresh on-site within 24 hours upon collection. Using either thawed or fresh

serum samples showed no significant difference in the performance of the developed assay (ANOVA p -value < 0.05).

Non-infected controls group were key components for the calibration of the positive and negative discrimination cut-off for both lateral flow and IgG gold standard ELISA. A total of 27 sera samples were used to set the cut-off of our assay, which were randomly selected from non-infected controls obtained in the 2001, 2003, 2012 and 2015 collections. These non-infected EVD negative sera samples were obtained from local individual volunteers, which were either EVD survivor family related or had no close contact with any Ebola survivors (all samples were tested negative by standard ELISA and PCR methods, and clinically showed no symptoms Ebola virus infection) [111].

The importance for the selection of this group of individuals for the determination of the cut-off value was a key component for the establishment of the specificity and sensitivity of our assay, since they represented the native condition of our target population.

All samples *known* and *unknown* tested in this chapter were also tested by standard ELISA for reference analysis.

The summary of results from screening of the *known* and *unknown* survivors groups is presented in Figure 3.5A and Table 3.1.

Out of the 91 survivors tested, regardless of the year of collection, 65 were determined to be positive to IgG antibodies against SUDV GP₁₋₆₄₉. These results were in line with previous studies and ELISA analysis. The fact that not all survivors tested positive demonstrated that some long-recovered SUDV survivors can maintain different levels of IgG humoral immunity against SUDV GP₁₋₆₄₉, whereas others demonstrate a complete lack of memory immunity [82].

Analysis of the results by collection year showed positive recognition of 14 out of 21 survivors' samples collected in 2001, 19 out of 25 from 2003, 12 out of 18 from 2012, and 18 out of 26 from 2015. All 32 non-infected control samples were also analysed and tested negative to immune recognition response against SUDV GP₁₋₆₄₉. An example of five the lateral flow strips is presented in Figure 3.5A.

Two formats of ELISA were utilised for reference analysis of sera samples. Chemiluminescence ELISA was used to test samples collected at 2001,

2003 and 2012. This format of ELISA was utilised to follow the analysis protocol previously performed on these samples by A. Sobarzo *et al*[82], [137]. Colorimetric ELISA was used for the analysis of the samples collected in 2015.

Samples collected during the 2015 collection were analysed on-site with the developed lateral flow assay in their fresh. To maintain these similar setting of the samples (without freezing and storing them) the ELISA was performed in the facilities of Uganda Virus Research Institute and in collaboration with J.J. Lotwanna. Due to absence of luminescent reader, colorimetric ELISA analysis was carried out for the analysis of these 33 samples.

The box plot analysis was performed on the tested samples with the developed lateral flow method and the chemiluminescence ELISA and presented in Figure 3.6. In this analysis samples from the collection of 2001, 2003 and 2012 were used. Fresh samples obtained during the 2015 collection were not included as these were tested with a colorimetric ELISA.

This result showed comparable distribution of the signal readout for both analytical methods strengthening the robustness of the developed assay.

Table 3.1. Summary of serum screening results of SUDV-GP₁₋₆₄₉ monoplex lateral flow assay of SUDV survivors and non-infected controls.

Year of Collection	Survivors¹		Controls²	Total
	Positive	Negative	Negative	
2001	14	7	16	37
2003	19	6	5	30
2012	12	7	6	25
2015	18	8	5	31
Total	65	26	32	123

¹ Previously infected and diagnosed EVD patients from the 2000-2001 SUDV outbreak in Uganda.

² Non-infected controls obtained from closely related (family, close contact) and non-related EVD survivors volunteers.

Comparison between the analysis of 123 samples with SUDV monoplex assay and GP₁₋₆₄₉ IgG colorimetric and chemiluminescence ELISA yielded with a correlation of 97% between the two methods. The SUDV monoplex demonstrated 96% sensitivity and 100% specificity in comparison to ELISA. There was no significant difference (ANOVA p -value < 0.05) in detection ability between the different collection years as shown in Figure 3.5B. Data analysis presented in Figure 3.6 shows the surveillance map that was generated during the 2015 sample collection field study in Uganda, where each entry corresponds to the location of the tested patient and the test result of the assay (positive or negative). The color intensity of the red pins correspond to the relative quantitative immune response to GP₁₋₆₄₉ of each tested individual, resulting within the range of 5.8 to 40 RTLI. The zoomed in map represents individuals that were tested at the same geographical location. Finally the spacing between these samples was produced artificially to display the information of each tested individual.

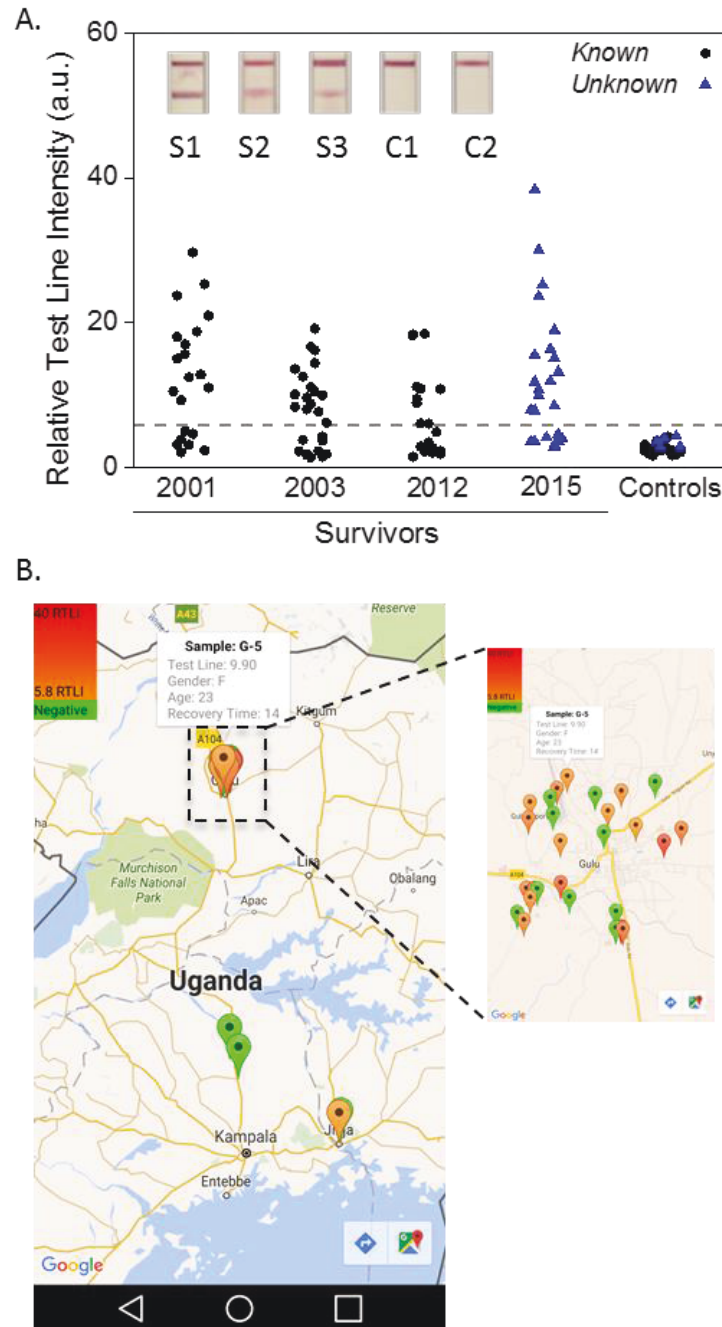


Figure 3.5. Monoplex SUDV-GP₁₋₆₄₉ lateral flow test. A. A summary of analysed sera for the detection of IgG response to SUDV GP₁₋₆₄₉ in survivors, grouped by collection year, and controls of non-infected local individuals. Known (black-circle) and unknown (blue-triangle) have been on-site collected and evaluated with strips. The dashed line represents the cut-off of the assay calculated from the average value of the 27 controls plus three times the standard deviations. In the insert, Lateral flow strips of three survivors (S1-3) and two non-infected controls (C1-2). B. Map generated by on-site testing of survivors of the 2015 collection in Uganda, red corresponds to positive and green negative. A tap on each entry displays patient details.

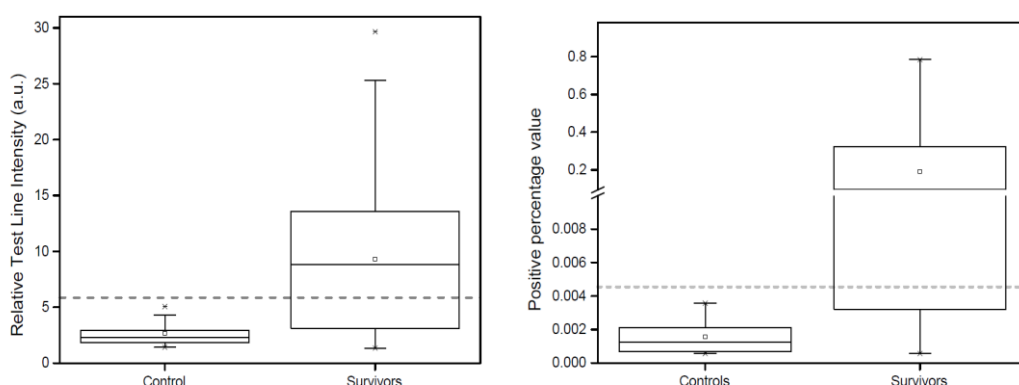


Figure 3.6. Samples readout comparison by ELISA and mono-SUDV lateral flow. Box plots of the lateral flow (left) showing the 25th, median, and 75th percentile for the known survivors and non-infected controls samples. Out of 60 analyzed survivors, 40 tested positive and all 27 controls tested negative (signal within the level of the background noise). Total of whiskers are drawn showing the 10th and 90th percentile, and the open squares indicate the mean lateral flow signal in each set, and dashed line represents the cut-offs. Box plot of ELISA (right) of the known samples, the signal presented in positive percentage. 41 out of 60 samples tested positive, all controls tested negative.

3.3.4. Evaluation of the SUDV multiplex assay with human sera

A multiplexed lateral flow format using SUDV viral proteins was developed for detection of IgG antibodies against VP40, GP₁₋₆₄₉ and NP. These viral proteins were selected based on previous research, which demonstrated the ability to elicit a strong IgG humoral response in SUDV survivors from Gulu district, Uganda [111].

Detection of multiple analyte provides a better understanding of the biological process occur as a result to viral infection system produce. Therefore, simultaneous screening for broader profile of humoral response to the viral infection was the key incentive for developing the SUDV multiplex assay. Such an assay for the detection of immune response fingerprints could potentially increase the assay sensitivity and allow the identification of more infected or recovered EVD patients rather than the monoplex format. In addition, the combination of several viral proteins might better mimic the host immune response to the whole viral antigen as it accrued during native infection.

In the multiplex format, three test lines were spotted with the three recombinant viral proteins in the following sequence (from the sample pad): VP40, GP₁₋₆₄₉ then NP. A total of 15 samples of SUDV survivors and 5 non-infected controls from the 2015 collection were analysed. The incentive for selecting these 15 survivors was based on their validated expression of neutralising antibodies to SUDV. This was validated from the results based on flow cytometry analysis and viral plaque neutralisation test. These experiments were conducted in collaboration with a team from the USAMRIID. Hence, it was decided to analyse only survivors that present immune response to EVD and the aim of this study was to classify their EVD specific antibodies to VP40, GP₁₋₆₄₉ then NP.

Furthermore, these samples were previously tested using GP₁₋₆₄₉ SUDV monoplex lateral flow format, as well as individual recombinant protein and full antigen chemiluminescence ELISA. SUDV multiplex assay positive results were determined based on visual appearance of at least one test line. A summary of results from the SUDV multiplex, GP₁₋₆₄₉ SUDV monoplex, and full-inactivated SUDV antigen ELISA, are presented in Table 3.2. The results of SUDV-multiplex shown positive recognition of 13 out of the 15 survivors, SUDV-monoplex showed 9, out of which 9, 10 and 7 samples were tested positive for GP₁₋₆₄₉, NP and VP40, respectively. Furthermore, 4 survivors tested positive to three proteins, 5 tested positive to two (out of which 4 to GP and NP and 1 to NP and VP40) and the remaining 4, presented positive immune response to a single protein as presented in the Venn diagram in Figure 3.7.

ELISA analysis performed with full-inactivated antigen detected 8 survivors out of 15. 9 survivors were detected via SUDV monoplex, which was in line with the survivors tested positive to GP₁₋₆₄₉. All non-infected controls shown negative result in the three assays. In addition, all samples were tested via three different ELISA assays with a different viral protein and the semi-quantitative results are summarised in Table 3.3. The strength of the immune response was demonstrated semi-quantitatively as a single +, ++ and +++ that exhibited readout above the cut-off, double cut-off and triple cut-off, respectively. These results were in line with the SUDV multiplex assay.

Data analysis of the SUDV multiplex test showed different levels of immune response against different viral proteins between the tested survivors, as expected and previously described[137]. In addition, by screening the samples with the developed SUDV multiplex test, a higher number of survivors was identified in comparison to ELISA with whole viral antigen. The multiplex viral protein assay exhibited 100% specificity. The clinical sensitivity was determined as the positively tested survivors out of the total tested survivors. This analytical value was evaluated compared between the developed point-of-care analytical methods, SUDV-multiplex and monoplex, 86% and 60% respectively. This increase in sensitivity in this cohort study demonstrated that simultaneous analysis of multiple analytes could improve the detection rate of EVD survivors.

Concentration measurement of purified recombinant proteins is standard, whereas determining the amount of each actual viral protein in the whole virus is challenging. The key hypothesis of this section was that the amount of each recombinant viral protein on the strips might be higher than in the actual inactivated antigen (used in ELISA), leading to an improved detection ability in the developed assay systems.

Subsequently, monoplex lateral flow test strips with a single test line of each previously used viral protein, GP₁₋₆₄₉, NP and VP40, were prepared. A total of 7 different and randomly selected survivors and 5 controls were tested separately against each viral protein using quantitative monoplex lateral flow and ELISA. Test results were classified as positive and negative to IgG response to each protein separately (NP, GP¹⁻⁶⁴⁹ and VP40) with an independent calibrated cut-off for each protein. Data analysis between two methods demonstrated 100% correlation, comparing, presented in Figure 3.8B.

Table 3.2. Summary of serum screening results of SUDV multiplex and monoplex lateral assays and full antigen ELISA of survivors and non-infected controls.

Method	Survivors ¹ (n=15)		Controls ² (n=5)
	Positive	Negative	Negative
Multiplex assay ^a	13	2	5
Monoplex assay ^b	9	6	5
ELISA ^c	8	7	5

^a SUDV-multiplex lateral flow test strips spotted with VP40, GP₁₋₆₄₉ and NP viral proteins

^b SUDV-GP₁₋₆₄₉ lateral flow test strips

^c Full inactivated SUDV antigen ELISA

¹ Previously infected and diagnosed EVD patients from the 2000-1 SUDV outbreak in Uganda.

² Non-infected controls obtained from closely related (family, close contact) and non-related EVD survivors volunteers.

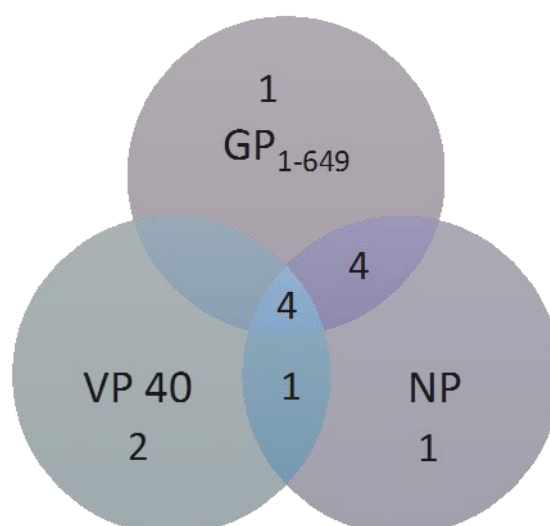


Figure 3.7. Venn diagram of the IgG immune-reactivity between SUDV recombinant proteins in human survivors detected via the developed SUDV-multiplex lateral flow test strip. Out of 15 tested survivors 9, 10 and 7 tested positive to IgG response against GP₁₋₆₄₉, NP and VP40, respectively.

*Table 3.3. ELISA serology screening of SUDV survivors** and controls** against multiple recombinant viral proteins, NP, GP₁₋₆₄₉ and VP40. Results are classified in a semi-qualitative approach for the presence of IgG antibodies against the tested protein.*

Sample	GP₁₋₆₄₉	NP	VP40
S1	+++	++	-
S2	+++	++	-
S3	-	+	++
S4	++	++	++
S5	+++	+++	+++
S6	+++	+	++
S7	+	+	+
S8	+++	++	-
S9	-	-	+
S10	-	-	-
S11	+++	++	-
S12	++	-	-
S13	-	-	+
S14	-	+	-
S15	-	-	-
C1	-	-	-
C2	-	-	-
C3	-	-	-
C4	-	-	-
C5	-	-	-

**Single + represents response above a single cut-off, ++ above double cut-off and +++ above triple cut-off.*

***15 samples were obtained from survivors (S.1-15) and 5 from non-infected controls (C.1-5) from the 2015 collection group*

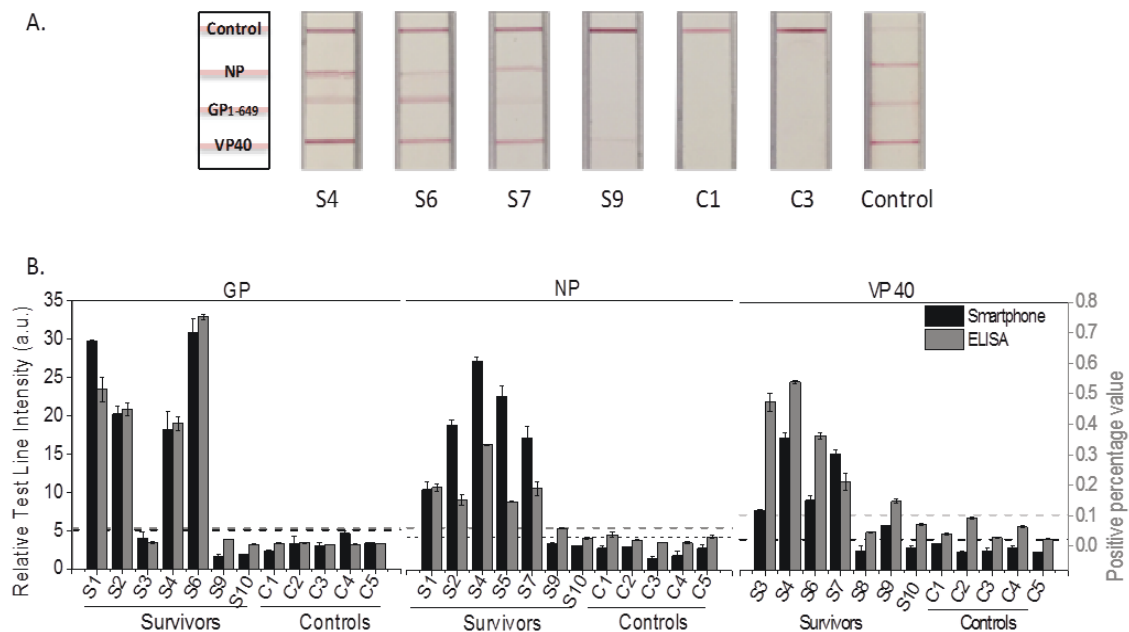


Figure 3.8. SUDV-multiplex test strip with three test lines for simultaneous detection of IgG response to SUDV EBO against VP40, GP and NP in sera. A. schematic presentation of the order of the spotted viral proteins upon ELF strip. Capture of tested sera obtained from survivors (S4, S6, S7, S9), non-infected controls (C1, C2) and monoclonal mouse anti-His₆ as a positive control. B-D. Quantitative analysis of sera samples using monoplex lateral flow with different viral proteins in each test (black) and ELISA (grey). B. Detection of IgG antibodies against viral GP, NP and VP40 resulted in 4, 5 and 5, respectively, out of 7 survivors tested positive and 5 controls tested negative. The multiple viral proteins analysis showed 100% correlation between monoplex lateral flow and ELISA. Black and grey dashed lines represent the ELF and ELISA cut-offs. The data was averaged over 2 measurements in triplicates for ELISA and error bars represent $\pm$ RTLI for results from ELF and $\pm$ %PP for ELISA (n=2). Samples numbering is independent from Figure 3.3A.

3.3.5. Species multiplex assay

Another multiplex configuration was developed and evaluated in this chapter. An EVD species multiplex test for the identification of the Ebola virus species that mainly affect humans, SUDV, BDBV and EBOV was developed using GP₁₋₆₄₉ viral proteins. To demonstrate the proof-of-concept of this assay, sera samples were tested from three survivors of 2000-1 SUDV outbreak in Gulu district, Uganda (S16, S20 and S21) two survivors from the 2007 BDBV (S17,

S19) outbreak in Bundibugyo district, Uganda, and one survivor from the 2014-5 EBOV outbreak in West Africa (S18). The results presented in Figure 3.9 showed that out of the six tested samples, three survivors demonstrated single Ebola virus species recognition (single test line), while the other three survivors were positive for two different species. Additionally, two non-infected control samples (C1, C2) were used as negative controls showed no recognition to any of the three viral subtypes as expected. These results were also confirmed via ELISA.

Interestingly, although S21 was reported as SUDV survivor, the immune response analysis revealed stronger immunity to BDBV species. This can be explained as S21 was originally from the Bundibugyo district and the sample was collected in 2014. Hence, this result implies that S21 might have been exposed to BVBD during the 2007-8 outbreak. Subclinical EVD infection is rare but possible and therefore S21 could be an example of such case [138].

