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Biophysical characterization of the interaction between HIV-p24 protein and the conjugate HIV- p24 derived aptamer

By

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DECLARATION

I, Philisiwe Fortunate MOLEFE (Student no: 201224182), solemnly declare that ***Biophysical characterization of the interaction between HIV-p24 protein and the conjugate HIV- p24 derived aptamer*** is my own work, submitted for the fulfilment of the Master of Science degree in the University of Zululand, has not been submitted for any degree or examination in any University, and that all the sources quoted therein have been acknowledged in detailed references.

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LIST OF ABBREVIATIONS

A_{600}	Absorbance at 600 nanometres
AIDS	Acquired immunodeficiency syndrome
Ala, A	Alanine
Amp	Ampicillin
APS	Ammonium persulphate
Arg, R	Arginine
Asn, N	Asparagine
Asp, D	Aspartic acid
Cv	Column Volume
Cys, C	Cysteine
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
FTIR	Fourier-transform infrared spectroscopy
µl	Microliter
°C	Degrees Celsius
Glu, E	Glutamic acid
Gly, G	Glycine
His, H	Histidine
HIV	Human immunodeficiency virus
Ile, I	Isoleucine
IPTG	Isopropyl β-D-thiogalactoside
KDa	KiloDaltons
LB	Luria Bertani broth
Leu, L	Leucine

Lys, K	Lysine
Met, M	Methionine
mM	MilliMolar
Nm	Nanometre
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
Phe, F	Phenylalanine
PMSF	Phenylmethylsulphonyl fluoride
SELEX	**S**ystematic **e**volution of **l**igands by **ex**ponential enrichment
UV/VIS	Ultraviolet-Visible light

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ABSTRACT

The Human Immunodeficiency Virus epidemic is a serious world health concern. Reports show that 75% of people, who are aware of this disease, live with it, and the remaining 25%, which constitute over 9 million people are still in need of access to HIV testing services, with some waiting for the window period to subside. Early diagnosis is imperative for mitigating viral transmission. Unfortunately, available rapid tools are serologically based and may not detect the virus in time and moreover other methods, such as PCR methods and p24 antigen tests, have limitations resulting in diagnostic delays amongst others. The HIV-p24 protein is overexpressed during the early and last stages of viral infection. This makes it an important biomarker, requiring sensitive and efficient point-of-care diagnostic tools, which are based on biological agents that do not elicit immune response and therefore can diagnose the virus, especially before seroconversion. In this study, two DNA aptamers (agents that bind to their targets with high affinity and specificity) have been successfully synthesized, *in vitro* through systemic evolution of ligands by exponential enrichment (SELEX) and studied in conjunction to the HIV p24 biomarker. Subsequently, bioinformatics was used to characterize the interaction between each aptamer and the target protein which displayed strong interactions. These interactions are crucial in the development of an alternative diagnostic tool which will detect the virus at early stage or window period.

Key words: HIV-p24, aptamers, interaction studies, diagnostics

CHAPTER

ONE

(Introduction and Literature Review)

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

The discovery of the Human Immunodeficiency Virus (HIV) during the 1980s in Africa (Williams *et al*., 2016; Wright *et al*., 2012; Hemelaar, 2012) led to the conception that HIV initially came from the Simian type of the virus, which is found in chimpanzees, known as Simian Immunodeficiency Virus. HIV is well-known for weakening the immune system, impairing its functionalities and thus making the body susceptible to opportunistic illnesses, which then result in a diseased phase known as Acquired Immunodeficiency Syndrome (AIDS) (Quaranta *et al*., 2012). HIV is distinctively separated into subtypes, which are formed as a result of mutations that occur during protein splicing, leading to viral behavioural changes that enable the virus to defy the combating effects of therapeutic agents. HIV-1 is the most prevalent around the world and has been noted for its extensive virulence when compared to HIV-2, which is less virulent and most common in the western part of Africa (Campbell-Yesufu and Gandhi, 2011). However, both eventually result in AIDS, depending on the well-being of an individual, as well as the viral type acquired. The progression of HIV to AIDS leads to premature death of affected individuals (Joshi and Joshi, 1996) and 1.1 million mortalities are still reported irrespective of the percentage (approximately 20 million people) of those with access to antiretroviral treatment www.unaids.org/factsheet (05 September 2017). HIV remains a serious problem and an important research issue https://www.cdc.gov (22 June 2017).