The identification of multiple viral species within a single sample, as demonstrated in the developed EVD species-multiplex assay, might be a result of cross-reactivate recognition between the viral species, as previously reported [139] and will be further explained in Chapter 4. Indeed, it is clear that the importance of Ebola virus species differentiation is critical for the identification of previously unknown pre-and subclinical infection of emerging areas of the disease.

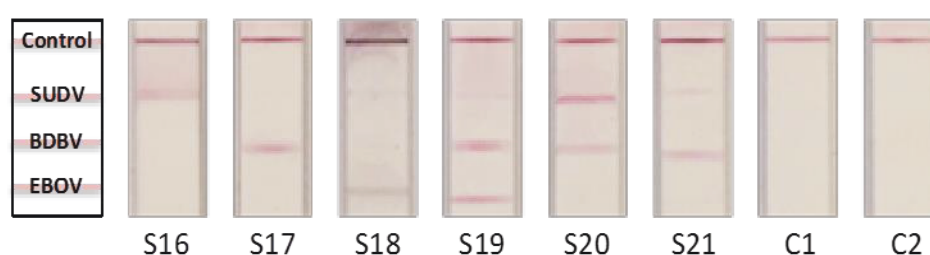


Figure 3.9. Photo of the tested samples with the EVD species multiplex lateral flow test strips for identification of the immune response against a specific EVD species. Each test line represents the detection of IgG response to specific viral species, control line spotted with human IgG and AuNPs conjugated to anti-human antibodies. Six samples (S16, S17, S18, S19, S20 and S21) obtained from survivors infected with three different species of EVD and two controls (C1 and C2) from non-infected individuals. Samples numbering is independent from Figure 3.3A.

3.4. Conclusions

The latest Ebola virus outbreak exhibited how the disease went from a rare, local and rural disease into a highly virulent worldwide threat. As a response to this outbreak, international organisations have encouraged the release to market of point-of-care diagnostic testing to improve epidemic control and patient management [108]. The standard approach for the diagnosis of Ebola virus combines both detection of viral genetic signatures as an early stage diagnostics (via either PCR or direct antigen detection) and detection of the humeral response via serology testing for treatment and surveillance monitoring [140].

In the light of increasing frequency and the magnitude of EVD outbreaks in the recent years, several point of care assays have been launched. These assay were targeted for detecting EVD viral antigen either through viral gene expression [97] [136] or direct antigen [102]. However, serology is still performed with standard ELISA within lab-based facilities. As most Ebola virus cases occur in rural areas, simple and portable tests, with a comparable performance to standard lab-based immunoassays, for the detection of the hosts' immune response is a key component for disease management [142].

This chapter describes the validation of a novel approach for detection of human humoral response to Ebola virus. The developed test is a smartphone-based lateral flow assay for detection of IgG specific antibodies in human SUDV survivors. In the first stage of the assay validation and evaluation, a total of 91 SUDV survivors and 32 non-infected controls were tested by using the monoplex format, out of which 65 survivors demonstrated positive IgG immune response, while all 32 negative controls showed no immunoreactivity.

The results of the assay are obtained within 15 minutes with 100% and 96% specificity and sensitivity when compared to standard ELISA. In addition, the on-site screening of the *unknown* samples demonstrated the ability of the developed system to be used as a point-of-care test.

In the second stage, a total of 15 SUDV survivors and 5 non-infected controls were tested with the SUDV multiplex assay. Detection of IgG immune response against multiple viral proteins during the viral infection may indicate

the stage of recovery from the disease and transfer from the acute to the post-acute stage. Furthermore, broader profiles of IgG antibodies might also reflect on the immunity and the neutralisation capability of an individual survivor [137], which could correlate with protection against second viral exposure. Such simultaneous multiple IgG tests are more complex and challenging with a standard ELISA, which require longer operation, trained personnel, and usually cannot be performed on-site.

The SUDV multiplex assay yielded the detection of IgG of a profile immune response in over 86% of the tested survivor, while all negative controls showed no immunoreactivity to the three tested viral proteins. This was significantly higher to the 60% survivors detection via the SUDV monoplex format. In addition, a proof of concept of the species multiplex assay was demonstrated by identifying the infecting EVD species of 6 survivors from SUDV, BDBV and EBOV. The species multiplex is especially useful for the identification of subclinical EVD infection, as demonstrated. Moreover, in this chapter the performance of the developed smartphone app was validated as a lateral flow reader alongside its connectivity and data communication features. The system enables portable and simple strip quantification and it facilitates ready data storage by sharing and real-time surveillance mapping. We envision that this feature in particular could provide a surveillance tool for rapid epidemiological studies of the disease spread at times of critical need such as demonstrated by the recent Ebola outbreaks in west Africa and Uganda [107].

The important benefits of this study include the possibility for real-time screening of populations in the infected areas, increased understanding of viral spread patterns with potentially assisting reservoir tracking and the source of infection. The Ebola virus point-of-care serology testing, that our assay system is designed to facilitate, can improve the efficiency of patient screening during acute and post recovery stages. Having a system for detection of human IgG antibodies against Ebola virus glycoprotein is also especially advantageous for vaccine development, which has increased dramatically in the wake of the 2014 outbreak in West Africa. Tremendous efforts are being made to fast track the development of vaccines based on immunisation against Ebola glycoprotein [83] [77].

The immunogenicity of these vaccines is measured using antibodies from antisera by ELISA, neutralisation assays for IgG response and intracellular cytokine staining for T-cell immune response [143]. Thus, point-of-care detection of IgG immune response against Ebola virus GP will potentially provide better evaluation of vaccine efficiency under outbreak condition. Furthermore, serological tests based on engineered recombinant viral proteins, as presented in this thesis, present the following advantages: lower costs, bulk production and clearance for use in biological safety facilities (BSL-2), which are more available in remote and rural areas. In contrast, tests involving complete antigen detection require advanced BSL-4, which there are very few in Africa [144].

The motivation behind this chapter was to address the critical need for faster, simpler and more portable serology tools for Ebola virus. The developed simple and portable lateral flow-based assay system has demonstrated the ability to detect IgG antibodies in Ebola survivors with high sensitivity and specificity under field conditions. Further work is needed to evaluate specificity and sensitivity in larger cohort groups, in vaccine evaluation studies and further work for shelf life and environmental condition are required. Nevertheless, the wake of the latest EVD outbreak in West Africa emphasised the unmet need for POC technologies for monitoring, diagnosis and prognosis. Indeed, such technologies are crucial for better control of the disease spread and patient management.

Chapter 4

Bundibugyo Ebola Virus Serological Screening and Viral Cross Reactivity

4.1 Introduction

4.1.1. Scope of the chapter

In this chapter, analysis of sera samples obtained from survivors Bundibugyo district in Uganda was described. These samples were obtained from survivors of the BDBV and non-infected controls. The analysis of these samples was produced with the smartphone-based lateral flow BDBV monoplex and ELISA, as reference analysis. The chapter also demonstrates the robustness of developed assay to be utilised for a different clinical study. Also to demonstrate the simplicity in translating the previously presented system to detecting IgG antibodies against BDBV species in the serum of previously infected human patients. During this work a BDBV human sera database was created using samples from previously infected patients and non-infected controls from the same district in Uganda. Finally, the collected samples were screened via BDBV monoplex and species multiplex assays.

BDBV is considered to have the highest cross reactivity to other viral species, as reviewed in this chapter. Hence, it was imperative to demonstrate the ability to detect this cross reactivity via the developed species multiplex assay. Hence, this chapter describes cross reactivity of the BDBV survivors immune response to three viral species, BDBV, EBOV and SUDV. For this purpose three recombinant GP₁₋₆₄₉ from each species were used to assemble to multiplex assay. In this chapter, the immune cross-reactivity and cross-protection of EVD survivors is detected and also quantified via developed multiplex assay.

4.1.2. BDBV outbreaks review

BDBV species was discovered in late November 2007 in the rural cities of Bundibugyo and Kikyo in the Bundibugyo District, Western Uganda. Although the initial alert was sent to Uganda Ministry of Health regarding this outbreak in early 2007 it took nearly half a year to confirm it. This delay occurred due to a limited access to diagnosis laboratories [73], demonstrating a need for improved EVD surveillance, reporting, and diagnostics, in endemic locations across Africa. A total of 149 cases, and up to 37 deaths, were reported during the first outbreak [145]. The second BDBV outbreak occurred in 2012 in the Democratic Republic of Congo affecting around 40 people with 15 reported deaths [146]. Figure 4.1 demonstrates the geographical locations and the magnitude of the two BDBV outbreaks. In this chapter, nearly 20 survivors from the 2007-8 outbreak in Bundibugyo district, Uganda were identified and recruited for the study. Nonetheless, as a result of logistic issues, only 14 samples were collected for this study.

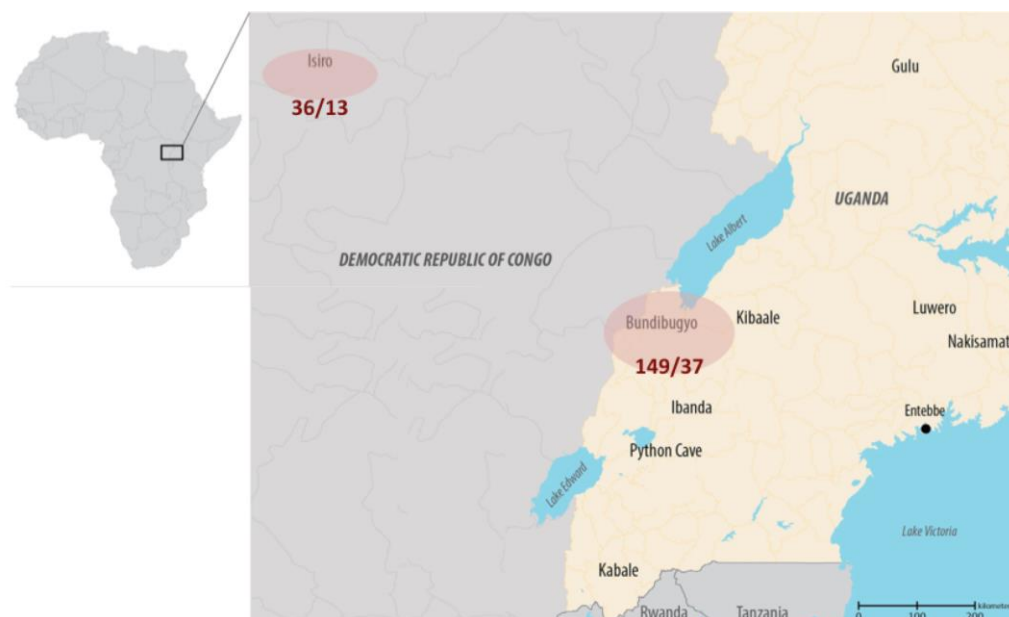


Figure 4.1. Map of the Democratic Republic of the Congo and Uganda showing the locations of BDBV outbreaks, each red ellipse represents the location of the outbreak and the Number of recorded cases/deaths. Adapted and modified from Albariño, C. G. et al [146].

4.1.3. Cross-reactivity between species

Currently there are a total of five species of Ebola virus have been identified, with genome differences of 30–45% between species. Four of these species are responsible for causing EVD in humans, and were discovered in Africa. Three of these species, including SUDV, EBOV and BDBV, were responsible for the major EVD outbreaks in the last 40 years. The fifth species, Reston Ebola virus, is capable of causing a similar disease in nonhuman primates and was transmitted to human in America [147]. As such, the species multiplex format of the developed assay in this thesis was constructed to identify BDBV, SUDV and EBOV, which outbreaks yielded in large population of human survivors. Indeed, identifying the nature of cross reactivity of the expressed antibodies following viral infection is imperative for diagnosis of EVD, patient management and development of future vaccines [148].

Previous work demonstrated that BDBV species exhibits similar symptoms to those of other EVD species, however it has relatively lower mortality rate, of 25%, compared to other species where up to 90% of cases were fatal [149]. Sequence determination of complete genomes of the EVD species has been studied extensively and their findings were crucial for the work of epidemiologists, ecologists, virologists, and taxonomists [150]. Together this has enabled better understanding of the molecular relationship between the different viral species, highlighting the close genetic origin of the BDBV species to the EBOV, as presented in a phylogenetic tree of Ebola virus [151]. Due to this genetic relationship, infected individuals with either EBOV or BDBV may have a level of cross reactivity in their immune response to both species [148].

Based on phylogenetic studies, Ebola virus strain has two main groups. The first group includes SUDV and Reston Ebola virus species. The access to the survivors from the second species is limited as it infected only few American lab employees nearly 25 years ago. The second group of Ebola virus consists of the EBOV species and another subgroup with BDBV and Cote d'Ivoire Ebola virus. The former species is responsible infecting a single patient back in the 80's, and as it was never considered as an outbreak, it was not included in this study of cross reactivity.

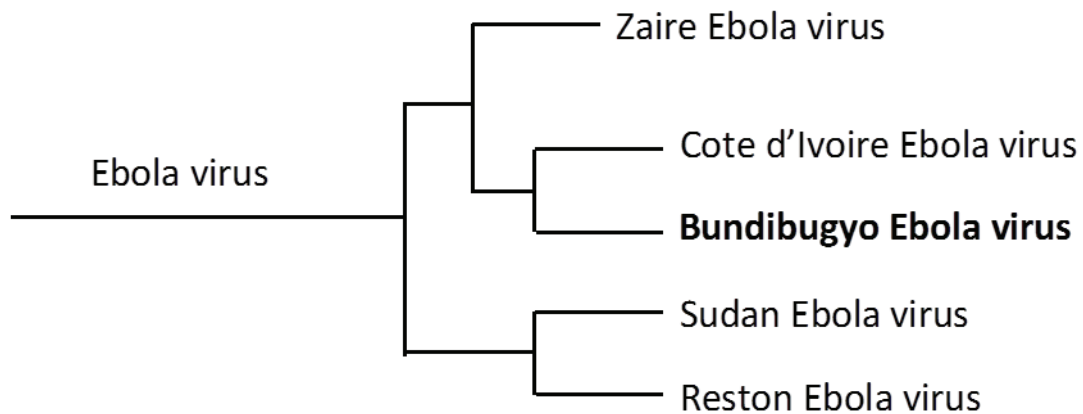


Figure 4.2. Phylogenetic tree presenting full-length genomes of Ebola virus species. BDBV is closely related to EBOV species. As described by Conlan, S. et al [151].

There is a persistent need for sensitive and reliable serological approaches for examining filoviral infections during and post outbreak. The identification of the specific viral species is crucial for potential antibody-based therapeutic; evaluation of future vaccines and epidemiological surveillance to assess the exposure of a population. As the genetic material from the pathogen is often missing, antibody detection methods are indispensable, especially for examining non-viremic patients and for disease surveillance [94], which is the key motivation for this study.

4.2. Materials and methods

4.2.1. Ethics statement

The study was approved by the Helsinki committees of the Uganda Virus Research Institute in Entebbe, Uganda (reference number GC/127/13/01/15), Soroka Hospital, Beer-sheva, Israel (protocol number 0263-13-SOR) and the Ugandan National Council for Science and Technology (UNCST) (registration number HS1332). Written informed consent as well as a personal health questionnaire was completed for each subject.

4.2.2. Samples collection

A total of 24 serum samples were obtained from survivors from the 2007-8 BDBV outbreak in Bundibugyo district, Uganda. 4 survivors samples was excluded from this study due to mistreatment of the samples. Subjects included confirmed individuals previously infected with BDBV and healthy local community members that were not infected, according to PCR and ELISA records from the outbreak [132]. The collected samples were divided into two groups; survivors, those that were infected with the virus during the outbreak, and controls, healthy volunteers that were not infected with the virus. All samples were gamma irradiated before use in assays.

4.2.3. Cloning and expression and purification of recombinant GP₁₋₆₄₉

Purified recombinant His-tagged (His₆) GP₁₋₆₄₉ viral polypeptide (lacking the transmembrane domain) of SUDV, EBOV and BDBV, was prepared as previously described in Chapter 2 Section 2.2.1 [111].

4.2.4. Lateral flow immunochromatographic strip test

4.2.4.1. Gold nanoparticles conjugation

Citrate coated AuNPs of 40 nm diameters were purchased from BBI Solutions, UK. A AuNPs conjugates were prepared with secondary anti human IgG antibodies (FC specific) purchased from Sigma Aldrich. The conjugation process was performed as described in Chapter 3 Section 3.2.4.1.

4.2.4.2. Strip assembly

Lateral flow test strip was assembled as previously described in Chapter 3 section 3.2.4.2 with a minor modification. The monoplex in this chapter was the control line was spotted with 1 mg/ml (in water) GP₁₋₆₄₉ from BDBV. The multiplex test lines were spotted GP₁₋₆₄₉ from three viral species in the sequence

selected in Chapter 2 section 2.3.9, SUDV, BDBV and EBOV, with a minimum distance between spotted lines of 2 mm.

4.2.5. Human sera testing with lateral flow strip

Two groups of samples were tested via lateral flow test strips: 1) samples that were obtained from previously infected patients and referred to as survivors, and 2) samples obtained from non-infected volunteers, close related to the survivors and referred to as controls. In this section all tested sera samples were collected in 2014 and stored in -80 °C. The operation procedure was performed as described in Chapter 3 Section 3.2.5.

4.2.6. Test strips analysis via smartphone application

In this chapter all samples screened with the lateral flow test strip were quantified using the smartphone app as described in Chapter 3 Section 3.2.7. In this chapter also the multiplex strips were quantified with the app.

4.2.7. Samples testing via chemiluminescence ELISA

The serum samples analysed in this chapter were validated with chemiluminescence ELISA as a gold standard using the procedure described in Section 3.2.8.1.

4.2.8. Data analysis of lateral flow strips and ELISA

The developed application was operated using a Samsung Galaxy S-4 smartphone to quantify the lateral flow test line. The resulting relative test line intensity was obtained by subtracting the average intensity of the test line from the average intensity of the bare membrane. The serum ELISA result is presented as a positive percentage (%PP), obtained as the normalised raw result divided by the positive control that contained antibodies against GP₁₋₆₄₉, 3C10,

6D8 and anti-BDBV. For both lateral flow and ELISA assays the cut-off, for decision as whether test positive or negative, was established based on the average of the 5 tested non-infected controls with addition of three times the standard deviation. Based on this cut-off the tested survivors' samples were categorised for semi-quantitative analysis as either positive or negative. The ELISA's double and triple cut-off (++) was the double and triple (+++), respectively, of the value of single. Overall D-sensitivity and D-specificity was determined as the fraction of total survivors that tested positive and the fraction of non-infected controls, which tested negative, respectively. Sensitivity and specificity values of the lateral flow assay were evaluated in comparison to the ELISA results and were determined as the ratio between the amounts of positively tested survivors and negatively tested non-infected controls, respectively.

4.3. Results and discussion

4.3.1. Sample collection

The humoral response of EVD survivors has been widely studied using serological tests. ELISAs are considered as standard serological tools for the assessment and diagnosis of the humoral response to EVD [94], [137], [148] [139] and therefore was used as reference for the evaluation of the developed smartphone-based lateral flow assay.

Sera samples were collected in 2014 from the Bundibugyo district in Uganda. In this chapter, sera samples were obtained from 10 previously infected and recovered BDBV patients. At the collection these survivors were 7 years after the recovery from BDBV. These survivors were diagnosed with BDBV at the acute stage of the infection by using standard methods, RT-PCR and ELISA (data not included). Additional 10 samples were obtained from healthy and non-infected volunteers that had EVD-free medical records. These individuals were originally from Bundibugyo district and were closely related to the survivors (e.g. family members, neighbors from the same village). The non-infected control group was splitted into two groups, the first group was used to calibrate both

ELISA and lateral flow assays and evaluate the cut-off (n=5) and the second group was tested verifying the calibrated system.

4.3.2. Sera characterisation via chemiluminescence ELISA

Three independent indirect serological ELISA formats were performed to analyse a total of 10 samples from survivors and 5 from non-infected controls. In each format, the IgG response against a different GP₁₋₆₄₉ from a different EVD species was tested, including BDBV, SUDV and EBOV. A semi-quantitative summary immune response of the tested survivors (S1-S10) and controls (C1-C5) against different species is presented in Table 4.1.

Venn diagram presented in Figure 4.3 summarised the cross reactivity between the three species identified within the tested subjects. In total 9 survivors demonstrated a persistent immune response against the GP₁₋₆₄₉ from the BDBV. A single survivor (S10) presented no immune response to BDBV GP₁₋₆₄₉, which is expected as not all survivors retain persistent immune responses to viral GP [82]. Moreover, out of these 9 survivors, 5 also tested positive to EBOV. This cross-protection and cross reactivity between the EBOV and BDBV might be due to the close genetic relationship between the two viral species (Figure 4.2).

Interestingly, a single survivor (S2) demonstrated the presence of specific IgG antibodies against all three tested viral species. There are several reasons to explain the immune cross-protection of S2. Firstly, the cross reactivity between BDBV and SUDV is thought to be rare but it occurs in up to 20% of the cases [152]. S2 had a relatively strong immune response to both EBOV and BDBV (+++). Perhaps the large amount of IgG might non-specifically bound also to the GP from SUDV, however is less likely as it was not the case for S1. Furthermore, this survivor might have been exposed to SUDV during the previous outbreaks, which occurred in similar regions.

In general the immune response of these 9 survivors was strongest against BDBV species, as expected. All 5 non-infected controls tested negative to all viral species.

Table 4.1. ELISA serology screening of BDBV survivors and controls against GP proteins of three viral species. Results are classified in a semi-qualitative approach for the presence of IgG antibodies against the tested protein.

Sample	GP₁₋₆₄₉ BDBV	GP₁₋₆₄₉ EBOV	GP₁₋₆₄₉ SUDV
S1	+++	+++	-
S2	+++	+++	++
S3	+++	+	-
S4	+++	+	-
S5	++	-	-
S6	+++	++	-
S7	+++	-	-
S8	+++	-	-
S9	-	-	-
S10	+++	-	-
C1	-	-	-
C2	-	-	-
C3	-	-	-
C4	-	-	-
C5	-	-	-

**Single + represents response above a single cut-off, ++ above double cut-off and +++ above triple cut-off.*

***10 samples were obtained from survivors (S.1-10) and 5 from non-infected controls (C.1-5)*

**** GP₁₋₆₄₉ - recombinant glycoprotein produced without the transmembrane domain and with His₆ tag.*

Humoral response against multiple viral species within individual survivors might suggest cross-reactivity between these species or present exposure of the survivors to multiple viral strains. Figure 4.3 demonstrated the overlapping between the circles represents the number of survivors that exhibit

immune response against GP₁₋₆₄₉ from Bundibugyo, Zaire and Sudan species. Out of the total 10 samples, a single survivor tested positive against all three species, 4 against Zaire and Bundibugyo. The other 5 samples presented an immune response against a single viral species, Bundibugyo.

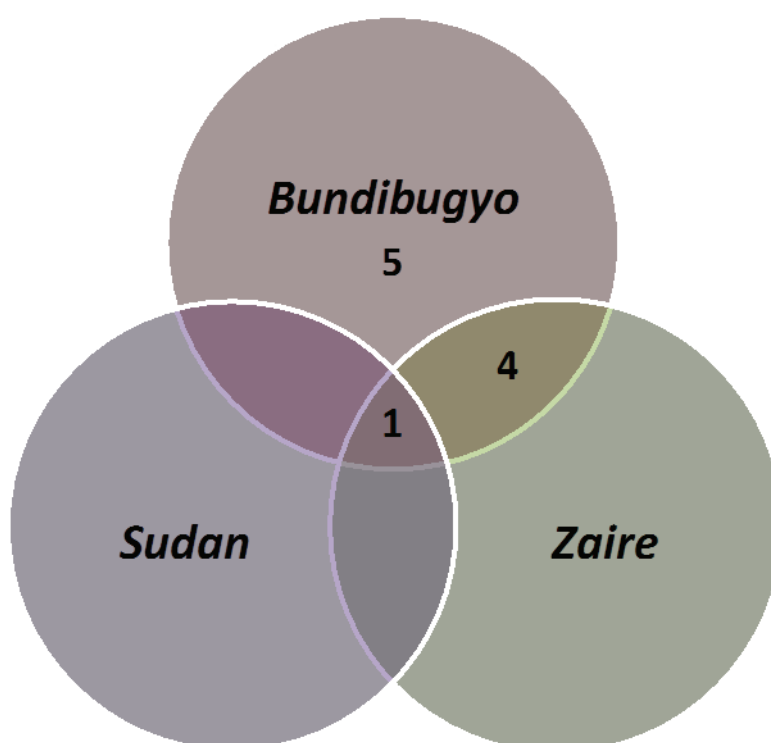


Figure 4.3. Venn diagram representation of positive immunoreactivity between Bundibugyo, Zaire and Sudan GP₁₋₆₄₉ of screened BDBV survivors. Each sample was tested against each viral species in three independent experiments.

4.3.3. BDBV Monoplex lateral flow assay

In this chapter, the previously presented monoplex format was modified and adjusted to detect human immune response to GP₁₋₆₄₉ from BDBV species. The BDBV monoplex test strip consisted of a single test line spotted with GP₁₋₆₄₉ from the BDBV and a control line with human IgGs. In addition, 40 nm AuNPs functionalised with anti-human IgG antibodies were used for colorimetric

labelling of the lines. The assay was calibrated with 5 control samples producing the cut off value for distinguishing positive and negative samples.

The same (from section 4.3.2) 15 samples were analysed via the lateral flow assay and quantified with the first version of the developed smartphone app (without the geographical tagging) and presented in Figure 4.4. A total of 10 samples were obtained from BDBV survivors (S.1-10) and 5 from non-infected controls (C.1-5). Examples of lateral flow test strips are presented in Figure 4.4A demonstrating differences in the intensity of the test line between the tested individuals. Supporting that levels IgG antibodies against GP₁₋₆₄₉-BDBV vary between survivors. The intensity of the control lines was comparable between all tested samples, highlighting the robustness of the assay.

Quantitative analysis of the tested samples as presented in Figure 4.4B showed several levels of the humeral immunity response between different survivors. Out of 10 survivors, 9 tested positive, and all non-infected controls showed a negative response. These results were in line with the analysis performed via ELISA. Sensitivity was determined as 90% for both analytical methods while specificity was determined to be 100%. In addition, 100% correlation between the monoplex assay and the ELISA was demonstrated.

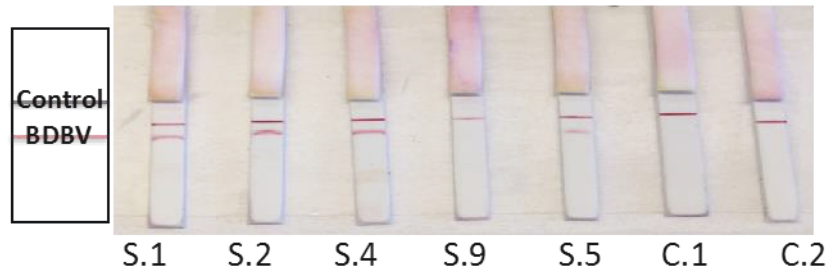
4.3.4. Multiplex analysis using EVD species multiplex assay

The multiplex species assay was designed to simultaneously detect and classify the IgG antibodies against three EVD species. The lateral flow test strip comprises three spotted test lines and a single control line. Each test line consisted of GP₁₋₆₄₉ of each species in the following sequence from the sample pad, EBOV, BDBV and SUDV. Different sequences of the order of the spotted viral proteins were tested and demonstrated similar results. The proof of concept of this format was demonstrated in Chapter 3. In this chapter this format was harnessed to identify cross reactivity of BDBV survivors immune response with other viral species.

A total of 5 (S.1-5) serum samples from survivors of the 2007 BDBV outbreak and 2 (C.1-2) from non-infected controls were analysed for proof of

concept of the multiplex lateral flow test strips. Other samples were excluded due to not-sufficient volume to conduct this study.

A.



B.

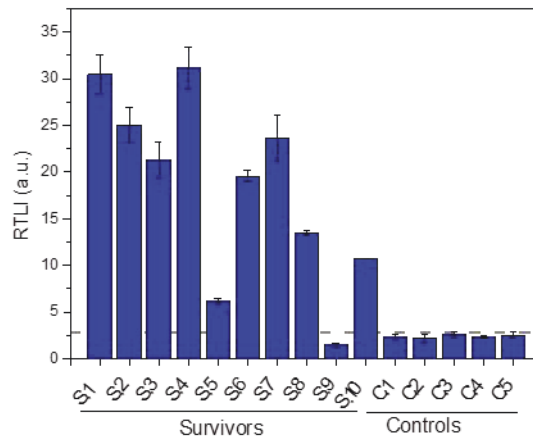


Figure 4.4. Lateral flow and smartphone quantitative analysis of BDBV samples. A. Lateral flow strips of three survivors (from left to right S1, S2, S4, S9 and S5) and two non-infected controls (C1-2) B. 10 samples obtained from survivors (S.1-10) and 5 non-infected controls (C.1-5) were tested to detect IgG antibodies against BDBV GP₁₋₆₄₉. The results are presented as Relative Test Line Intensity (RTL). The dashed line represents the cut-off of the assay calculated from the average value of the 5 controls plus three times the standard deviations. Standard deviation represents independent repeats (n=2).

Representative test strips of the analysed samples are presented in Figure 4.5A. In addition, quantitative analysis was performed using the smartphone app, in order to eliminate any subjective interpretation. Obtaining quantitative analysis was especially important for the classification of samples with relatively low signal intensity (e.g. S5).

Simultaneous detection of multiple antibodies within the same sample is a key advantage of the multiplex configuration, as it enables detection of cross-reactivity between the human immune response to all three viral species. However, the intensity of the readout may differ between the monoplex and multiplex format. This is mainly related to the total amount of AuNPs that were applied and had to label multiple test lines in the multiplex format. Also, the antibodies that cross-react with two species will be split between two test lines in the multiplex, whereas in the monoplex most of them will bind to a single test line.

As such, the multiplex assay had to undergo an independent calibration process for each test line. As such, cut-off for each viral species in the multiplex was evaluated by using two different control samples. This cut-off was a key component for the classification of the tested samples via the multiplex assay.

Figures 4.5B-F present the results of the quantitative analysis for survivors 1-5, respectively. All survivors tested positive to persistence humoral response against BDBV GP₁₋₆₄₉. The magnitude of the BDBV test line correlated to the monoplex results.

Survivors 1-3 also tested positive to IgG antibodies against EBOV GP₁₋₆₄₉. All survivors tested negative to SUDV species. Also, the immune response of these survivors was significantly higher to BDBV than EBOV, as previously described. The non-infected controls tested negative to all three species.

The magnitude of the intensity of the control line was comparable throughout all the tested samples, demonstrating the consistence of the assay.

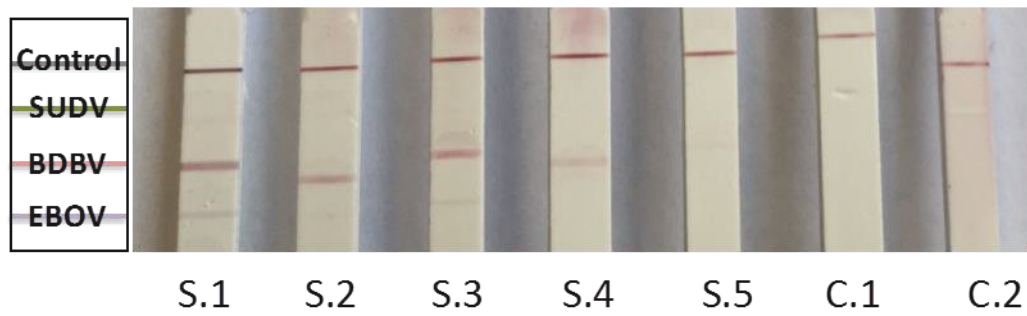
The results of all samples analysed with the strip, except S2, aligned with the resulting analysis performed by the standard ELISA. When tested with the multiplex assay, S2 had no detectable immune response against SUDV, as identified in the ELISA analysis. This might be related to the fact that ELISA has better limit of detection when compared to lateral flow. The lateral flow assay has no amplification steps, which makes it significantly less sensitive compared to the ELISA. As such, the inability to detect the cross-reactivity of S2 with SUDV was addressed to insufficient sensitivity of the developed assay.

Analytical parameters of the assay were evaluated in comparison to the ELISA. The developed multiplex lateral flow smartphone-based assay exhibited

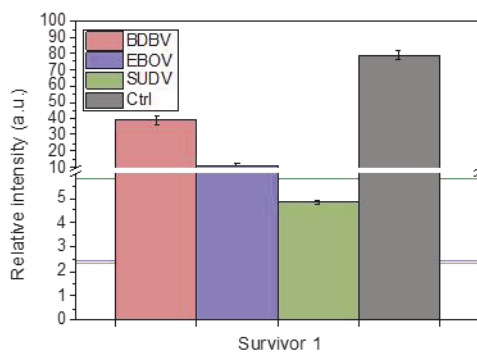
100% sensitivity and 100%, specificity to the BVBD and EBOV species. Indeed, the developed assay can be operated on-site generating a results within only 15 minutes, whereas the ELISA is a laborious method, the operation of which requires up to 5 hours.

The smartphone analysis provided quantitative analysis that aligned with the analysis performed by ELISA and can be performed under field conditions without need for complex laboratory equipment.

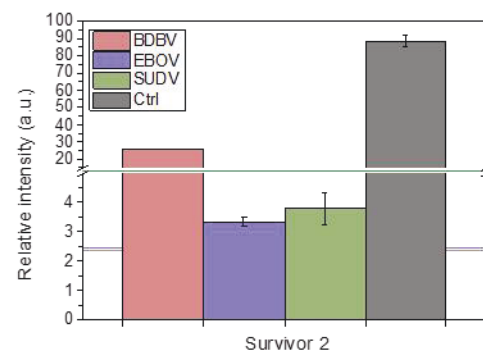
A.



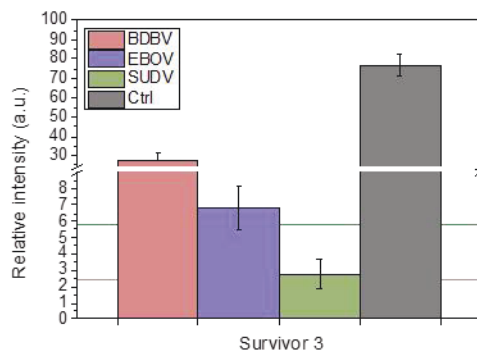
B.



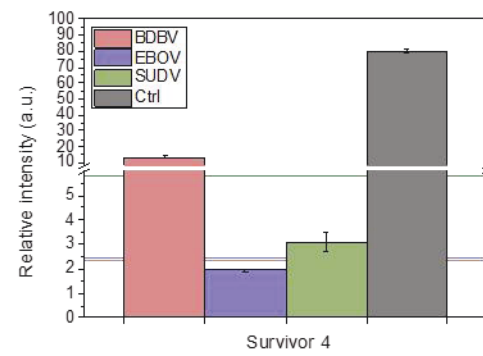
C.



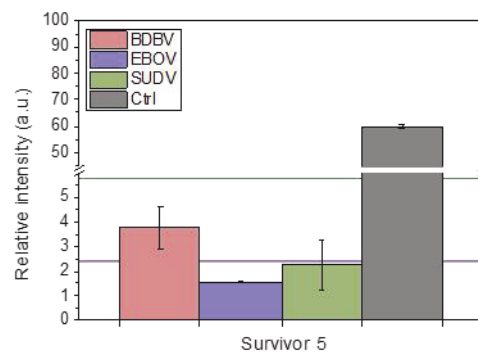
D.



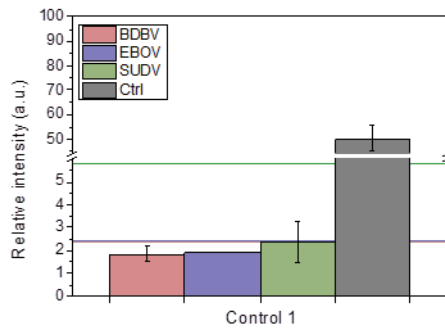
E.



F.



G.



H.

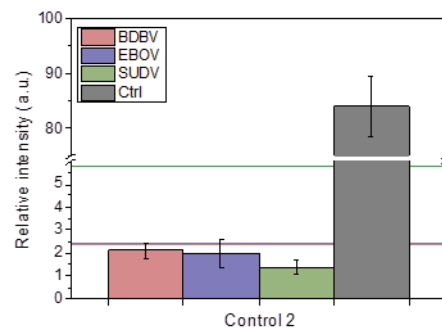


Figure 4.5. Screening BDBV samples with EVD species multiplex assay. A. Example of resulting strips upon BDBV survivors' analysis including S1, S2, S3, S4 and S5 and non-infected controls (C1 and C2). Illustration described structure of the strips with three lines spotted with GP₁₋₆₄₉ from SUDV, BDBV and EBOV. Visual test line represents the detection of IgG response to a specific species. B-F. Quantitative analysis of S1-S5, respectively. G-H. Quantitative analysis of C1 and C2, respectively. The readout is presented in RTLI units corresponding to the detected immune response to GP₁₋₆₄₉ from (from left to right) BDBV, EBOV, SUDV. Red, purple, green lines represent the cut-off values for BDBV, EBOV and SUDV, respectively. The data was averaged over 2 experimental repeats in triplicates for the ELISA and error bars represent $\pm$ RTLI for results.

4.4. Conclusions

Studies of the viral species cross reactivity and the cross protection of EVD survivors is crucial for the development of effective vaccine and targeted therapies[153]. These studies usually focus on the investigation of immune response in survivors. The work presented in this chapter expended the usage of the new serological assay for cross reactivity studies of survivors' immune response. Here, the assay was also used to test different population of survivors that were infected to additional viral species, BDBV. For these reasons this work was presented in an independent chapter.

Briefly, this chapter demonstrated the evaluation of the smartphone-based lateral flow assay for the detection of immune response against BDBV. This assay utilised recombinant viral GP as the recognition elements. This viral protein was selected to support the cross reactivity and cross protection studies,

which suggest the following. Firstly, viral GP is currently the best known neutralising antibody to EVD, which detection is imperative for the development of potential viral therapeutic [139]. Secondly, the ongoing vaccine development focuses on the immunisation against GP [154]. Crucially, the majority of long-recovered EVD survivors can maintain persistent and strong IgG humoral immunity against the viral GP [139], also proved in Chapter 3.

As previously, the assay was developed to target the immune response to the GP viral protein [155] by presenting a recombinant version of this protein on the test line/s of the lateral flow strip.

In order to develop and evaluate the novel BDBV assay, human samples from BDBV survivors and non-infected controls were obtained. These samples were initially characterised with serological ELISA tests for the detection of IgG response against GP₁₋₆₄₉ for BVBV, EBOV and SUDV. In total almost half of the survivors exhibited cross-reactivity between both BDBV and EBOV, as expected [139], [148]. In addition, 10% of the survivors lost/had no persistent immune response to the targeted viral protein. This lack of immunity could be expected since it was previously demonstrated that not all survivors of EVD develop a humoral immune response against GP [139].

The BDBV monoplex lateral flow strip was calibrated by using sera samples obtained from non-infected volunteers originally from the Bundibugyo district in Uganda. The origin of these individuals was a key component for the specific and sensitive calibration of the developed assay as these samples exhibited the same immune exposure as the survivors. Quantitative analysis of the 15 samples (survivors and controls) via the BDBV monoplex demonstrated 100% correlation with the standard ELISA and was performed within 15 minutes.

A proof of concept monoplex assay for each species was demonstrated by testing a total of 7 samples. The simultaneous detection approach performed via this assay presented cross-reactivity between the immune response of survivors of BDBV to EBOV and SUDV. As expected almost half of the tested survivors exhibited immune also to the EBOV species. However, overall reactivity of the IgG antibodies tended to be stronger for autologous rather than heterologous GP₁₋₆₃₉.

This chapter proved that BDBV survivors exhibit cross reactive immunity against other viral species. The next step would be to test the cross protection of these survivors via viral plaque neutralisation assays.

To sum up, the mono/multiplex smartphone-based lateral flow assay demonstrated high reproducibly, robustness and it could be easily adjustable to testing other EVD viral species. This assay exhibits significant benefits compared to the standard ELISA method that requires laborious conditions, longer operation time and not portable.

Finally, this assay can be an effective tool for the therapy and vaccine development, as well as for epidemiologic and epizootiologic investigations of the Ebola virus.

Chapter 5

Detection of Ebola Virus Nucleic Acids using SNA and Enzymatic Target-Recycling Amplification

5.1 Introduction

Chapters 2, 3 and 4 review indirect detection of Ebola virus through the detection of IgG antibodies. In contrast to the previous approach, this chapter presents the approach for direct detection of Ebola virus. Herein, viral nucleic acids are targeted as the detection analyte of the assay. As previously reviewed, PCR is the standard method utilised for the detection of viral genome. With PCR, the recognition is performed through thermal amplification of the targeted nucleic acids, which requires expensive specialised equipment.

Hence, the motivation for the work presented in this chapter was to produce an alternative assay for detecting Ebola virus nucleic acids. The aim was to design simpler and potentially temperature-independent assay that requires basic laboratory equipment.

The design, development and optimisation of a solution-based biosensing system for Ebola virus nucleic acids are described. This system operates with an optical transducer and fluorescence readout. In particular, the sensing of the Ebola virus oligonucleotide is mediated and amplified with AuNPs and Exonuclease III enzyme (Exo III), respectively. Furthermore, incorporating computer simulations for *In-silico* design of the assay enables specific differentiation between three viral species. Detailed description of the operation is presented in Section 5.3.1.

5.1.1. Spherical Nucleic Acids

Spherical Nucleic Acids (SNA) particles are used to facilitate the biosensing of the Ebola virus nucleic acids. In general, SNA are an inorganic NPs core densely functionalised with a self-assembled monolayer of oligonucleotides.

AuNPs are usually used as the core onto which surface the functionalising oligonucleotide bound. These functionalising oligonucleotides usually contain three regions: target recognition segment, spacer region, and a chemically attached thiol. The functionalisation of AuNP with these oligonucleotides occurs through thiol and gold atom bond [156]. Once the oligonucleotide binds to the surface of AuNP, additional functional groups such as florescent dye molecules, quenchers, modified bases, and drugs can be hybridised to the recognition sequence of the oligonucleotide.

Mirkin *et al.* [157] originally developed the method for the preparation of SNA. In this method, thiolated DNA forms thiol-gold bonds with the surface of 13 nm core AuNPs while exchanging with the original citrate groups, as schematically presented in Figure 5.1. The accumulation of a highly dense self-assembled monolayer of thiolated DNA is then achieved through a gradual increase of salt concentration, also called salt aging process.