Hence, the diagnosis of HIV during the early stages of infection is beneficial because it allows for early administration of treatment, which then promotes immune recovery and therefore prevents viral progression to AIDS (Zhang *et al.*, 2011). However, available diagnostic tools have failed to detect the virus during its early stages (Zhang *et al.*, 2016) until after the

window period, thus giving the virus enough time to replicate. Such challenges heighten the importance of studying viral proteins, especially HIV-p24; the central focus of this study, also known as the capsid protein, that encloses the viral RNA and its enzymes. Like all other HIV proteins, p24 also participates in the HIV life cycle. All HIV proteins are expressed during viral progression and assemblage into new virions. However, it is noteworthy to mention that the excess levels of HIV-p24 in the blood, during the early stages of infection makes it a biomarker and imperative to research (Tang *et al.*, 2010). In this study, HIV-p24 was studied in conjunction with its derivative DNA aptamers. Aptamers are short oligonucleotide sequences that bind to target non-nucleic acid molecules with high affinity and specificity (Schutze *et al*., 2011). Although their synthesis is through **S**ystematic **E**volution of **L**igands by **Ex**ponential enrichment (SELEX), which is an *in vitro* process that involves multiple steps before development of the final product (Szeto *et al*., 2013), aptamers can be addressed as both chemical and biological agents, since they can be synthesized using either biological (involving biological enzymes) or chemical (addition of chemicals for modification) methods (Mayer, 2009; Famulok and Mayer, 2014). Aptamers are scientifically trending molecules (Jayasena, 1999) used in medicine for both diagnostics as well as for therapeutic purposes. This is due to their structural flexibility, stability, manageable synthesis and modification which enhance them for any intended function (Xi *et al*., 2014). Although they exhibit functions like those of antibodies, aptamers are non-immunogenic and most of all preferably adaptable (through post modifications). They can be readily synthesized chemically, rendering their production as trouble-free and cost effective (Yan and Levy, 2009).

In this study, the expressed, purified and characterized HIV-p24 protein was conjugated to the HIV p24-derived aptamer. Thereafter, the interaction between these bio-conjugates was predicted and characterized, during molecular docking by bioinformatics, to determine the binding scores and area of contact between the aptamer and the target protein. Knowing these interaction parameters is crucial because strong interactions set grounds for further confirmatory studies and thereafter, may potentiate the application of the bioconjugates

towards the development of a novel and alternative diagnostic tool. This will provide immediate diagnosis, even during the window period, since the available lateral flow devices in the market today has failed to detect the infection during this phase.

1.2. Epidemiology

Human Immunodeficiency Virus is a *Lentivirus* by genus, a descendant of a *Retroviridae* family and a subfamily of *Orthoretrovirinae* (Blood GAC, 2016). HIV is distinguished into two major types, HIV-1 and HIV-2 (Fanales-Belasio *et al*., 2010). HIV-1 is related to Simian viruses found in the chimpanzees and gorillas of Central Africa, whereas HIV-2 is somewhat analogous with viruses found in primates called sooty mangabey of West African origin (Sharp and Hahn, 2011; Blood GAC, 2016). HIV-1 is alienated into four groups; M (main), N (non-M, non- O), O (outlier) and P (Santoro and Perno, 2013). Groups N, O and P are only found in western Africa (Maartens *et al*., 2014), whereas M is widely distributed and is further divided into 9 subtypes ranging from A-J. These subtypes include circulating recombinant forms (CRFs) and multiple unique recombinant forms (URFs), which are all found across different geographic regions (Hemelaar, 2012; Cappy *et al*., 2017). Consequently, such diversity of Group M contributes to the high prevalence, morbidity and mortality rates. HIV-1 is well-known for its high virulence, drug resistance as well as for vast dispersion and transmissibility world-wide. HIV-2 is less transmissible, less virulent and mostly found in West Africa. Despite all the similitudes between HIV-1 and HIV-2, both eventually progress to AIDS (Robertson *et al.*, 1995). In 2012 and 2013, about 35 million new cases were reported. Despite the decreased incidence rates, sub-Saharan Africa remains the 'hotspot' for the highest number of people infected with HIV (see figure 1.1; Maartens *et al*., 2014; Blood GAC, 2016; Bystryak and Acharya, 2016).