The salt aging step screens between the negative charges of the DNA strands to reduce their repulsion and enables dense packing onto the surface of the gold. The formed SNA retain all the optical properties of bare AuNPs, including, size-tunable localised SPR and the ability to quench nearly located fluorescence dyes. The second optical property was applied in the transduction mechanism of the developed assay.

The use of SNA in molecular diagnostics of nucleic acids offers many advantages over regular NPs. Many SNA-based biosensing systems have been developed for either in-vitro diagnostics [158] [159], cellular imaging [160] or drug delivery [161].

The SNA exhibit high colloidal stability in complex matrix such as body fluids[162]. Furthermore, the functionalised oligonucleotides on the surface of

the SNA have been exploited as a form of biological recognition element for selective detection of analyte [157] [161].

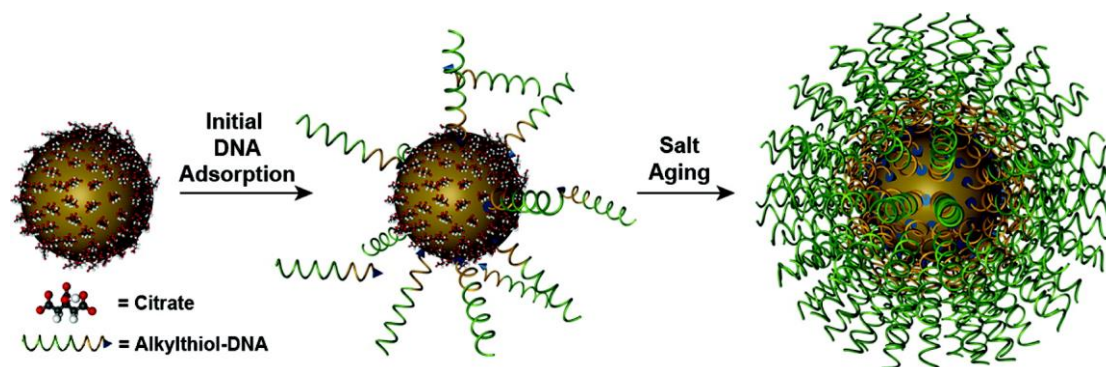


Figure 5.1. Schematic illustration of the synthesis method of SNA. Citrate coated AuNPs are incubated with DNA modified with thiol in its terminus to form low-density monolayer. Introducing a salt gradient that varies from 0.15 – 1 M for over 5 enables formation of high-density SNA shell. Adapted from Mirkin et. al [162]. Copyright 2012

The structure of SNA possesses a better thermal stability than a free DNA. In particular, the disassociation, or “melting”, of free oligonucleotides duplex in solution occurs over a broader temperature range (~ 20 °C). The duplex assembled with SNA exhibits melting transition over a narrower temperature range (~ 2 – 8 °C). Moreover, the disassociation temperature (T_m) of SNA-duplex is significantly higher than the T_m of free-duplex [162]. This increase in T_m enables binding complementary oligonucleotide with higher specificity and provides better stability to the formed SNA-duplex.

5.1.2. Fluorescence dye quenching as the analytical signal of the assay

A fluorescent dye (or fluorophore) is a chemical entity emitting fluorescence light upon light excitation, which is an optical property reviewed in the introduction Section 1.2.2. These optical properties are usually related to the chemical structure of the dye contains several aromatic groups or cyclic molecules with several π bonds. Each dye absorb light energy of a specific

wavelength, also called excitation wavelength, and re-emits a light at a longer wavelength, referred as emission wavelength.

Cyanine 5 (Cy5) was used as a signal transporter of the presence of target analyte. Cy5 is a synthetic dye belonging to family of polymethine fluorophores. The molecular structure of Cy5, as presented in Figure 5.2A, comprises short aliphatic chain and four aromatics rings that are responsible for its fluorescent properties. Cy5 can be excited maximally at 645 nm and emit fluoresce maximally at 665 as presented in absorbance and emission spectra in Figure 5.2B.

Close distance of up to 10 nm to AuNPs causes quenches the emission of Cy5. In this chapter, an oligonucleotide conjugated to Cy5 was designed to hybridise to the SNA, quenching the emission of the dye. Because at a close proximity between the dye and the particle, the particle absorbs the emission wavelength and reduces the intensity of the fluorescence emission of the system, also known as Fluorescence Resonance Energy Transfer (FRET) [20]. This property was used as the analytical readout of the developed assay in this chapter [164].

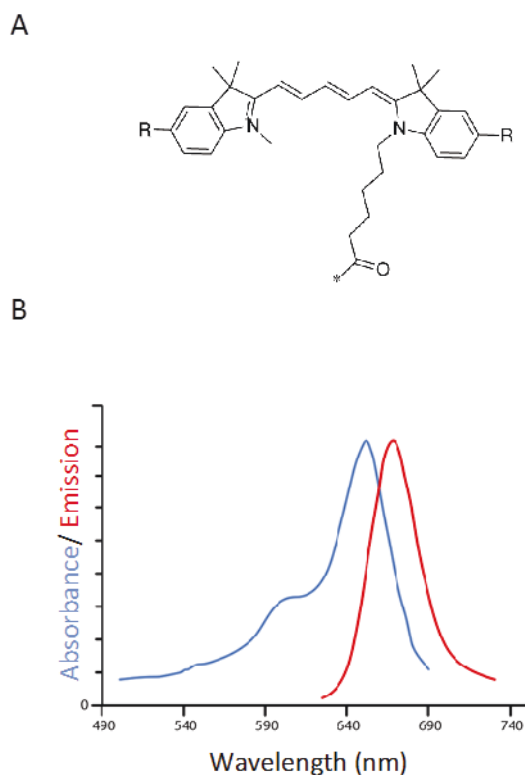


Figure 5.2. Cy5 (A) chemical structure and (B) Optical properties. Absorbance spectrum is in blue and emission spectrum is in red.

5.1.3. Target-recycling with Exonuclease III

A limitation of traditional NPs-based nucleic acids assays is the non-sufficient limit of detection of the target oligonucleotide in clinical samples. Amplification can be used to increase the amount of target oligonucleotide to the detection range of the assay. Target-recycling is a relatively novel strategy to obtain this amplification. In this process, the target is used multiple times in the biosensing cycle. In this chapter, target-recycling strategy is performed with Exo III enzyme [165].

Exo III is a class of enzymes belongs to the *exonuclease* family. This enzyme has a specific activity of one by one removal of mononucleotides from a blunt 3'-hydroxyl terminus of double stranded DNA. This cleavage activity of Exo III specific to a double stranded DNA and cannot process free single strands [166]. Critically, Exo III has no specific recognition site and irrespective of the sequence available with a blunt end [167].

5.1.4. Using *In-silico* simulation for the design of the recognition oligonucleotides

The recognition elements of the assay design was performed with *in-silico* simulation analysis of the Ebola virus genome [168]. This analysis uses reference sequence (RefSeq) database and Basic Local Alignment Search Tool (BLAST). The algorithm was designed to produce 60 mer sequences with high difference between the three species and has no complementary target in the human genome. The selection of 60 mer was a result of restriction in length of sequence that can form SNA [162].

RefSeq database is created by the National Center for Biotechnology Information (NCBI). This database is a comprehensive collection of non-redundant well-annotated sequences, describing the reference genomes of a large number of select taxonomically diverse organisms [169].

RefSeq includes submitted genomic data, as well as sequences transcribed and translated from these references computationally and using

manual curation.

RefSeq is accessible via direct downloads, but usually searchable via keyword searches on gene/protein annotations and via homology searches using BLAST and related software [169] [170].

NCBI BLAST is a collection of alignment tools that looks for sequence similarities between query DNA/RNA/protein sequences and underlying sequence databases [171].

BLAST is significantly faster than pairwise alignment techniques, which consider each pair of sequences individually. However, it produces only local alignments and is not guaranteed to find optimal alignments or to pick up all homologs found in the database [172].

5.2. Materials and methods

5.2.1. *In-silico* simulation of Ebola virus oligonucleotide probes

Sequences of the EBOV, SUDV, and BDBV species were downloaded from the NCBI Ebola database [173]. Sequences from each species were aligned with MUSCLE. Next, the alignments were manually curated to remove loci containing very rare insertion events, and sequences, not covering a significant proportion of the alignment. 60-mer sequences specific to each species were selected from the full genome. A list was derived of unique 60-mers appearing within sequences of the alignment. 60-mers containing gap or ambiguous nucleotides were removed. The remaining 60-mers in the list were then scored and ranked according to how many sequences in the alignment contained each 60-mer.

The top 500 most commonly appearing 60-mers were then evaluated against the three alignments. Given a sequence and a 60-mer, the minimum Hamming distance between the 60-mer and any consecutive 60 nucleotides in the sequence was calculated.

For an alignment, the mean value of this statistic was calculated across all sequences in the alignment. Thus, for each 60-mer and alignment, the mean minimum Hamming distance was calculated across the alignment, indicating the

extent of the match between the 60-mer, and the “average” sequence of a specific strain. A similar calculation was performed to find the mean difference between the 60-mer and two Marburg virus sequences.

Then, the 60-mer sequences held were compared to identify common regions between species. The consensus sequence of each strain alignment was found. The resulting three sequences were aligned using Muscle. 60-mers were then derived from the consensus sequences of this alignment. For each 60-mer, the mean minimum Hamming distance was calculated for each of the three strain alignments, and the Marburg sequences.

Each of the sequences was tested via Standard Nucleotide BLAST[174] against the human genome. 100 sequences with lowest similarity to the human genome were selected.

Output files:

Specific 60-mers:

Zaire_specific.out;

Sudan_specific.out;

Bundibugyo_specific.out

These contain sequences that are specific to the respective viral strains. Files show 60-mer sequences and statistics, giving the mean minimum Hamming distance from each of the three specific strains, and the Marburg sequences

Common 60-mers: Common.out. Contains sequences that are close to all of the respective viral strains. The file shows 60-mer sequences and statistics. The “Total” value is the sum of the mean minimum Hamming distances from each of the three strains aligned sequences in fasta format with a code written in C++.

5.2.2 In-silico analysis of secondary structure of DNA oligonucleotides

A *nupack.org* database was used to analyse the probability to form secondary structures between the obtained Ebola virus target oligonucleotides. The sequences were analysed using the analysis at 25 °C and 37°C, with following parameters:

Number of strand species: 2

Maximum complex size: 2 strands

Ionic strength: 50 mM NaCl

The analysis output included prediction of the free energy to form secondary structures and a diagram plotting the potential internal secondary structures. The selection criterion for the selected oligonucleotide was to have free energy of the secondary structure lower than -10 kcal mol⁻¹.

5.2.3. Melt curve analysis of the designed oligonucleotides

Custom DNA oligonucleotides were designed and purchased from IDT DNA. DNA oligonucleotides were diluted to 4 µM in phosphate-buffered saline (PBS) pH 7.4 and validated with Nono-drop using the oligonucleotides program.

A 5 µl of each DNA oligonucleotides and 2 µl of 2x SYBR-green was added to a final volume of 20 µl into 125 µl PCR tubes PCR tubes. The samples were analysed via One-Step PCR using the melting curve procedure starting at 25 °C to 90 °C with 0.25°C/sec.

The fluorescence intensity melt curve was converted into first derivative melting-curve, d/dT. The peak of the d/dT curve was estimated as the melting temperature of the tested oligonucleotides.

5.2.4. Gold nano particles synthesis

79 mg of H₂AuCl₄*4.3H₂O dissolved in 5 mL of H₂O (sigma 520918 lot# MKBR0958V) prepared in a glass vial. 10 mL of 2 (wt%) trisodium citrate dehydrate in water prepared (BDH 301284C lot# K91568338 233). Heated to 70 °C (over about 10 minutes).

A 180 mL of milli-Q water in a 250 mL RBF (2-neck) brought to reflux and stirred gently. The H₂AuCl₄ is then added once the water is at reflux and stirring speed brought up as high as possible. Within 5 minutes the trisodium citrate is added by syringe (at 70 °C, no needle for fast injection) to the H₂AuCl₄.

The solution was left for 5 minutes at reflux and under fast stirring. It was then removed from oil bath and allowed to cool to room temperature. The key is to have the sodium citrate hot when added, to add it fast and with very strong stirring.

The gold solution should only be added just before the sodium citrate to avoid formation of hydroxides. The particles are then allowed to cool and stored at 4°C indefinitely.

5.2.5. SNA Synthesis

SNA consisted of AuNPs and thiolated recognition probes. The SNA were prepared by conjugating thiol-modified oligonucleotides with previously synthesised 13 ± 1 nm citrate-capped AuNPs (10 nM) the synthesis was carried out in DNAase and RNAse free water (18.2 MΩ).

A total of 18 µl of 250 µM thiolated-DNA were added to 1.5 ml of AuNPs and incubated for 1 hour at room temperature. Next, 1.5 ml Tween 20 (Sigma-Aldrich P9416), in phosphate buffer (pH = 7.4) was added to a final concentration of 0.01%, 5 mM, respectively. After a 2-hour incubation period, ionic strength of the solution was gradually increased with additions of sodium chloride (NaCl) over 5 hours to bring the final NaCl concentration to 300 mM to complete the functionalization process.

Finally, the solution containing the conjugates was centrifuged (13,000 rpm, 25 minutes) and resuspended in water three times to produce the purified SNAs. The prepared SNAs were stored Eppendorf® Protein LoBind microcentrifuge tubes DNA/RNA, volume 1.5 mL at 4°C.

The concentration of the SNAs was determined via UV-visible (UV-vis) spectroscopy measurements. The measured absorbance was related to the concentration of gold particles using Beer's Law at the SPR peak,

$$A = \epsilon \cdot l \cdot C$$

where,

ϵ = Extinction coefficient of AuNPs in aqueous solution ($2.77 \times 10^8 \text{ M}^{-1} \text{ cm}^{-1}$),

l = Path length of the light through the material sample (1 cm),

C = Concentration (M),

A = Absorbance.

5.2.6. SNAs optical characterization

UV-Visible absorption spectra were recorded from 400-700 nm using a Perkin-Elmer Envision plate reader and fluorescence emission spectrum was recorded from 620-700 nm with excitation at 600 nm for Flare conjugated to Cy5. The analysed SNA were diluted 1:10 from stock into water or buffer. The absorbance intensity was normalised to the SPR peak.

5.2.7. Dynamic Light Scattering

The size of the prepared SNA was determined with DLS using a ZetaSizer Nano ZS (Malvern Instruments). The sizing measurements were performed in disposable low volume cuvette, the SNA samples were diluted to be 1 OD at the SPR wavelength.

Highly concentrated samples (>5 OD) lead to inaccurate sizing due to viscosity effects and can cause multiple scattering (i.e. scattering from one particle is re-scattered by another particle), which in turn can lead to an artificially low particle size.

The measurements were carried out at 20°C with an equilibration time of 5 minutes, and 3 measurements for each sample with 3 runs of 1 minute for each measurement. The data was presented in size distribution by volume and intensity.

5.2.8. Transmission electron microscopy

SNA were diluted 1:10 from stock in water. Samples were prepared by depositing 2 µL SNA on the top of carbon-coated copper grids and tried in falcon tube to prevent accumulation of dust for approximately 5 hours.

TEM images were produced using JEOL 2010 transmission electron microscope operated at an accelerating voltage in the range of 50 to 150 mV. Analysis of SNA size was performed by measuring SNA diameters directly from the images.

5.2.9. Flare titer

A flare is a short fluorescently labelled reporter strand capable of displacing longer oligonucleotides. Fluorescence emission range of Cy5 flare was acquired using a 96 wells Corning® Costar® Ultra-Low attachment multiwell plate. Excitation and emission were recorded at 640, 665 nm, respectively of Cy5 flare using the following range of concentrations: 40K, 10K, 500, 250, 25, 10, 1 and 0 nM.

5.2.10. Flare hybridisation onto SNA

Varying concentrations of Cy5 flare strands were hybridised to complementary and non-complementary SNA. SNA (1 nM) were mixed with different concentrations of Cy5 flare concentration: 50, 20, 10, 8, 6, 4, 2, 1, 0 (nM) in PBS pH 7.4. To facilitate the hybridisation, the samples were heated to 90 °C for 10 minutes and slowly cooled down to room temperature overnight.

Then, samples were transferred into a black 96 well Corning® Costar® Ultra-Low multi well plate and the fluorescence emission was recorded out with Perkin Elmer plate reader using 640 nm for excitation and 665 nm as the emission. A non-complementary SNA was used as a control, which was synthesised with a scrambled DNA oligonucleotide.

5.2.11 Assay dose-response

1 nM SNA and Cy5 flare were mixed with a 8-fold excess of the flare in PBS and heated to 90 °C for 10 minutes. Then, the duplex was cooled down overnight to room temperature.

Fluorescence experiments were performed using a black 96 wells Corning® Costar® Ultra-Low attachment multiwell plates and Perkin Elmer plate reader with the following settings: 500 flashes, 640 excitation and 665 nm emission. Prior the measurement SNA were hybridised with Cy5 flare as described in Section 5.2.9. Then, a range of concentrations of non-labelled 60 mer oligonucleotide target was mixed with the SNA-flare hybrid using occasionally

PBS as 1:5 diluted CutSmart® Buffer, 50mM Potassium Acetate, 20mM Tris-acetate, 10 mM Magnesium Acetate, 100 µg/ml BSA, pH 7.9. The mixture was incubated for 15 minutes prior to recording of fluorescence spectrum.

5.2.12 Assay dose response with Exo III

SNA was hybridised with flare as described in Section 5.2.10 with a small modification. The components were concentrated by a factor of 10 in PBS and then prior assay run diluted with CutSmart® Buffer to achieve final dilution of 1:5. Exonuclease III (*e.coli*) M0206S was purchased from Biolabs. Then, 2 µl of Exo III, previously diluted 1:45, was added to the assay mix.

Prior to target addition, the initial set point signal was recorded. The cut-off for determination of the limit of detection was evaluated as the average emission intensity of the system with no added target - referred as I_0 , with addition of three standard deviations. Upon target addition, all ingredients were equilibrated as the plate was incubated at room temperature for 1 h. The readout was recoded using 640 nm excitation and 665 nm emission.

5.3 Results and discussion

5.3.1. Scope of the assay

Ebola virus genome comprises single stranded RNA. Upon extraction, the stability of RNA sequences significantly decreases. Hence, all PCR kits for Ebola include reverse transcription step to produce complementary DNA (cDNA)[69]. cDNA is more stable against degradation than the original RNA. The assay developed in this chapter targets SUDV viral cDNA template as the analyte.

Recognition elements of the assay were designed based on the SUDV sequences available on NCBI RefSeq. Other viral species, including BDBV EBOV, and phylogenetic related Marburg virus [175] were used as control sequences to produce high specific and selective assay.

The design of recognition elements was performed in the first part of this study and was produced through *in-silico* simulation. Computer-based analysis

enabled processing the whole viral genome (19,000 bases) and identified regions that exhibit high difference between the viral species. These selected sequences were then used to design the recognition elements of the assay.

The second part of this chapter describes the steps for development and optimisation of the assay, which is schematically described in Figure 5.3. The assay comprised SNA, which formed with the first recognition oligonucleotide. The second recognition element, the flare, comprises two complementary regions and Cy5 in the 5' terminus. The first region binds to the thiolated oligonucleotide on the surface of the SNA and the second to the target analyte.

SNA and the flare form a duplex through the first complementary region of the flare. This significantly quenches the fluorescence emission of Cy5. Quenching occurs upon the hybridisation as the Cy5 comes to close proximity of the AuNPs. The hybrid formed on the surface of the SNA presents a toehold region. The target analyte, cDNA fragment of the SUDV genome, binds to this toehold and disassociated the flare from the surface of the SNA to form a duplex with the analyte. This disassociation of the flare increases the fluorescence emission of the system. Here, the quantitative readout of the assay corresponds to the change in fluorescence emission of Cy5, which is linearly proportional to the amount of target SUDV.

The target recycling amplification step is performed with Exo III. This enzyme recognises the blunt 3' terminus of the flare and target duplex. Stepwise removal of the flare results in release of the Cy5 and the target. Finally, the free target is available to remove another flare from the surface of the SNA increasing fluorescence readout.

5.3.2 *In-silico* design of detection probes

Ebola virus has an average of 19 000 bases of a single-stranded RNA. This length is similar between the main three viral species, including SUDV, BDBV, EBOV and the closely related Marburg virus [176] . In this section, the design of the recognition elements for the assay is described.

An algorithm for *in-silico* simulation was designed and developed with the assistance of Dr Eleanor Gray from UCL and Dr Chris Gray from Cambridge

University. The algorithm processed the whole viral genome and identified fragments specific to SUDV compare to other viral species. The simulation exported the whole RNA sequence for SUDV, BDBV, EBOV and Marburg virus from NCBI RefSeq viral database [173] [175]. Then, it converted the RNA to cDNA. A 60 mer cDNA was selected as the output fragment. This length was restricted by the synthesis properties of the SNA.

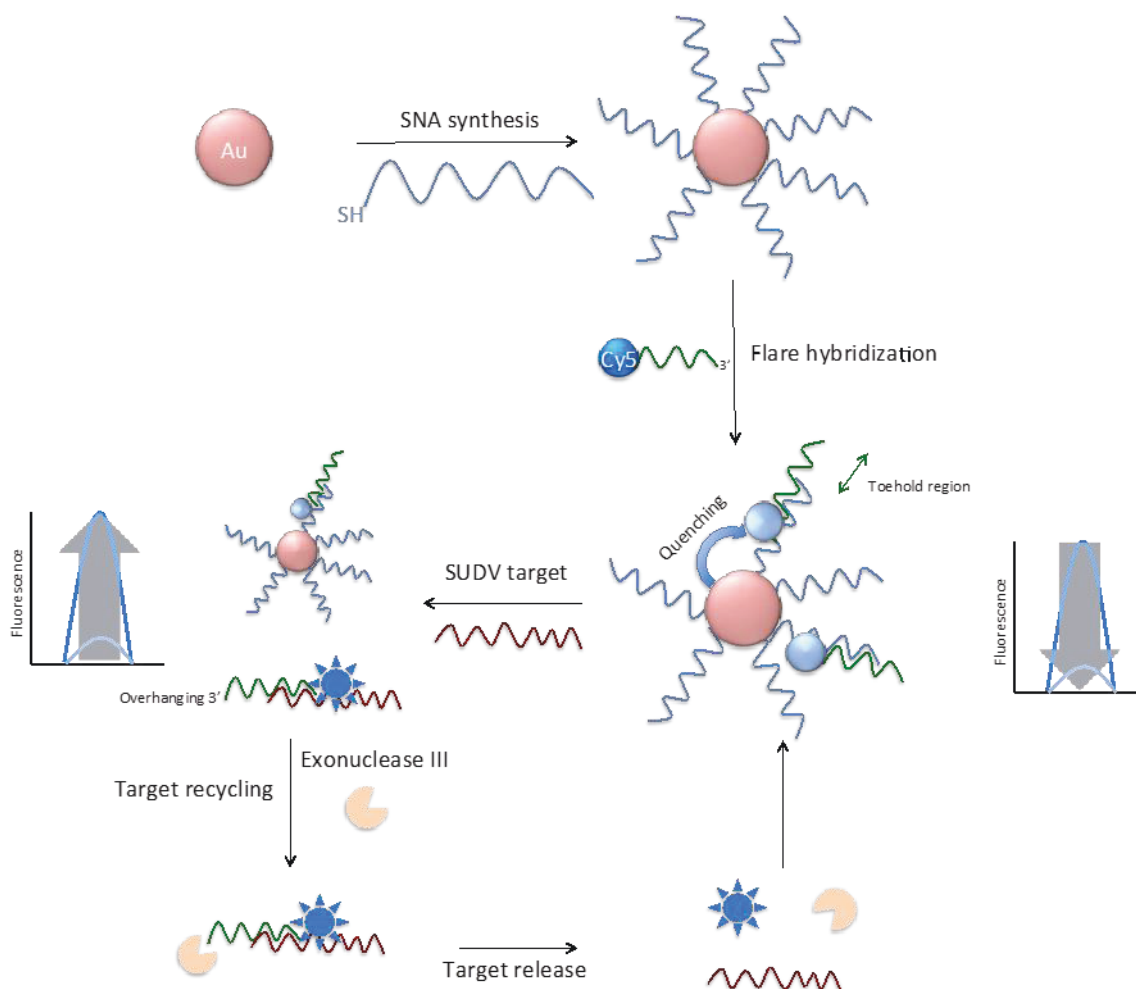


Figure 5.3. Schematic illustration of the assay. From top right, the pre-assay steps consist of preparation of the SNA by conjugating AuNPs to thiolated recognition sequence and flare hybridisation to the DNA sequence onto the surface of the SNA. The closely located AuNP quenches fluorescence emission of the flare. The SNA-flare complex is introduced to the target oligonucleotide, which removed the flare from the surface of the SNA through the toehold region. The toehold is on the same flare strand as the dye. Following this step, the fluorescence emission of Cy5 is increasing. Target/flare duplex presents an overhanging 3' terminus on the flare strand specific to Exo III. Target recycling is achieved by digestion of the flare

sequence with Exo III. Then, the free target removes additional flare and the fluorescence readout of the system increases.

In principle, this assay was designed as an early stage diagnosis test for Ebola virus in human. Hence, to exclude any potential cross reactivity with other components of the human sample, the identified 60 mer sequences were compared with the human genome. This comparison was included in the simulation that produced BLAST analysis.

The *in-silico* simulation analysis yielded in 100 sequences for each viral species with four scores. Each score, in the scale from 0 to 32, corresponded to the level of difference between the identified sequence and the whole cDNA genome of other viral species. The higher the score corresponded to higher difference.

The top 5 scored sequences for BDBV, SUDV and EBOV are presented in Table 5.1, 5.2, and 5.3, respectively. In addition to BDBV and EBOV, Marburg virus was selected and used as a control species for the design of the system. Marburg virus genome and symptoms are similar and closely related to Ebola virus it is therefore essential to design a system that has the ability to differentiate between Ebola virus and Marburg virus.

Table 5.1. BVDV top scored 60 mer sequences

	Sequence (5'-3')	Difference score			
		BVDV	SUDV	EBOV	Marburg
1	TTTTACGAACTTTATCCGGGGAGGGCTTATG CCCTGTCGAACC AAAGTGCAGCCAATTC	0	31.5	24.03	30
2	TTTCAGGATTAAGGCTGCTAGTGCCTCAATC AGAAGATACCGAGACCTCAACCTACACCG	0	31.5	19.03	31.5
3	TTCATCTTTTGTAGCTCCACTCTGAGTCACA ACATAATTGAGTTTTTGTCTCAGAACAGTT	0	31.5	30.98	29
4	TTTATCCGGGGAGGGCTTATGCCCTGTCGAA CCAAAGTGCAGCCAATTCTTAGATGAAAT	0	31.1	18.04	31.5
5	TTTTGTAGCTCCACTCTGAGTCACAACATAA TTGAGTTTTTGTCTCAGAACAGTTATCAGG	0	31	29.02	29.5

The identified sequences via the *in-silico* simulation were then analysed to predict their secondary structure. Formation of secondary structures may reduce the availability of the recognition elements to bind the target and therefore reduce the efficiency of the assay. As such, the aim was to select the oligonucleotide with the lowest probability to form secondary structures.

In-silico secondary structure analysis was performed on the previously selected sequences. This analysis predicted the free energy of each sequence to form any secondary structure. Additionally, equilibrium map that predicted the likelihood to form these secondary structures with the required free energy was produced.

The sequences that presented the lowest likelihood to form secondary structures and had the highest free energy were selected. The secondary structure maps for the selected sequences for BDBV, SUDV and EBOV are presented in Figure 5.4, 5.5 and 5.6, respectively. The selected sequences are labelled in red in Table 5.1, 5.2 and 5.3. For BVDV, sequence number 5 exhibited the highest free energy ($-6.37 \text{ kcal mol}^{-1}$). For SUDV, sequence number 2 exhibited the highest free energy ($-4.76 \text{ kcal mol}^{-1}$). For EBOV, sequence number 2 exhibited the highest free energy ($-6.52 \text{ kcal mol}^{-1}$).

All assay components were designed based on sequence number 2 for SUDV sequence and presented in Table 5.4. Thiolated probe and flare were the recognition elements of this assay. The former one was in the synthesis of the SNA. The 15 mer flare included two complementary sequences to hybridise to the SNA (labelled in red) and to form a duplex with the target (labelled in purple). The complementary region of the flare/target was longer than flare/probe, and this enabled disassociation of the flare in presence of the target. Finally, the 5' terminus of the flare was conjugated to Cy5 and a blunt 3' terminus for the digestion of Exo III.

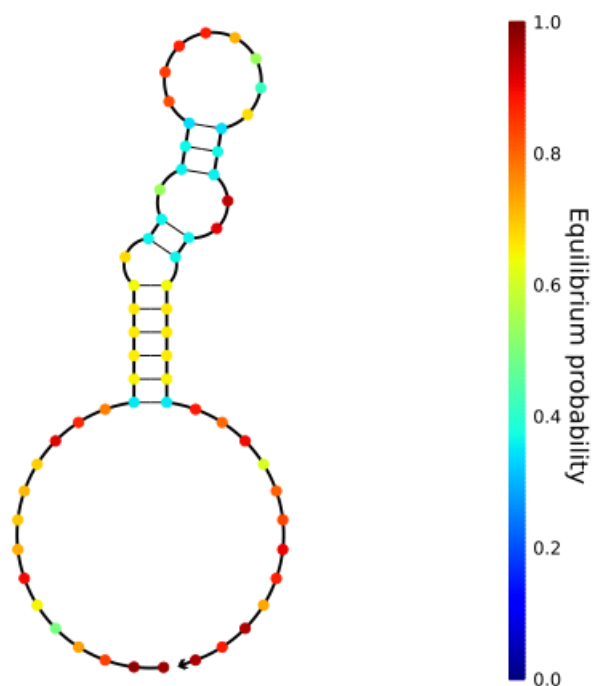


Figure 5.4. In-silico prediction of secondary structure of the selected BVDV DNA oligonucleotide at 25°C and 50 mM NaCl. Predicted free energy of the secondary structure -6.37 kcal mol⁻¹.

Table 5.2. EBOV top scored 60 mer

	Sequence (5'-3')	Difference score			
		BVDV	SUDV	EBOV	Marburg
1	TGATGTACCAGTGGCGACATTGCCCATAGAT TTCATAGTCCCAATTCTTCTCAAGGCACT	23	20.5	0	30
2	GTGATGTACCAGTGGCGACATTGCCCATAGA TTCATAGTCCCAATTCTTCTCAAGGCAC	23	20.5	0	30.5
3	AGTGATGTACCAGTGGCGACATTGCCCATAG ATTCATAGTCCCAATTCTTCTCAAGGCA	22	20.5	0	30
4	TGTTATAAATTATGCAGTTGCACTGTTCTGA TATTAAATTTAGAAACACTGAGGCTACAGA	21.2	23	0	28.5
5	GCCAAATAACAGACATAACAAATCCTACTT GGTTTAAAGACCAAAGAGCAAGGCTACCTA	21	20.4	0	26.5

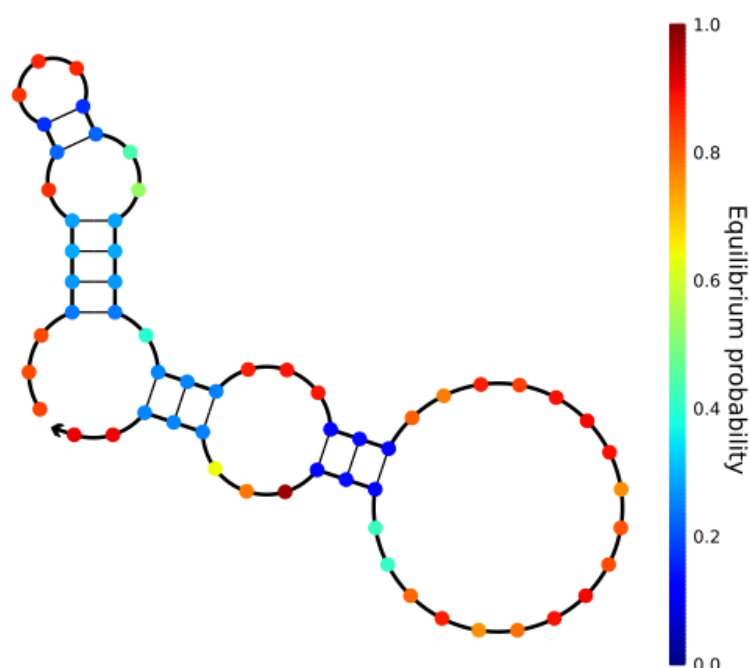


Figure 5.5. In-silico prediction of secondary structure of the selected EBOV DNA oligonucleotide at 25°C and 50 mM NaCl. Predicted free energy of the secondary structure -4.76 kcal mol⁻¹.

Table 5.3: SUDV top scored60 mer sequence

	Sequence (5'-3')	Difference score			
		BVDV	SUDV	EBOV	Marburg
1	TTTACCTGAGAGCCTACAACATGGATAAAC GGGTGAGAGGTTTCATGGGCCCTGGGAGGAC	32	0	29.99	30.5
2	GTTTACCTGAGAGCCTACAACATGGATAAA CGGGTGAGAGGTTTCATGGGCCCTGGGAGGA	32	0	30.96	30.5
3	GAGAGCCTACAACATGGATAAACGGGTGAG AGGTTTCATGGGCCCTGGGAGGACAATCTGA	31.8	0	29.98	30.5
4	TTCCAAGATTGACTTCAATCCAAACACCTTG CTCTGCCAATTTTCATCTCCTTAAGATAT	31	0	30.01	29
5	TTACCTGAGAGCCTACAACATGGATAAACG GGTGAGAGGTTTCATGGGCCCTGGGAGGACA	31	0	29.97	30

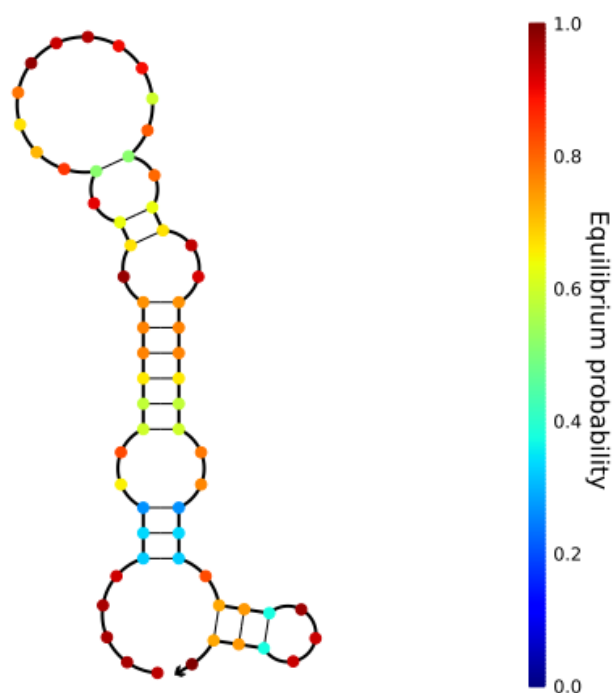


Figure 5.6. In-silico prediction of secondary structure of the selected SUDV DNA oligonucleotide at 25°C and 50 mM NaCl. Predicted free energy of the secondary structure -6.52 kcal mol⁻¹.

The formation of SNA is conditional to the length of the functionalised thiolated oligonucleotide [162] and therefore the thiolated probe was constructed of 20 bases. This sequence included a complementary region of 13 base pairs with the flare sequence. In addition, seven adenine base pairs were included to provide a spacer between the surface of the AuNP and the binding region. A 20-30 mer length is the restriction of an oligonucleotide to form stable SNA[162].

Prior detecting a full-length target, a 60 mer target was utilized for the development of the assay in order to simplify the system. Two 60 mer control targets were designed for BDBV and EBOV. The short target probe was used to optimise the assay prior the addition of the Exo III.

Table 5.4. Probes for the SUDV nucleic acid assay. Purple colour corresponds to the hybridisation regions between the sequences (1), (2) to (4). Red region represents the hybridisation region between (3) and (4).

Sequence name	DNA Sequence
1 SUDV target	5' - GTT TAC CTG AGA GCC TAC AAC ATG GAT AAA CGG GTG AGA GGT TCA TGG GCC CTG GGA GGA - 3'
2 Short SUDV target	5'- ACA ACA TGG ATA AAC GGG TGA GAG GTT C-3'
3 Thiolated probe	5' - GGG TGA GAG GTT C - (A) ₇ - C3- propylthiol - 3'
4 Flare	5'- Cy5- GA ACC TCT CAC CCG TTT ATC CAT GTT GT -3'
5 BVDV control	5' - TTT TGT AGC TCC ACT CTG AGT CAC AAC ATA ATT GAG TTT TTG CTC AGA ACA GTT ATC AGG - 3'
6 EBOV control	5' - GTG ATG TAC CAG TGG CGA CAT TGC CCA TAG ATT TCA TAG TCC CAA TTC TTC TCA AGG CAC - 3'
7 Thiolated control probe ('other')	5' - TCG TCG ACA ACG GCT CCG GCA T- (A) ₇ - C3- propylthiol - 3'

5.3.3. Hybridisation test of the selected probes

The specificity of the hybridisation between the designed oligonucleotide probes is described in this section. Melt curves of different combinations of probe duplexes were recorded in presence of SYBR-green and presented in Figure 5.7. In principle, a melt curve represents change in the fluorescence emission of SYBR-green as a result of duplex confirmation change (i.e. double, single stranded), which occurs as a result of temperature change.

Figure 5.7 displays the melt curves of the following pairs for oligonucleotides: (3) with (4), (3) with (2) and (4) with (1). As the temperature increased and the double stranded molecules of DNA began to dissociate and released the intercalated SYBR-green molecules. Release of the dye reduced the fluorescence emission of the system and generated a dissociation curve.

Two inflection points were identified at 64 °C and 77 °C for (3)/(4) duplex and (4)/(2) duplex, respectively. At these temperatures, 50% of the duplexes were disassociated. As a control, the thiolated probe (3) was tested with the target (2) and the resulting curve exhibited an absence of inflection point corresponding to the inability of these two strands to form a duplex. This result confirmed that non-specific interaction might occur between the target and the SNA.

The melting temperature of the tested duplexes was calculated as the first negative derivation of the melt curve. The maximum points in the curves in Figure 5.8 correspond to the melting temperature of each tested duplex. The recorded melting temperature were 64 °C, 32 °C and 77 °C for the tested duplexes, including thiolated probe + flare, thiolated probe + SUDV target and flare + SUDV target, respectively.

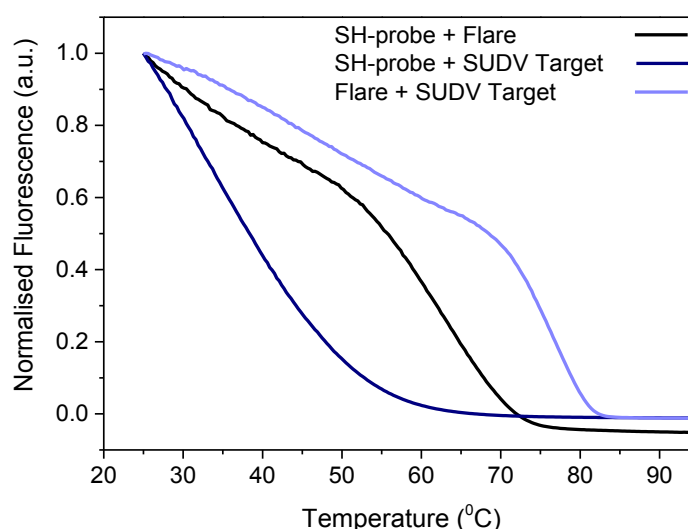


Figure 5.7. Melt curves show the relation between fluorescence and temperature of free DNA oligonucleotides strands, thiolated-probe, flare and SUDV target. Samples were mixed with SYBR-green; excitation of the samples at 498 nm and florescence emission was recorded at 525 nm. Reduction reported in fluorescence during increase of the temperature through the dissociation of the double stranded DNA. Inflection points in the curves correspond to the duplex disassociation temperature.

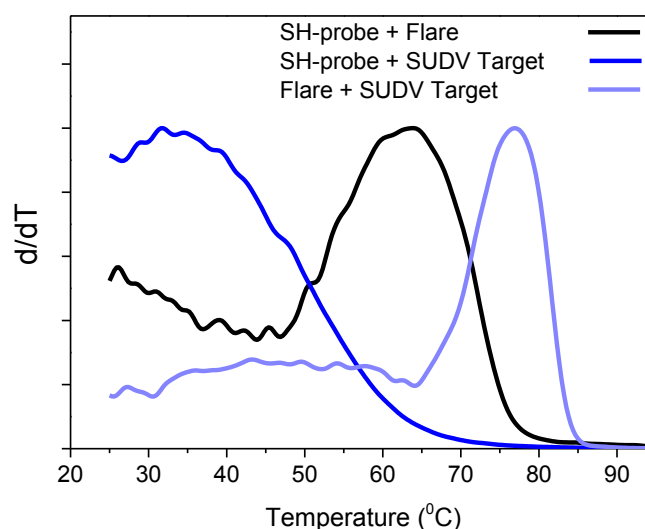


Figure 5.8. Derivatives melt curves show the relation between the negative first derivative of the melting curve and temperature of free DNA oligonucleotides strands, thiolated-probe, flare and SUDV target. The peak of the curve corresponds to the temperature of dissociation of double stranded DNA, defined as 50% dissociation, T_m .

The melting temperatures (T_m) of the possible duplexes in the assay were tested and are presented in Figure 5.9. The sequences of the oligonucleotides in the assay were designed so that the SUDV target and flare duplex had the highest affinity to hybridise and therefore demonstrated the highest T_m .

The sequences of the thiolated probe and flare duplex exhibited high stability with a T_m significantly above room temperature. The resulting T_m of the duplex comprised the thiolated probe and flare (64 °C) was lower than the T_m of the duplex formed with the flare and SUDV target (77 °C). This temperature difference confirmed that the flare/thiolated probe might be disassociated in presence of target SUDV.

The specificity of the flare to SUDV target was tested against BDBV and EBOV targets. The binding of the flare to EBOV and BDBV exhibited relatively low T_m , 26.5°C and 32.5°C, respectively, suggesting a low possibility to bind. The melt curve analysis demonstrated that the hybridisation of the flare to target was selective over other control targets. Moreover, the SUDV target and

thiolated probe duplex exhibited at T_m of 31.96 °C and therefore had low possibility to form.