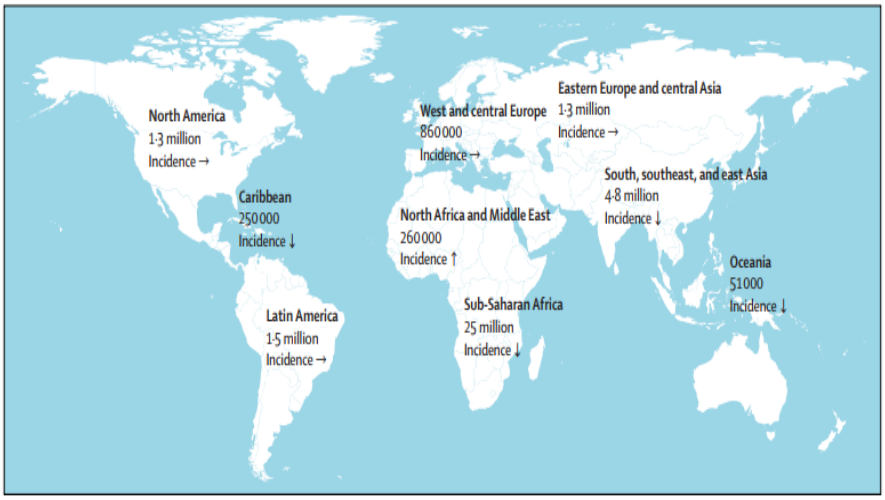

Figure 1.1: Incidence rates of people infected with HIV in 2012. The figure shows the distribution of HIV infection across the globe from 2001 to 2012. (Maartens et al., 2014)

1.3. Genome and characteristics of HIV

HIV is composed of a lipid, bilayered membrane of approximately 120 nm in diameter, which encloses the viral genome. The genome consists of two identical RNA strands, with long terminal repeat sequences (LTRs) affixed on both 5' and 3' ends. The 5' LTR region codes for the promoter responsible for the transcription of viral genes in the 5' to 3' direction (Blood G.A.C, 2016; Cappy *et al*., 2017). These genes are known as gag, pol- and env- and they are produced as polyproteins (Montagnier, 1999; Turner and Summers, 1999). They are well-known signature molecules in all retroviruses (Steffy and Wong-Staal, 1991) coding for the viral structural components. The gag group is responsible for the production of the major structural proteins inside the virus, where it is cleaved during viral maturation to form the following domains; a matrix protein (p17), a capsid protein (p24) and nucleocapsid (p7) protein (King, 1994). Pol accounts for viral enzymes such as reverse transcriptase (RT), which is required for the transcription of DNA from viral RNA, RNase H, Integrase (IN) for integrating viral DNA into the host cell genome and Protease (PR) (Votteler and Schubert, 2008). The virus also consists of regulating elements such as Tat and Rev, which control reverse transcription and the expression of viral proteins respectively and a minimal amount of other proteins, namely Nef, Vpr, Vif and Vpu, which are needed for replication (Mushahwar

et al., 2006; King, 1994). Nef is needed for increased infectivity and apoptosis, Vpr for viral replication, Vif for the synthesis of infectious viruses in lymphocytes and Vpu which is specific only to HIV-1 is needed for $CD4^+$ degradation through the ubiquitin-protease system and facilitation of released virions from infected cells (Montagnier, 1999).

1.4. Structural molecules of HIV

1.4.1. Envelope Proteins

The HIV envelope is composed of a viral glycoprotein 160 (gp160) that is cleaved by intracellular proteins, such as furin protease, to form three homotrimers of glycoprotein 120 (gp120) and glycoprotein 4 (gp4); these heterodimers extend from the viral membrane forming spikes (Hoorelbeke *et al*., 2013; Merk and Subramanian, 2013; Sellhorn *et al*., 2012; McCune *et al.*, 1988). The heterogeneity of the envelope and its ability to transform is a result of the flexibility of the glycoproteins present, causing immune recognition to be quite a hurdle (Galimidi *et al.*, 2015) and thus playing a critical role in viral replication (Wilen *et al.*, 2012). Viral dissemination is initiated by the binding of the surface protein (gp120) on target cells, followed by successive events that trigger the trans-membrane protein (gp41) to fuse the viral and the host cell membranes, allowing entry of the viral genome (Blumenthal *et al.*, 2012; Tran *et al.*, 2012 and Kim *et al.*, 2008).