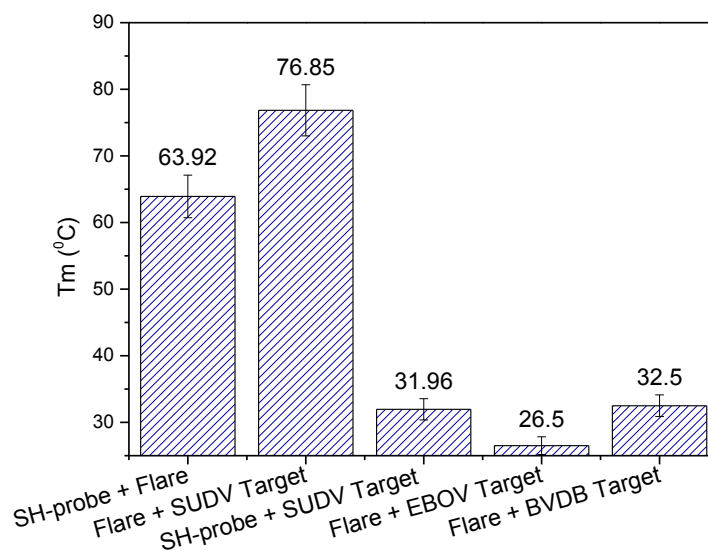


Figure 5.9. T_m of sets DNA oligonucleotides obtained from melt curves represents the temperature of the dissociation of the 50% of the double stranded DNA.

5.3.4. Synthesis and characterisation of SNA

The synthesis of the SNA was carried out with thiolated oligonucleotide (sequence 3) and 13 nm AuNPs. Through the salt aging procedure, the concentration of NaCl was gradually increased to 300 mM, which changed the colour of the solution from red to purple. Such change colour usually corresponds to reversible or irreversible aggregation of AuNPs. Here, reversible aggregation occurred as a result of high salt concentration. High salt concentration reduced the repulsion between oligonucleotides and induced hybridisation regardless to their sequence. Because these oligonucleotides were functionalised on the surface of AuNPs, this hybridisation resulted in cross linkage between the particles. The washing procedure confirmed this explanation, as multiple washes of the SNA into water changed the solution colour back to red, as demonstrated in Figure 5.10. Essentially, disassociating the bound oligonucleotides released the links between the particles.



Figure 5.10. Image of SNA synthesised with thiolated oligonucleotide probe (3), from right to left: SNA in synthesis solution (purple), 300 mM NaCl, PBS pH 7.4 and 0.01% Tween 20, SNAs in water (red).

Absorbance spectra of the synthesised SNA after each washing step were acquired and are presented in Figure 5.11. After three washing steps, the shape of the absorbance spectrum narrowed and shifted back to the blue range. This indicated the disassociation of the cross-linked particles and confirmed the aggregation was reversible.

Three washing steps were sufficient, as the absorbance spectra remained similar between the 3rd and the 4th wash. After three washes, the SNA exhibited similar optical properties as the bare AuNPs in water. The SPR peak was recorded at 529 nm, a shift of 3 nm compare to bare AuNPs (525 nm). This shift corresponded to the conjugation of the thiolated oligonucleotides onto the surface of the particles.

The SNA synthesis yield was found to be 90% and was performed by comparing the resulting concentration of the prepared SNA to the stock concentration of AuNPs. Small loss of particles occurred during multiple washing steps, as expected.

Additional SNA was prepared by using the thiolated control probe, (sequence 7) and referred as other SNA. The absorbance spectrum of SUDV SNA

was compared to the spectrum of other SNA and presented in Figure 5.12. The SPR peak of SUDV SNA (in PBS), other SNA (in PBS) and bare AuNP (in water) was at 529 nm, 527 nm and 525 nm, respectively.

The control probe had additional 10 bases compare to the SUDV probe. This difference in SPR between the SUDV SNA and other SNA might as a result of change in the length of the oligonucleotide. However, it would have been expected that the SUDV SNA exhibit shorter SPR wavelength than other SNA. This may be explained by the fact that sequences above 30 bases cannot be used for SNA synthesis due to high probability of forming secondary structures. Hence, the other probe consisted of 30 bases that might have bound in a non-monolayer configuration. Such configuration prevented the oligonucleotides from being linear which may reduce the SPR peak.

The colloidal stability of the synthesised SNA was studied by recording their size distribution. DLS distribution by intensity was recorded for the SUDV SNA, other SNA and bare AuNPs, as presented in Figure 5.14.

Single peak indicated on a single particles population with PDI of 0.242, 0.196 and 0.263, for SUDV SNA, other SNA and bare AuNPs, respectively. Z-average was obtained for SUDV SNA, other SNA and bare AuNPs, 24.73 nm, 27.69 nm and 21.77 nm, respectively. Finally, the diameter of the particles was also calculated using the DLS volume formula producing the following diameter the 44.31 nm, 64.45 nm and 30.12 nm for SUDV SNA, other SNA and bare AuNP, respectively.

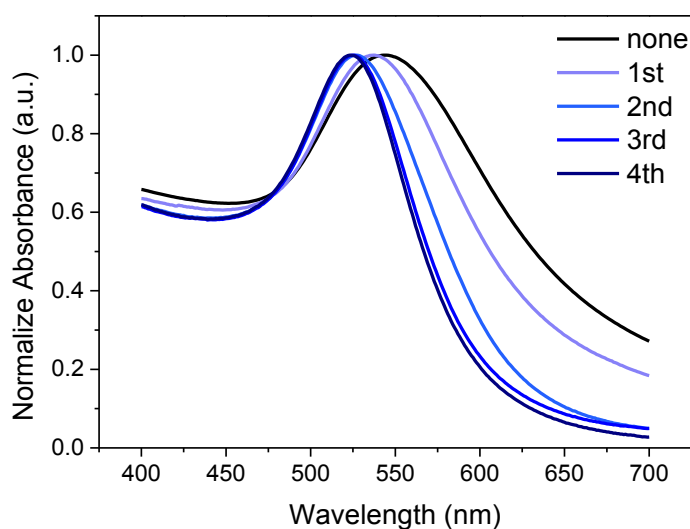


Figure 5.11. Normalised absorption spectra of SNA synthesized with SUDV thiolated oligonucleotide (3) after each wash into water from the synthesis solution. Blue shift of the spectra occurs through the washing process.

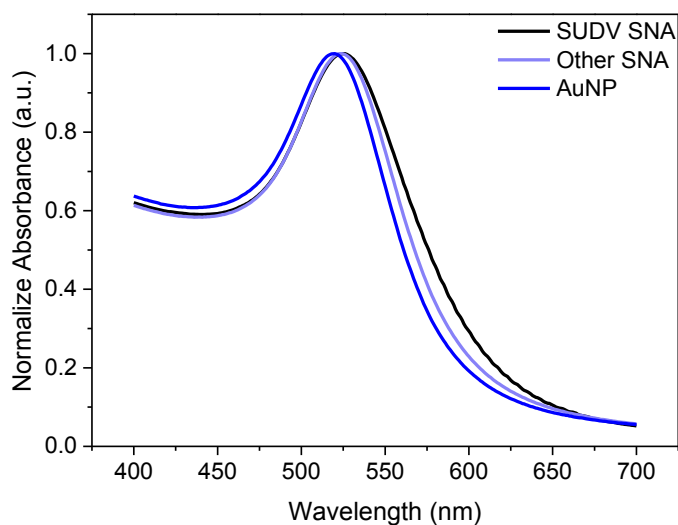


Figure 5.12. Normalised absorption spectra of two populations of SNA synthesized with (3) and (7) and bare AuNPs used for the SNA synthesis.

The size difference between the SNA and the bare AuNPs represented a confirmation for the functionalisation of the oligonucleotide probes onto the surface of the bare AuNPs. Moreover, the diameter of the other SNA was almost twice larger than the SUDV SNA. This may confirm the previous hypothesis that

the sequence forms a non-monolayer configuration significantly increasing the size of the particle.

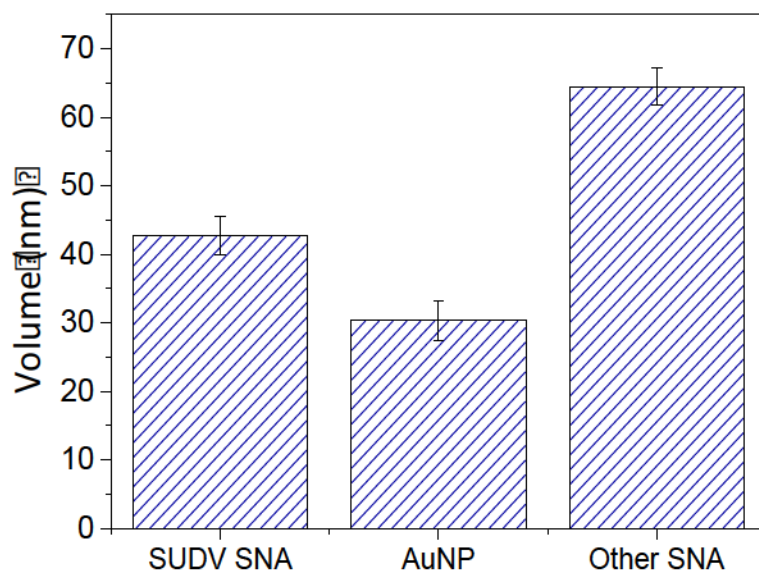


Figure 5.13. Particles average volume measured by dynamic light scattering of bare AuNP, SNA synthesized with SUDV thiolated oligonucleotide (3) and SNA synthesized with control thiolated oligonucleotide (7). Data obtained from three independent experimental particles' synthesis repeats, error bars represent $\pm$ diameter in nm ($n=3$).

The morphology and topography of the synthesised SUDV SNA was investigated with Transmission Electron Microscope (TEM) and is presented in Figure 5.15.

Sample preparation of the SNA was achieved by drop casting and drying the sample at room temperature. As such, sample drying may induce artefacts, including nanoparticles aggregation [177]. However, the presence of a thin film around individual particles is related to the drying artefacts of the sample. For these reasons, the diameter of the SNA was not measured with TEM technique. Alongside with the size measurements, the TEM images illustrate the successful formation of SNA and show a single size population as confirmed from observations of the DLS analysis. The obtained TEM images demonstrate the spherical shape of SNA with a consistent morphology. The grid observed in Figure 5.15D corresponded to gold lattice plane as often observed in high-resolution TEM images.

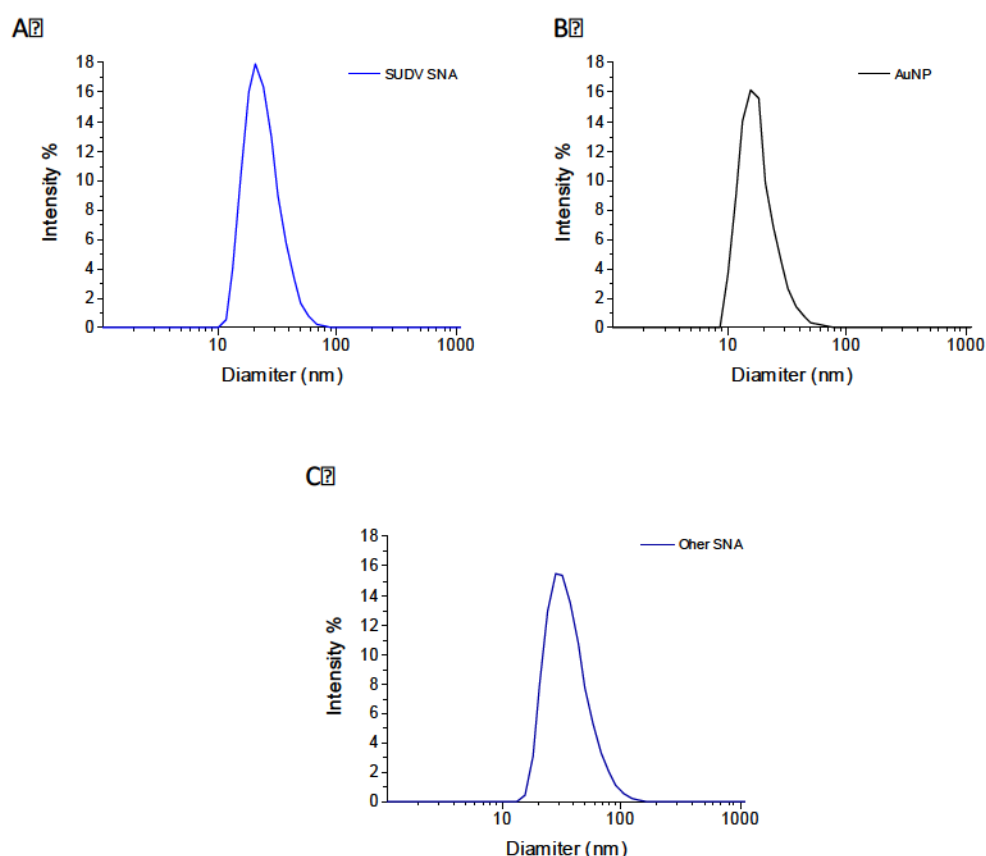


Figure 5.14. Dynamic light scattering intensity distribution curves of (A) Bare AuNPs, (B) SNA synthesized with SUDV thiolated oligonucleotide (3) and (C) SNA synthesized with control thiolated oligonucleotide (7).

5.3.5. Assay development and assembly

In the assay FRET between Cy5 and AuNPs was designed to be the reporting signal correlated to the amount of target. Distance between the FRET components is imperative for efficient energy transfer. Hence, SNA/flare hybrid was designed as such to bring Cy5 as close as possible to the surface of the AuNPs.

Figure 5.16 demonstrates the absorbance spectra of SUDV SNA, the flare and the fluorescence emission spectrum of the flare. The overlap between the dashed black spectrum and the blue spectrum demonstrates the quenching ability of the Cy5 with 13 nm AuNPs. In addition, Cy5 spectra corresponded to

the literature values, and confirm that binding the dye to the flare sequences retained its optical properties.

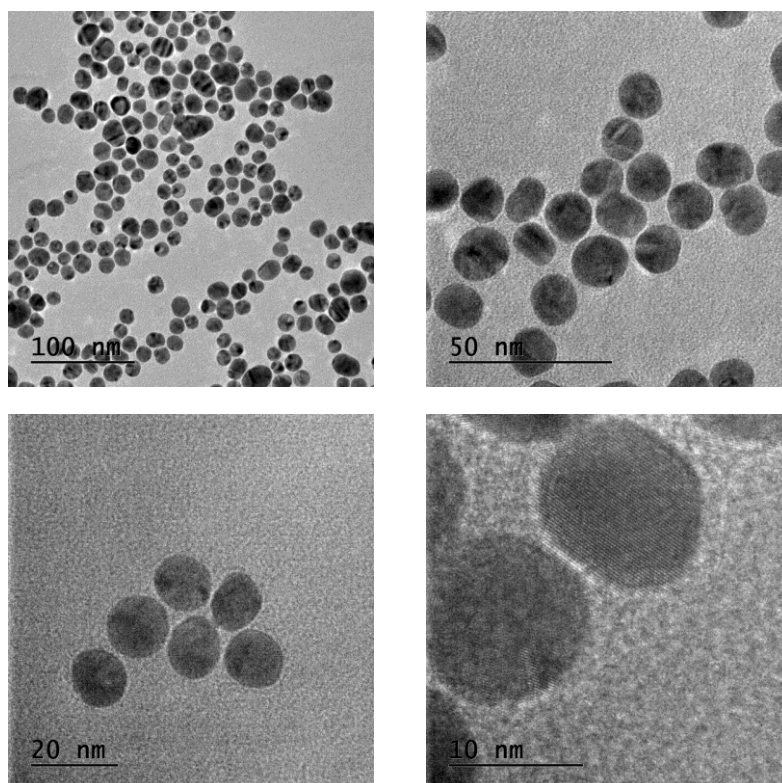


Figure 5.15. TEM images of SNA synthesised with SUDV thiolated oligonucleotide, using four different magnifications. Scale bar from top left, 100 nm, top right 50 nm, bottom left 20 nm and bottom right 10 nm.

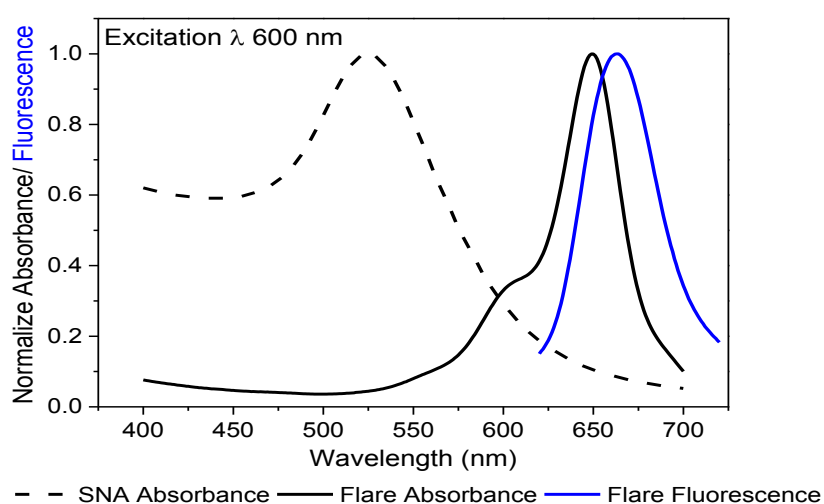


Figure 5.16. Normalised absorption (black) and fluorescence emission (blue) spectra of SNA (dashed line) and Cy5 flare (solid lines) in PBS. Excitation at 640 nm.

To study of the emission range, fluorescence emission of the flare was recorded in the range from 40 μM to 1 nM, and presented in Figure 5.17. Interestingly, the fluorescence emission of higher concentrations, 40 μM and 1 μM , was observed to be lower than the emission obtained from 500 nM. This may occur as a result of self-quench. Self-quenching is usually observed in high concentrations of fluorescence dyes, such concentrations form short distances between dye molecules that causes self-FRET [178].

The maximal fluorescence emission was recorded at 500 nM of flare. However, quenching 500 nM of flare might require high amount of SNAs, which might reduce the sensitivity of the assay. As such, the concentration range of the flare for the assay optimisation was determined between 0 nM to 50 nM.

A characterisation study to identify the optimal amount of SNA used in nucleic biosensing assays was conducted by Mirkin *et. al*[163]. Based on this study, 1 nM of SNA was used thought out this chapter. Here, 1 nM of SNA was mixed with an excess of flare in the following ratio 1:50, 1:20, 1:10, 1:8, 1:6, 1:3, 1:2 and 1:1. These ratios of flare were within the previously selected detectable range.

As a control the flare was also tested in presence of non-complementary SNA that consisted of oligonucleotide of a similar length to SUDV probe, referred as complementary SNA. Upon hybridization between the flare and the SNA, the fluorescence emission of the system was recorded at 665 nm after excitation at 640 nm and presented in Figure 5.18.

The fluorescence emission of Cy5 was significantly reduced in presence of SUDV SNA. The quenching ratio varied between different ratios of the flare and was the lowest at 50 fold excess of flare. The control SNA induced a significantly lower quenching. These results suggested and confirmed that the flare was specifically binding to SUDV SNA. Nonetheless, the quenching effect was more significant when the concentration of the flare was below 50 nM. This might imply that the surface of SNA could accommodate below 50 copies of flares.

Finally, 8 fold excess of flare produced sufficiently low quenching outcome of the flare and therefore were used throughout this chapter.

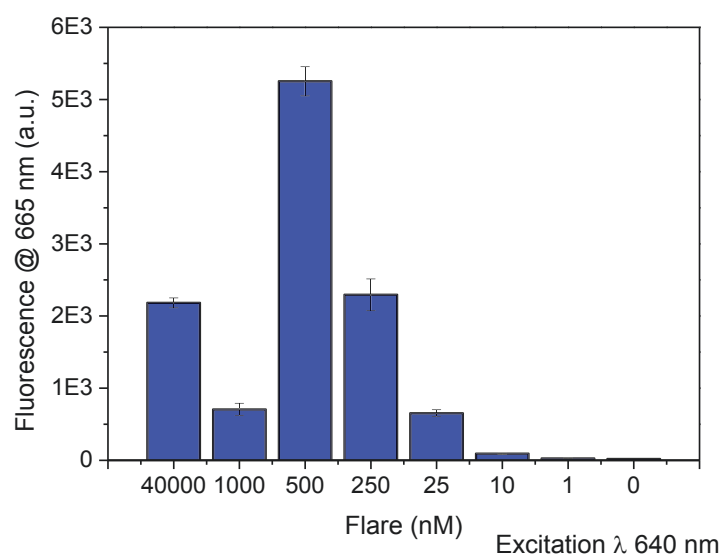


Figure 5.17. Absolute fluorescence emissions at 665 nm of different concentrations of Cy5 flare in PBS. Data obtained from three independent experimental repeats in triplicates. Error bars represent $\pm$ a.u. ($n=3$). Excitation at 640 nm.

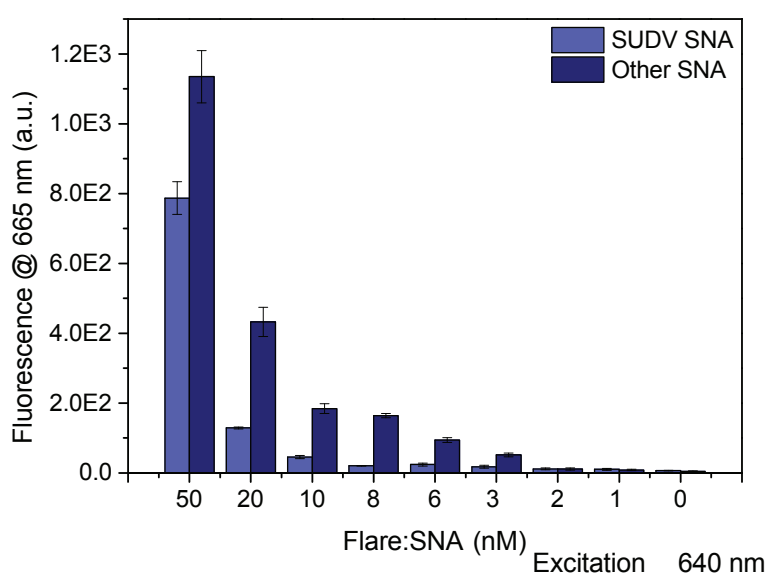


Figure 5.18. Absolute fluorescence emissions at 665 nm of different titer ratios of Cy5 flare and SNA (1 nM) with complementary oligonucleotide (light blue) and non-complementary oligonucleotide (dark blue). Data obtained from three independent experimental repeats in triplicates. Error bars represent $\pm$ a.u. ($n=3$). Excitation at 640 nm.

5.3.6. Detection of short target

The assay operation was initially investigated with simplified version of the SUDV target, short of 30 mer target. The longer target was designed to enable the target recycling amplification. Prior addition of the target, the SNA and the flare were hybridised and the fluorescence emission of the mix was obtained and referred as the set point at $t=0$.

Free SNA and free flare were used as negative controls to investigate any non-specific changes in the fluorescence upon target addition. Hybridising the flare onto the surface of the SNA resulted in nearly 78% quenching. This was obtained from the set point analysis and calculated as the ratio between flare/SNA duplex and free flare, see Figure 5.19. Free SNA was referred as the background of the system.

Upon addition of target SUDV oligonucleotide, the target was expected to disassociate the flare to form target/flare duplex. Removal of the flare from the surface of AuNP restored the fluorescence emission of Cy5 and, therefore resulted in emission increase. Hence, the intensity of the emission was directly proportional to the amount of the added target. Different concentrations (10000 nM, 1000 nM, 100 nM, 10 nM, 1 nM, 0.1 nM, 0.01 nM and 0 nM) of short SUDV target were applied to a mix of SNA/flare, free flare and free SNA.

A dose response curve was produced and is presented in Figure 5.20. As a control target was also added to free flare and free SNA, whose fluorescence emission remained constant. Fluorescence emission of free flare was referred as the maximum readout value.

Fluorescence was increased with the amount of added target when mixed with flare/SNA duplex. Above 1000 nM, the assay reached the *plateau* region rapidly. However, concentrations below 10 nM retained low fluoresce after a longer incubation time. In practice, less than 10 nM analyte was not sufficient to result in any readout. Hence, the lowest concentration to be detected within this system was evaluated to be 10 nM.

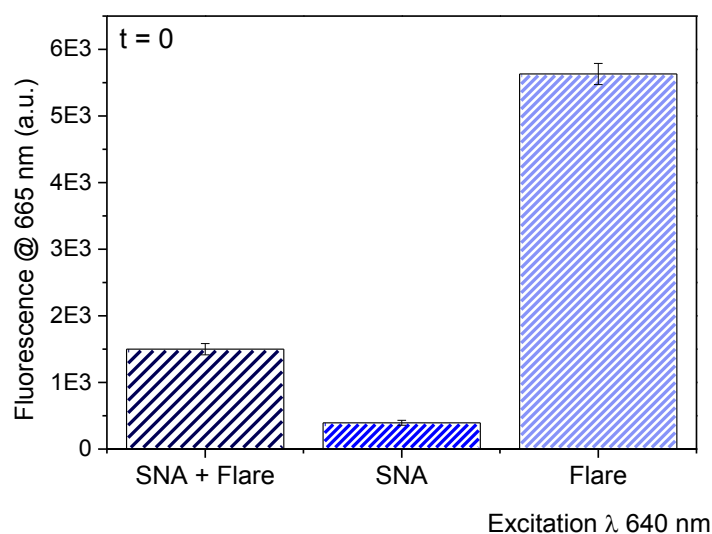


Figure 5.19. Initial set point of the assay prior target oligonucleotide addition. Absolute fluorescence end-point measurements obtained from three independent experimental repeats in replicates of $n=8$ independent experiments. Error bars $\pm$ a.u. Excitation at 640 nm.

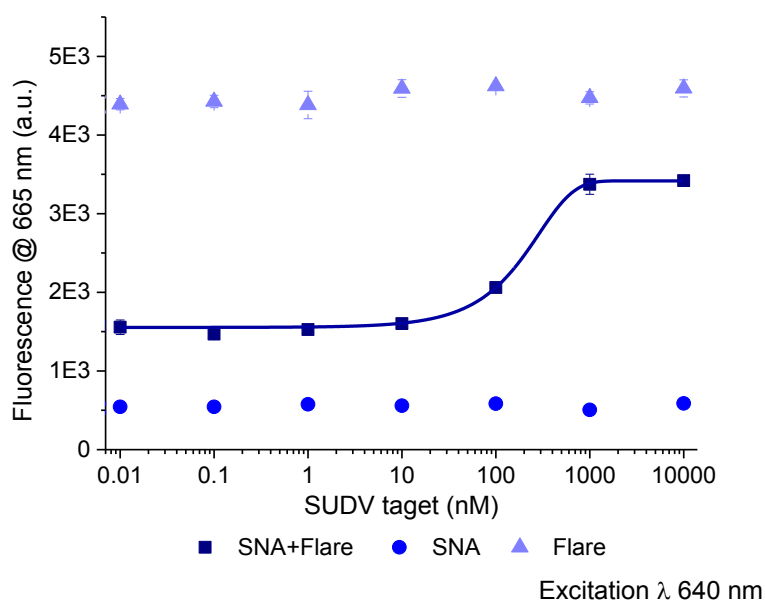


Figure 5.20. Logarithmic dose response curve. Absolute fluorescence emission recorded at 665 nm upon 60 minutes incubation of the target SUDV oligonucleotide (1) with initially hybridised SNA + flare (square), SNA (circle) and free flare (triangle) in PBS at room temperature. Measurements obtained from three independent experimental repeats in triplicates, error bars represent $\pm$ a.u. Excitation at 640 nm. Dose response fitting, Model SGompertz ($y = a \cdot \exp(-\exp(-k \cdot (x - x_c)))$), $R^2 = 0.99276$. Error bars $\pm$ a.u. Excitation at 640 nm ($n=3$).

The hybridisation kinetics was investigated to determine the required incubation time for the assay. Reaction completion was defined as the time takes to reach the *plateau* region. A 10000 nM of target caused reaction completion within less than 2 minutes, whereas 1000 nM required nearly 30 minutes. Lower concentrations did not reach completion. 10 nM was saturated after 56 minutes and therefore the 1 hr was determined as incubation time at room temperature.

5.3.7. Detection of full 60 mer SUDV target

The quantitative readout of the developed assay was calculated as de-quenching ratio of the Cy5, by subtracting and dividing the fluorescence of the blank sample from the fluorescence emission of tested sample. The de-quenching readout was directly proportional to the amount of target.

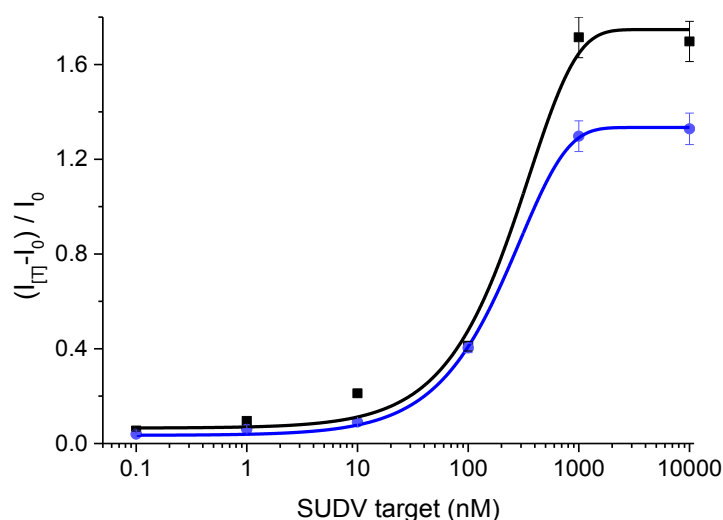


Figure 5.21. Comparison of binding of short SUDV oligonucleotide target (1), blue and full length SUDV oligonucleotide target (2), black to SNA (synthesised with thiolated SUDV oligonucleotide (3)) previously hybridised to flare in PBS. Normalised logarithmic dose response curves of different target concentrations after 30 minutes incubation at room temperature. The data presented as the fluorescence emission at 665 nm of each measurement normalised by black. Target added Nonlinear Curve Fit (ExpDecay1), with $R^2 = 0.9982$ (black) and 0.98497 (blue). Measurements obtained from three independent experimental repeats in triplicates, error bars $\pm$ a.u. Excitation at 640 nm. Bars indicate mean $\pm$ standard deviation ($n=3$).

The detection of long and short target sequence was compared and is presented in Figure 5.21 in relative de-quenching units. The limit of detection was 10 nM for both the short and long sequence.

This dose response exhibited that the developed assay can detect specifically both 60 and 28 bases target. As such, the operation of the assay for detection of 60 mer targets was similar to shorter target.

5.3.8. Calculation of the required clinical range for the assay

Ebola virus nucleic acid signatures are usually detected with real-time and quantitative PCR. The result of these assays is reposted in viral RNA load units. Viral RNA can be detectable starting from 24 to 48 h from the onset of the Ebola virus infection. During the 2000-1 SUDV outbreak in Uganda, Jonathan S. Towner *et al.* [97] tested infected patients and determined that at least 6,200 RNA load per ml was present in serum of acute patients [142]. To estimate the required clinical range the viral loading was translated into the molarity of the target using Equation 1,

Equation 1:

$$\text{Viral RNA (M)} = \frac{\text{Length} \cdot 500 \text{ (g mole}^{-1}\text{)} \cdot C \text{ (molecules ml}^{-1}\text{)} \cdot 10^3 \text{ (ml L}^{-1}\text{)}}{6.0221 \cdot 10^{23} \text{ (molecules mole}^{-1}\text{)}} ,$$

$$\text{cDNA (M)} = \text{Viral RNA} \cdot \frac{660}{500} ,$$

$$\text{60 mer DNA target (M)} = \text{cDNA} \cdot \frac{60}{\text{Length}} ,$$

where,

Length – length of SUDV viral genome (18,875 bases),

C= Threshold of viral RNA copies in serum, of infected patients (6200 copies).

Based on the above calculation, the limit of detection of the assay should be around $4 \cdot 10^{-13}$ M (pM-aM range) and an amplification stage is required to be included in the assay.

5.3.9. Addition of Exonuclease III to the assay

Target recycling was selected as an amplification step to improve the limit of detection of the developed system. Exo III was selected to perform the target recycling through removal of the flare from the target/flare duplex. Exo III binds to the 3' blunt ended DNA duplex 3' terminus and then digests that DNA strand. The oligonucleotides of the assay were designed such to enable the digestion of the flare from the flare/target duplex.

To achieve the stability and catalytic activity of the enzyme, special conditions must be applied in the assay operation. These conditions include presence of enzymatic co-factor, Mg^{+2} , and suitable buffers. CutSmart® Buffer is recommended to provide such conditions [179]. Moreover, testing the activity of Exo III in PBS exhibited no catalytic products, implying that PBS inhibited the catalytic activity of the enzyme.

5.3.9.1 SNA stability in CutSmart® Buffer

The colloidal stability of the SNA was tested in different dilutions of the CutSmart® Buffer and the absorbance spectra is presented in Figure 5.22. SNA aggregation occurred when the dilution of the buffer was above 1:8, suggesting that the stability of the SNA significantly reduced under the conditions of this buffer.

The SPR peak was red shifted and the spectra was broaden in presence of the CutSmart® Buffer. Nonetheless, the SNAs maintained their optical properties (SPR peak) and colloidal stability when the buffer was diluted by a factor of 5. In addition, Figure 5.23 presents the kinetics of the SNA aggregation. Complete aggregation in non-diluted CutSmart® Buffer occurred instantly. Although dilution by a factor of 5 of the buffer caused partial and slower aggregation of the particles enabling the operation of the assay in 1:5 dilution of CutSmart® Buffer over 1 hr incubation.

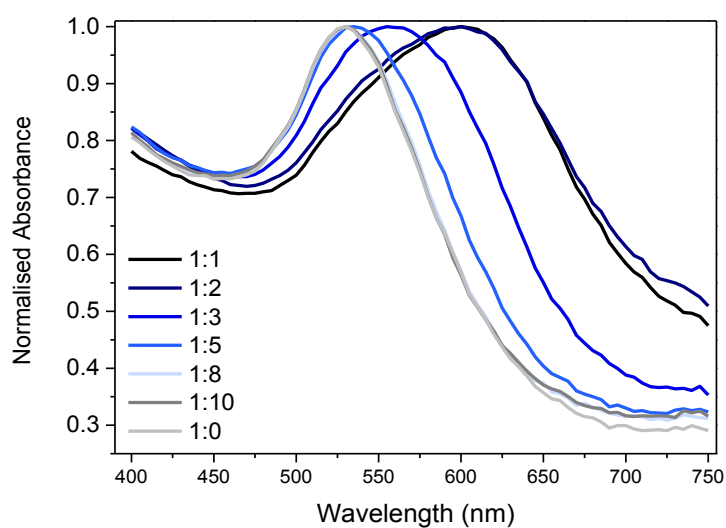


Figure 5.22. Normalised absorbance spectra of SNA in different dilutions of CutSmart® Buffer in water. Spectra obtained after 1 hr incubation at room temperature.

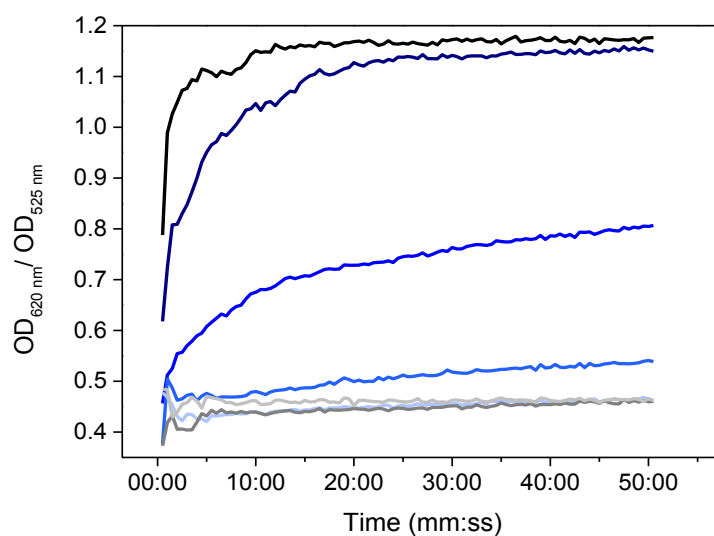


Figure 5.23. SNA aggregation kinetics over 50 minutes in the following dilutions of CutSmart® Buffer and water, from top line to bottom line: 1:1, 1:2, 1:3, 1:5, 1:8, 1:10, 1:0. Data obtained from the ratio of the absorbance at 620 nm and 525 nm at room temperature.

5.3.9.2 Dose-response and specificity test

To reduce the interaction time between the SNA particles and CutSmart® Buffer, SNA/Flare duplex was prepared in PBS and diluted with 1:5 diluted CutSmart® Buffer prior the assay operation. This preparation procedure improved the stability of the particles.

Different concentrations of target sequences were added to the mix. As a control BDBV and EBOV target sequences were used. Target dose-response curves, see Figure 5.24, were obtained testing target concentrations varying from 10 μ M to 1 pM.

The analytical readout signal was presented as the de-quenching, corresponded to the difference between the fluorescence intensity of the tested concentration, I_T , and no target (blank), I_0 , divided by blank. Increase in the readout signal was recorded above 10 nM, of target, as previously described.

The dose-response curves with BDBV and EBOV remained below the assays cut-off, suggesting no non-specific binding of targets of other viral species to the flare.

The overall readout signal of the assay was proportionally higher in 1:5 CutSmart® Buffer compare to PBS.

This result strengthens that previously designed recognition oligonucleotides demonstrated high specificity for the detection of target and differentiation ability between the three viral species.

5.3.9.3. Dose response upon enzyme addition

The desired clinical range of this assay was evaluated to be at the pM scale. Addition of the target-recycling step was aimed to improve the limit of detection to this scale. Using Exo III enabled target-recycling amplification. This provides linear release of flares and increases the overall fluorescence.

To understand the kinetics of the system, Exo III was incubated with SNA/flare duplex for 10 hr. The fluorescence emission was measured and normalised by the background noise produces by a free SNA and presented in Figure 5.25.

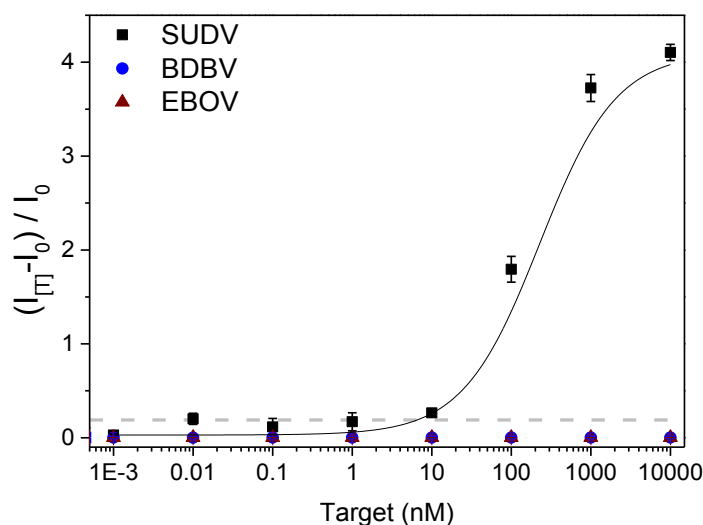


Figure 5.24. Selectivity of the assay between three Ebola virus species. Dose response between the concentrations of subtype-specific 60 mer sequences and the relative fluorescence intensity. Range of concentrations, 10 μ M, 1 μ M, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM and 1 pM, of SUDV, BDBV and EBOV were tested after 1 h incubation with SNA/Flare hybrids in 1:5 dilution of CutSmart® Buffer. Cut-off was calculated as the average of three independent measurements of SNA/Flare with three standard deviations. The limit of detection was determined as the lowest concentration presenting readout signal higher than the cut-off. No non-specific interaction occurred between the BDBV and EBOV targets and the SUDV SNA/Flare construct. Nonlinear Curve Fit (ExpDecay1), with $R^2 = 0.9767$. Bars indicate mean $\pm$ standard deviation ($n=3$). Excitation produced at 640 nm and emission recorded at 665 nm.

The normalised fluorescence of SNA/flare/Exo III was recorded at the initial set point ($t=0$) and after 10 hr incubation at room temperature. The normalised fluorescence doubled during the incubation period. As controls, the normalised fluorescence of SNA/flare and free flare without Exo III were also at the same time points. The analytical readout of the samples without Exo III remained constant upon 10 hr. Moreover, the constant emission of free flare suggested low photobleaching over the tested time.

This study demonstrates that Exo III has non-specific activity to non-blunt 3' terminus, as it digests the bound flare to the SNA surface. To reduce

this non-specific activity, different amount of Exo III were tested and exhibited similar results.

Long incubation period can significantly improve the efficiency of target-recycling. Nonetheless, in this assay rather short incubation period (1 hr) was crucial to control the non-specific activity of the enzyme.

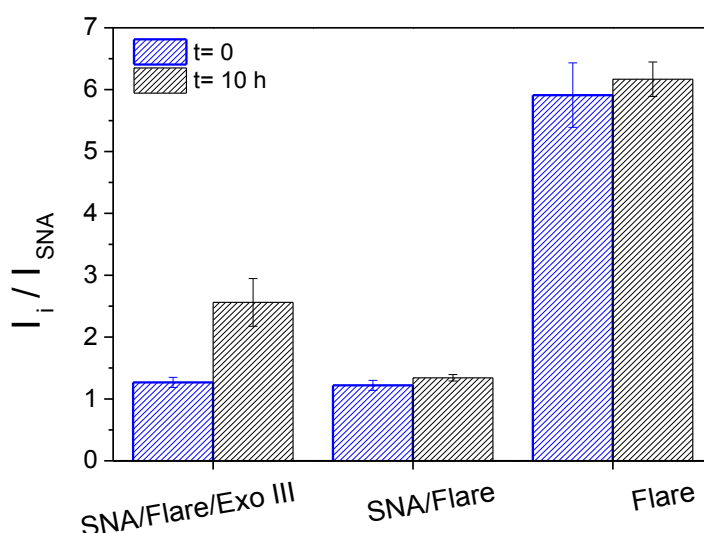


Figure 5.25. Normalised readout of SNA/flare/Exo III, SNA/flare and free flare at the set point (dash blue) and after 10 h (dash black) incubation at room temperature. The readout presented at the ratio between the fluorescent emissions of the tested sample to the background noise produced by free SNA. The readout of DNA/Flare/Exo III doubled over 10 h while SNA/Flare and free flare remained constant. Bars indicate mean $\pm$ standard deviation ($n=3$). Excitation produced at 640 nm and emission recorded at 665 nm.

Target dose response curve was produced with titer of a range SUDV target concentrations, from 10 μ M to 1pM. The target was incubated for 1 h with a mix of SNA/flare/Exo III. As a control target was also added to SNA/flare, the readout was presented as de-quenching intensity, see Figure 5.26.

The background noise of the samples containing Exo III was significantly higher than the background noise without the enzyme, supporting the previous conclusion that non-specific activity of Exo III occurs. As such, cut-off for the two systems was evaluated independently. Having Exo III in the assay improved the

limit of detection of the assay from 10 nM to 1 nM, a relatively low improvement that was below the desired clinical range. Nonetheless, this increase in the limit of detection advocated that the conditions of the assay were suitable for the target-recycling mechanism.