1.4.2. Matrix proteins (p17)

There are about 2000 copies of the matrix shell found lining the viral membrane. Apart from encapsulating the viral core, the matrix protein plays an important role in inducing natural killer (NK) cell production and the release of cytokines, which normally play their role as the first line of defence in the host cell's immunity. Interestingly, it provides a conducive environment for HIV leading to the rapid proliferation of the virus (Fiorentini *et al*., 2006; Vitale *et al*., 2003). It also interacts with the lipid membrane and is known for its involvement in redirecting viral assembly (Turner and Summers, 1999). The interaction with lipids is facilitated by the N-terminal domain of the matrix protein, which consists of myristic acid and

potentially changes its moiety to allow for lipid binding and for the integration of gp120 and gp41 into newly formed particles.

1.4.3. Capsid protein (p24)

Capsid p24 is a post-translational splicing product of the gag polyprotein (Pr55), which is composed of a matrix protein (MA), nucleocapsid, P6 and the two spacer peptides, SP1 and SP2 (Thenine-Houssier and Valente, 2016). HIV-p24 is a spheroidal core-protein, composed of pentameric and hexameric structures that assemble to form an orderly arranged capsid as delineated in figure 1.2. It consists of a C-terminal domain (CTD), as well as an N-terminal domain (NTD). These domains form associations through a linker region that facilitates dimeric termini to termini interactions responsible for the formation of both immature and mature capsids (Bhattacharya *et al.*, 2014; Scholz *et al*., 2005). The NTD bares the location of the capsid binding pockets, known as protein interfaces involved in nuclear entry. The spread of the virus depends on whether the capsid unravels or becomes nuclear imported, however the exact timing of the uncoating process remains unknown. The nuclear importation has been found to be linked to the capsid protein-protein interfaces to which nuclear cofactors bind and enable entry of the capsid to the nucleus (Price *et al*., 2012). Although the interface site is used to increase viral infection, it is also vulnerable to inhibitors such as the cleavage and polyadenylation specific factor 6 (CPSF6) which binds to reduce the capsid dependence to nuclear cofactors (Price *et al*., 2012; Blair *et al*., 2010). This affects the shuttling of the capsid from the cytoplasm to the nucleus and decreases viral contagion within the host. However, this inhibition is not sustained because p24 can mutate the site to prevent CPSF6 binding (Lee *et al*., 2012; Price *et al*., 2012).

The un-coating and assembly of the capsid protein during entry and viral replication are also held accountable for the propagation of infection (Bhattacharya *et al.*, 2014). Moreover, this accentuates the importance of the capsid to studies directed towards antagonising viral dissemination. Approximately 1000 – 1500 copies of the capsid protein are found in the blood

post-infection (Cabrera *et al.*, 2015). The up-regulated expression of this protein, during the early and late stages of HIV/AIDS and its abundance in the blood during these stages, does not only make it a target for diagnostic means but also for the development of a drug or vaccine (Zhang *et al.*, 2011; Blair *et al.*, 2010). The capsid detection is imperative for early diagnosis and for improving the control of HIV, thereby reducing the window period. HIV-p24 becomes available from as early as 10 to 14 days, and at 3 weeks, its concentrations start to subside when antibodies develop against the virus. Hence, some kits fail to detect its presence during the very early and declining phases, as highly sensitive kits are needed to detect small quantities (Tang *et al.*, 2010).

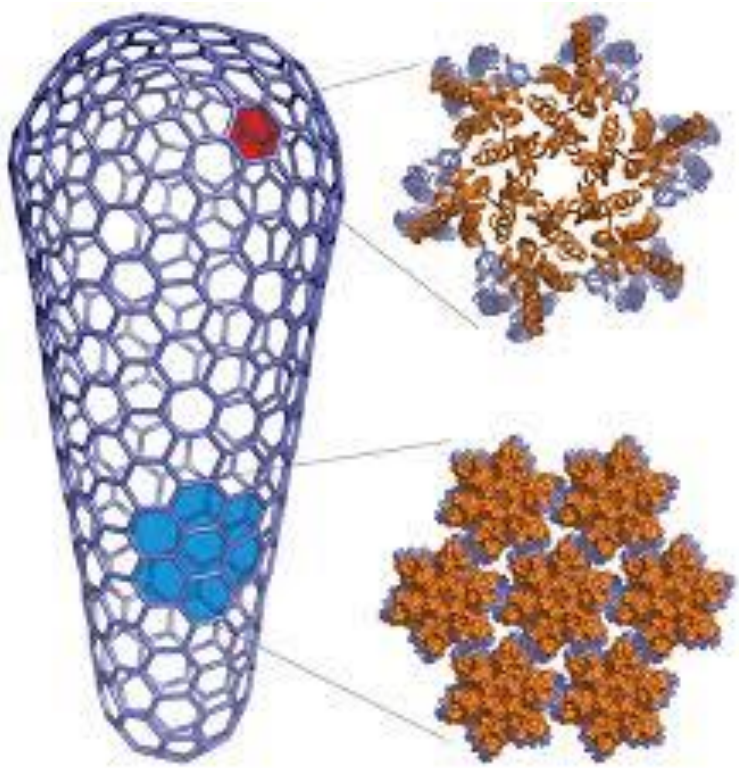

Figure 1.2: HIV capsid. The icosahedral structure of HIV-p24, delineating its pentameric and hexameric structures. Graphics by Owen Pornillos, Barbie Ganser-Pornillos, Kelly Dryden and Mark Yeager. Obtained online at: http://www.hivandhepatitis.com/recent/2011/0128_2011_d.html [Accessed 18 April 2018].

1.4.4. Nucleocapsid (p7)

The HIV-nucleocapsid is one of the proteins formed as a result of protease cleavage of the precursor protein. The nucleocapsid encapsulates viral RNA, consisting of two zinc finger motifs (CX2CX4HX4C) that are known for participating in nucleic acid binding as well as in performing chaperoning functions (Muriaux *et al*., 2010). These serve to protect genomic nucleic acids by modifying them in a cascade of events which involves binding, aggregation and destabilization which is ATP-dependent. Such events are the reason they finally assume thermodynamically stable conformations (Darlix *et al*., 2011). Among all its functions, the nucleocapsid is critically important in ensuring that reverse transcriptase is specific as well as preventing it from stalling, or being involved in directing, viral integration into the host chromosome which is important in the virus life cycle (Levin *et al*., 2010). The nucleocapsid is also involved in the assembly and packaging of the virus (Dawson and Yu, 1998). Its ability to facilitate genome recognition, with its zinc knuckle and packaging of the virus is an important target by inhibitors as it impairs normal functioning (Amarasinghe *et al*., 2000).

1.4.5. HIV-1 protease

HIV protease comprises 99 amino acids located within the viral core (Brik and Wong, 2003). It forms part of the gag-pol precursor and is responsible for catalysing the cleavage of the precursor polyprotein which then results in the production and maturation of the viral structural proteins, an important function in the replication of the virus (Purohit and Sethumadhavan, 2009; Chatfield *et al*., 1998). The HIV protease is active as a homodimer (Choudhury *et al*., 2003) and is classed as an aspartyl-protease, which is a group of enzymes, such as pepsin and chymotrypsin, that uses a catalytic triad (Asp-Thr(ser)-Gly) found at their substrate binding sites to perform catalytic functions (Weber and Agniswamy, 2009). This is done if there is a change in pH leading to the protonation of the 25^{th} residue of aspartate, which facilitates proton donation and interaction with the water molecule, causing a nucleophilic attack of the scissile peptide bond (Robbins *et al*., 2010). Although there are

known functions of the viral protease, there is still outstanding knowledge on the exact mechanism used in participating in the HIV life cycle, thus rendering it as an intensive research target like all viral proteins.