The specificity of the assay with Exo III was investigated by testing the assay performance with 10 nM of SUDV target compare to a higher concentration of BDBV and EBOV target. The controls targets were applied in a concentration 1000 higher than the actual target to investigate the selectivity and potential non-specific interactions with non-complementary sequences.

The readout of both BDBV and EBOV was below the cut-off, presented in Figure 5.27. Hence, these results exhibit the selectivity of the assay between 10 nM of SUDV target compare to non-complementary 10 μ M of BDBV and EBOV target. This implies that the developed assay can distinguish between Ebola viral species regardless to their concentration. The sensing mechanism is only sequence-specific, which was the aim of the recognition elements design part.

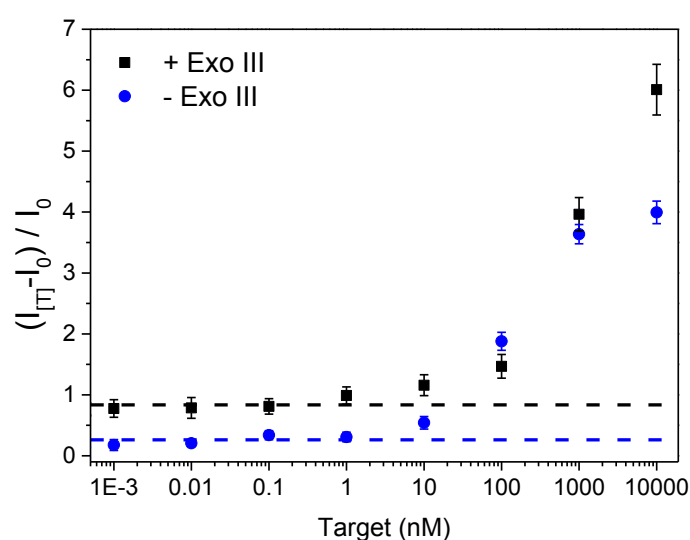


Figure 5.26. Dose response curves of the assay with and without Exo III. Dose response of the relative intensity at different SUDV target concentrations, 10 μ M, 1 μ M, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM and 1 pM, with (black) and without (blue) Exo III. Relative intensity evaluated as the difference between the fluorescence emission of the tested target and the blank normalised by blank. Bars indicate mean $\pm$ standard deviation (n=3). Excitation produced at 640 nm and emission recorded at 665 nm.

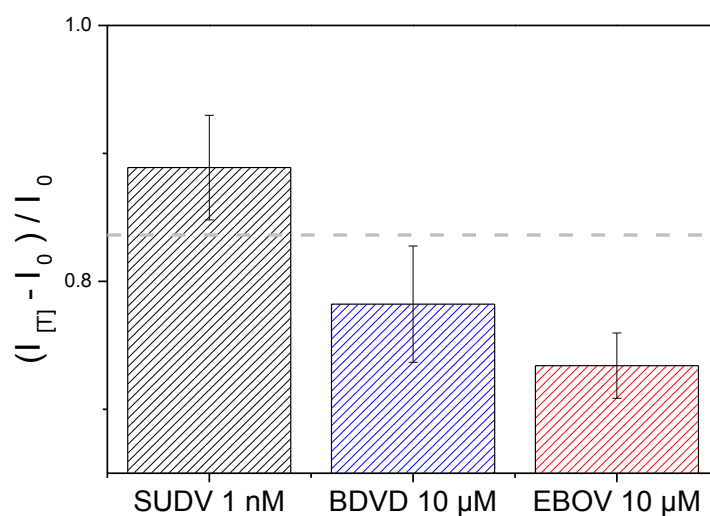


Figure 5.27. Specificity of the assay: SNA/Flare/Exo III incubated with 1 nM SUDV (black), 10 µM BDBV (blue) and 10 µM EBOV (red) for 1 hr. Both EBOV and BDBV readout was below the cut-off of the assay (with Exo III). Relative intensity evaluated as the difference between the fluorescence emission of the tested target and the blank normalised by blank. Bars indicate mean $\pm$ standard deviation ($n=3$). Excitation produced at 640 nm and emission recorded at 665 nm.

5.4. Conclusions

This thesis reviews the development of two assays. The first assay implements affinity recognition elements and dry paper platform to detect antibodies. The second assay utilises multiple nucleic acid elements for target recognition and operates in solution. As previously reviewed, the diagnosis of Ebola virus requires detection of host's immunity and direct detection of viral components (e.g. antigen and nucleic acids). Hence, this thesis addressed this requirement through the development of two complementary assays.

This chapter reviews the design, development and optimisation steps for the nucleic acids assays. The novelty of this study was the integration of SNA particles with Exo III to produce an amplified nano-bio sensing concept. Moreover, application of *in-silico* simulation analysis to design Ebola-specific recognition elements was never presented elsewhere.

Briefly, the assay comprised recognition oligonucleotide on the surface of SNA and hybridised to the fluorescent flare oligonucleotide. The target binds to the toehold region of SNA duplex removing and forming hybrid with the flare. Once flare/target formed, Exo III recycles the target by digesting the flare.

The operation conditions (e.g. 1 hr incubation, room temperature, dilution of 1:5 of CutSmart® Buffer) were selected to enable Exo III enzymatic catalysis and retain the colloidal stability of the SNA.

The limit of detection with Exo III was improved from 10 nM to 1 nM. However, the clinical relevant concentration of the target was estimated to be in between the pM to aM. Also, Exo III had a certain level on non-specific activity that increased the background noise of the assay.

Three requirements were identified prior the designing process and were to be delivered with the final configuration of the assay, included:

- (i) Production of specific assay capable to differentiate between Ebola virus species.
- (ii) Development of an assay that can be operated with basic laboratory equipment, such as the simple fluorescent reader (in contrast to PCR method).
- (iii) An assay that has sufficient limit of detection within the clinical concentrations range of target analyte.

The final state the assay achieved the first two of these requirements but struggled to facilitate the final one.

In particular, the *in-silico* approach for the design of the recognition elements resulted in high specificity to the target species. Here, the recognition oligonucleotides were specific for the detection of SUDV. Using computer-simulation enabled to process the whole viral genome and also identified a wide range of distinctive sequences between the viral species. These sequences were further analysed against human genome to prevent any potential non-specific interactions with the samples' matrix.

The specificity was experimentally investigated and exhibiting assay's immunity to bind any non-complementary sequence, even when tested is 1000

times above the concentration of the target. Furthermore, the assay exhibited high selectivity between other main Ebola virus species, BDBV and EBOV.

An optical analytical transduction was selected to simplify the required instrumentation. Indeed, simple fluorescent reader with the basic set of filter was sufficient for the operation of this assay. For this reason, Cy5 dye and AuNPs were used, as any basic reader includes set of filters to analyse this dye. The change in the fluorescence emission of Cy5 was the analytical readout, which was proportional to target levels [180].

Target-recycling amplification concept was included for the same reason. Compared to a standard PCR amplification, target-recycling is performed at constant room temperature with no need for specialised instrumentation. The limit of detection of the developed assay was not sufficient to fit the clinical range of target concentration and therefore needs to be further addressed. Potentially a different amplification approach should be explored to improve the sensitivity and reduce the background noise. Indeed, some features of this assay can be adapted. For the improvement of other standard assays, for instance PCR primers can be designed based on the identified sequences herein. This adaptation may significantly improve the specificity of standard PCR methods [96] [151] [101].

Although this assay was tested with fragments of DNA targets, it has the full potential to detect real RNA target as the recognition elements were designed against non-complementary DNA (ncDNA).

Chapter 6

Conclusions and outlook

6.1. Conclusions

This thesis presented the application of nanotechnology for the development of two systems with potential applications in medical diagnosis. The breadth of implementations of nanotechnology extends traditional concepts of technology. The advent of nanotechnology has brought many advances to the biomedical field. More than ever, nanomaterials have been translated from other areas of science and implemented in bionanotechnology. Thanks to the rapid growth of the biosensing technologies in the medical field, diagnostics have become less invasive, more affordable, faster and accurate.

The objective of this thesis was to build a comprehensive, simple and cost effective methodology for the detection of Ebola virus. The standard diagnosis of EVD consists of two components. In particular, the development and application of two biosensing projects for the detection of biomarkers related to Ebola virus was described. In the first project, a novel serological POC was developed and, in the second project, a nucleic acids bioassay based on SNA was built. These two types of tests are complementary and equally necessary for the diagnosis of EVD. The SNA-based bioassay is usually used for early stage diagnosis while the serological test can be used to predict recovery and survivor.

The multidisciplinary work presented in this thesis can be classified into two disciplines, engineering and virology. The engineering work included the design, development and optimisation of generic biosensing systems. The virology study included the implementation of the built systems for the detection Ebola virus. The work presented herein was produced during the time of the largest EVD outbreak (2014) and was addressing the reported global need for better diagnostics [100]. Hence, the key impact of this thesis was to develop

a set of tools for the detection of Ebola virus during the pandemics.

Chapter 2 presented the design, development and optimisation of generic lateral flow and smartphone immunoassay for the detection of Ebola virus antibodies. In the course of this study, genes for multiple viral proteins, including SUDV – GP₁₋₆₄₉, VP40 and NP – and GP from both BDBV and EBOV, were cloned with His₆, expressed with their full length and purified in collaboration with the Laboratory of Virology and Immunology, Ben-Gurion University. These proteins were then characterised and printed on the test lines as recognition elements of the lateral flow test strip.

Next, the AuNPs functionalisation process was optimised and characterised with DLS, UV-vis and TEM to determine the colloidal stability in complex media and the presence of the secondary antibody on the surface of the AuNPs. The conditions for optimal assay performance were further determined and included using BSA as blocking agent, operation time, serum as specimen, sample volume of 20 µl and the sequence of the spotted test lines in the multiplex format. Finally, the work presented in this chapter included optimisations of two formats for the assay for a single analyte and up to three analytes detection.

A complementary Android smartphone application was developed to produce quantitative analysis of the strips. Smartphone technology was selected over a standard reader because they are usually cheaper and can be used portably and independently from source of power [181]. Moreover, additional features, such as geotagging, Cloud data storage and share, of the smartphone can be employed for the test.

Following the assay development, a serum database was established from collected samples of the outbreak in 2000-2001 in Uganda, and from human survivors in the years following infection. SUDV was selected as model viral species due to a relatively recent and large outbreak that yielded well documented records of survivors [132]. This database included sample of survivors and non-infected controls closely related to the survivors.

The work presented in Chapter 3 described a study focused on characterising the human humoral immune response against individual viral protein of SUDV (GP₁₋₆₄₉). Samples from this database were used for the

evaluation of the developed test. In addition, Chapter 3 included fieldwork that was performed in 2015 in Uganda during which more samples were collected and tested on the spot. In this case the smartphone analysis was imperative as it produced a geographical map presenting all the test samples of this study.

In the first part of Chapter 3, a total of 91 SUDV survivors and 32 non-infected controls were tested via the monoplex assay, out of which 65 survivors demonstrated positive IgG immune response, while all 32 negative controls showed no immunoreactivity. The results of the assay were obtained within 15 minutes with 100% and 96% specificity and sensitivity when compared to standard ELISA.

In the second part of Chapter 3, the multiplex was also evaluated in a small pilot study to examine the profile of humoral immune-reactivity of SUDV survivors. A total of 15 SUDV survivors and 5 non-infected controls were tested with the SUDV multiplex assay. This yielded the detection of IgG immune response in over 86% of the tested survivor, while all negative controls showed no immunoreactivity to the three tested viral proteins.

In the third part of Chapter 3, a proof of concept of the species multiplex assay demonstrated the identification of the infecting EVD species of 6 survivors from SUDV, BDBV and EBOV.

Chapter 4 demonstrated that such technological platform could be readily translated to another Ebola virus species. In particular, the developed assay was adjusted for BDBV. Then, the assay was used to analyse the humoral immune response of 10 survivors and 10 controls from the 2007-8 BDBV outbreak in Uganda. In addition, this chapter explored the immune cross reactivity of BVBD survivors against the three viral species. A total of 5 samples were examined with the multiplex assay showing the nearly 50 % of the BDBV survivors exhibit cross reactivity to EBOV. The results were comparable to the ELISA analysis with a simultaneous, simple, robust and accurate detection of immune cross reactivity.

Chapter 5 reported the development steps of a solution based biosensing assay for nucleic acids. SNA were used as the interface for the detection of target oligonucleotide and target-recycling amplification was introduced via Exo III. This chapter described the design, optimisation and characterisation of the

assay and its components. The recognition elements were designed with *in-silico* simulation analysis of the Ebola virus genome to identify regions with high specificity to each viral species. Using computer-based simulation enabled to process the whole viral genome of three species by comparing the resulted sequence between the species and to Marburg virus. The SUDV specific SNA were synthesised and characterised for their physic-optical properties and colloidal stability with UV-vis, DLS and TEM.

The limit of detection of the assay with the target-recycling amplification was improved from 10 nM to 1 nM target oligonucleotide with ultra high specificity to SUDV. Despite the improvement, this detection ability was not sufficient for the clinical range of the target analyte, which was estimated at pM-aM range. The non-specific activity of the Exo III might be responsible for reducing the sensitivity of the assay and needs to be further addressed.

6.2. Outlook

In Chapter 2, the characterisation of the performance of the developed strip was carried out with a single population of antibodies. Although it is acceptable for research purposes, to yield a sound diagnostics it will be useful to analyse a larger population of antibodies (polyclonal and monoclonal). This can be achieved through the recently formed collaboration with the laboratory of USAMRIID that have the facilities for the production of such antibodies. Also as the result of the latest outbreak there are more EVD antibodies available for purchase.

The evaluation of the assay, developed in Chapter 3, was performed only with samples obtained from EVD survivors, further evaluation must include testing samples from acute patients. Moreover, the specificity of the assay needs to be further validated with other viral diseases from the same region. It is particularly important to confirm the absence of nonspecific or cross-reactive detection of serum antibodies in survivors of Marburg virus, Lassa fever, Crimean-Congo hemorrhagic fever, Yellow fever, malaria, all of which are endemic in Africa and likely develop clinical symptoms similar to EVD.

The assay is designed to measure only IgG, what might impair its use in

early acute situations, therefore the detection of the virus would be more suitable, until the humoral immune response is detectable. However, extending the concept of the developed assay to detect IgM antibodies might make the test suitable for acute situations.

Because of the flexibility of the platform, data regarding host immune response to Ebola virus could be collected from a large sample of individuals and could provide information regarding suitable treatment and maintenance of infected individuals. Surveillance screening for humoral response to Ebola virus is crucial for epidemiological studies to understand the patterns of disease spread and in rapidly identifying its source. Furthermore, the rapid identification of relative Ebola virus specific-IgG in convalescent patients can provide potential options for evaluating the suitability of a survivor as a plasma donor for therapeutic purposes [139].

This system could also be adapted for studying Ebola infection in animals. Ebola is a zoonotic disease, and transmission from infectious non-human reservoirs is a severe risk and impediment to Ebola disease control. However, many unknowns remain with respect to Ebola reservoirs. Making a simple and cost-effective serology tool available to field researchers studying non-human Ebola virus diffusion could yield excellent results in elucidating Ebola virus behavior in its entirety and could support field veterinarians working in non-human primate conservation [182].

In this work, the critical need for faster, simpler and more portable serology tools for Ebola virus has been addressed. This new simple and portable lateral flow-based assay system has demonstrated the ability to detect IgG antibodies in Ebola survivors with high sensitivity and specificity under field conditions. Further work is needed to evaluate specificity and sensitivity in larger cohort groups, in vaccine evaluation studies and further work for shelf life and environmental condition are required.

The technology described in Chapter 5 represents a proof of concept for a generic and easily modifiable nucleic acids detection platform. In order to transfer this system from a laboratory-based process to a user product, three major improvements should be implemented. The reagent preparation needs to be scaled up to analyse a large amount of samples and the operation of the assay

needs to be automated. Indeed, it is currently based on multiple manual steps (eg. Particles washes, SNA/flare hybridisation procedure). Moreover, the tolerance of the assay to biological media should be thoroughly tested for diagnostic purposes, and, if necessary, it should include additional steps to improve the stability of the components of the assay, such as SNA through surface modification.

NPs-based biomarker detection methods are emerging an alternative to traditional methods of detection. These solutions often offer generic design that can be easily translated from one application to another. In this thesis, two such technologies for the detection of Ebola virus were presented. The first technology can be classified as a POC test for single or multiple analytes on-site serological analysis. The second assay was designed for ultra-specific detection and differentiation between nucleic acids of different viral species. Although the second technology is laboratory-based, it was engineered to utilise simple, cheap and accessible laboratory equipment. It is clear that the development and validation of these systems as presented in this thesis is essential and needed. A fact that has been starkly emphasised in the wake of the 2014 Ebola virus West African outbreak.

Reference

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Appendix A

Biosensing

Biosensors are constructed with the following components:

1. Analyte – The target substrate that is desired to be sensed.
2. Biorecognition element or probes – The distinguishing feature that detects the target analyte of the biosensing system. This element determines the specificity and the sensitivity of the system[183]. The classification of these bimolecular sensing constructs depends on their biological entity and grouped in the following categories:
 - i. Biocatalytic Systems – Biological groups with catalytic properties, mainly enzymes. Generally, their enzymatic activity depends on the presence of a substrate, which is directly linked to the analyte. The recognition of the target analyte in these systems is normally within the concentration range of 10^{-1} – 10^{-7} M[184].
 - ii. Affinity Systems – One of the most commonly exploit biorecognition systems is through antibodies and antigens. This system possesses a great advantage of relatively high sensitivity with the ability to detect analyte concentration in the range of 10^{-6} - 10^{-10} M. Antibodies classified as either polyclonal, monoclonal, recombinant, fragmented, single chain or nanobodies[185]·[186]·[187]·[188].
 - iii. Whole-cell systems – Bacteria, yeast and Eukaryotic cells response to the environment can be used as the recognition systems as long as their environmental response is specific to the target analyte of the sensing system. Whole cells can be employed as a real time monitoring of small molecules, hormones and chemical changes [189]·[190]·[191].
 - iv. Nucleic acids - Nucleic acid-based systems are designed to detect their target either through hybridisation with a complementary sequence of bases, a non-linear structure of DNA or RNA or aptamers[192]·[193]·[163].

- v. Synthetic Biomimetic Systems – Synthetic probes offer cheaper, simpler and faster production of recognition elements than natural biomolecules. Imprinted polymers are often used as artificial receptors for proteins[194].
3. Signal generating interface - This component translates the biological recognition into an analytical signal, proportional to the amount of the detected analyte. As such, this feature is usually directly bound to the recognition element and may produce optical, electrochemical, magnetic and other bioanalytical readouts.

This thesis focuses on molecular diagnosis of biomarkers through the development of novel systems for the detection of viral analytes. Chapters 2-4 describe biosensing strategy that is based on affinity construct that use the specificity of antigens to the adaptive immune response of infected individuals. Chapter 5 demonstrates detection of viral nucleic acids via hybridisation to DNA probes.

The detection of any type of analyte requires a mechanism of signal transduction. This thesis will cover one class of signal transduction that is performed via an optical signal (colorimetric and fluorescence). For the purpose of optical signal transduction, a material with changeable emission or absorbance properties must be selected. The detection of the desired analyte is correlated with spectroscopic changes of these interface elements.

Appendix B

Ethics approval for collection and analysis of sample



המרכז הרפואי האוניברסיטאי סורוקה
SOROKA UNIVERSITY MEDICAL CENTER
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Dr. Leslie Lobel
Department of Virology
Faculty of Health Sciences
Ben-Gurion University

July 27th, 2015

Prof. Claris Risenberg
Head of the Infectious diseases unit
Soroka University Medical Center

Dear Prof. Risenberg and Dr. Lobel

This is to confirm that your research proposal entitled "Longitudinal Study of Immune Responses Using Serum and PBMC's of Filovirus Survivors" was approved by our board.

Please be advised that the revised informed consent form relating to this study was also approved.

Prof. A. Danon, Helsinki Committee Chair
The Ethics Committee (IRB) of the Soroka Medical Center.
FWA 00000721
IPF# 3005801-03
Approval number: 0081-14-SOR

Appendix C

Questioner and consent to participant in the serum database.

-CONFIDENTIAL-

Form No.

Personal Questionnaire.

Please fill up the following information:

1. Name: _____ Age: _____

Please mark the correct checkbox:

2. Gender: Male ☐ Female ☐

3. Are you currently showing any illness signs (headache, nausea, a runny nose, muscle pain): Yes ☐ No ☐ **If yes** please elaborate: _____

4. Are you currently taking any medical treatment (pills, antibiotics):
Yes ☐ No ☐ **If yes** please elaborate: _____

5. Have you been hospitalized or visited any medical clinic in the last month:
Yes ☐ No ☐ **If yes** please elaborate: _____

6. Have you ever been diagnosed as **HIV** positive Yes ☐ No ☐ **If yes** when _____

7. Have you ever been diagnosed with an autoimmune disease (Celiac, Crhone, Rheumatoid arthritis, else) Yes ☐ No ☐ **If yes** please elaborate _____

8. Do you have any chronicle diseases (Diabetes, Hepatitis, else):
Yes ☐ No ☐ **If yes** please elaborate: _____

9. Have you ever been diagnosed with a mental disorder (schizophrenia, Parkinson, Depression, else) Yes ☐ No ☐ **If yes** please elaborate (which, and what medication are you taking) _____

10. Have you ever been diagnosed with any type of cancer: Yes ☐ No ☐ **If yes** please elaborate (which cancer and treatment) _____

11. Have you ever undergone any medical surgery: Yes ☐ No ☐ **If yes** please elaborate (which and treatment) _____

12. Have you ever been diagnosed with Anemia Yes ☐ No ☐ **If yes** when _____

Version 1.1
Date 22 June 22, 2014