1.4.6. Reverse transcriptase, RNase H and integrase

Reverse transcriptase (RT) is an allosteric enzyme which is also known as an RNA dependent DNA polymerase. This enzyme catalyzes the synthesis of double stranded proviral DNA from a single RNA strand (Matamoros *et al*., 2011; Madrid *et al*., 2001). Reverse transcriptase exists as a dimer which comprises of two subunits; namely p66 and p51 (derived from p66). Both subunits independently consist of four subdomains which are commonly denoted as fingers, palm, thumb as well as connection (Schauer *et al*., 2014; Ivetac and McCammon, 2009). This is due to the incontestable comparability of p66 to the human right hand. Apart from housing the active site (where DNA is polymerized) and the allosteric site targeted by inhibitors, p66 also consists of an RNase H subdomain (Sluis-Cremer *et al*., 2004; Madrid *et al*., 2001) which is responsible for RNA degradation when using RT to synthesize DNA (Betancor *et al*., 2015). Sequences that are synthesized by reverse transcriptase are prone to mutations because RT lacks proof reading properties. Infidelities caused by its actions contribute to drug resistance and are important in the HIV life cycle (Bernado and Silva, 2014; Li *et al*., 2000). Immediately after the action of reverse transcriptase and RNase H, integrase then attaches itself to the viral DNA while it is nuclear imported and then integrates itself into the host cell chromosome.

1.5. HIV pathogenesis

Mechanisms by which HIV progresses within human cells are important in studies based on the expedition to obtain extremely sensitive diagnostic tools as well as therapeutic agents to conquer HIV (Naif, 2013). HIV transmission, by way of mucosal membranes, is spread through contact with body fluids such as infected blood and ejaculatory fluids. This virus

escapes the cells' defence mechanisms by impairing the host immunity. This occurs after viral entry, fusion and during replication.

Binding and entry of the virus is carried out by the actions of two important proteins, gp120 and gp41 (Schön *et al*., 2006). HIV gp120 binds primarily to $CD4^+$ T-lymphocytes of the host, which triggers conformational changes that expose other sites on gp120. These sites are specific to binding chemokine co-receptors, of which the most common are CCR5 or CXCR4 of the host membrane (Maartens *et al*., 2014; Simon *et al*., 2006). Once gp120 is bound to CCR5, gp41 then penetrates the host's membrane, bringing the viral membrane closer to the host's, allowing for their fusion and entry of the virus through endocytosis (Finales-Belasio *et al*., 2010). Upon entry, the virus un-coats, followed by digestion of both the matrix and the capsid protein, which then causes a release of the virus genetic material as well as its enzymes into the cytoplasm. Thereafter, reverse transcription occurs, whereby an RNA-dependent DNA polymerase catalyses the synthesis of proviral DNA from the RNA genome, which is then attached and carried by the viral integrase to nuclear pores and integrated in the host genome. At this point, the virus may remain latent or rather uses RNA polymerase to synthesize mRNA which codes for gene expression. This mRNA is subsequently used to produce viral proteins and the immature virus buds are then shuttled by ribosomal machinery to the cell surface and out of the host cell. At this stage, it is not infectious until HIV protease cleaves the polyprotein, allowing for individual assembly of the protein and resulting in a mature virion that is ready to infect other cells.

The HIV targets CD4+ cell receptors found in the peripheral blood, lymph nodes, macrophages and on the surface of T-lymphocytes and dendritic cells to facilitate host invasion (Weber, 2001). HIV progression occurs simultaneously with CD4+ and CD8 + cell depletion in all stages of infection; however, the decline is not constant across all the stages (Brenchley *et al*., 2004; Weber, 2001). HIV infection can be categorized into 4 stages: the primary or acute stage of HIV infection, the latent phase, the chronic phase and the late stage

of AIDS. The primary stage of HIV infection (PHI) is characterized by a significant reduction of CD4+ T-cells with little or no antibody development against the virus. This favours HIV replication and spread all over the body, thus explaining the high viremia during this phase, and the increased risk of viral transmission. Infected individuals often present with symptoms like those caused by influenza and virus infection; however, the onset response to the infection and seroconversion varies with individuals (Simon *et al*., 2006). The second (latent) deceiving stage is an asymptomatic phase where the virus continues to silently replicate, and the infected individual may not even be aware of the infection. During this phase, the presence of the virus may only be discovered or proven by serum testing. The third phase is the chronic phase which presents a drastic decline of the CD4+ T- cell population. However, the cause of CD4+ cell destruction, by apoptosis of the infected cells or by the lytic nature of the virus, is not clearly known (Weber, 2001). Nevertheless, the observatory CD4+cell decline, with increases in blood viral load, may support the cell destructive behaviour of the virus, and this decline renders the host (infected individual) highly immunocompromised and vulnerable to opportunistic pathogens leading to the AIDS stage, where the immune system can barely fight against infection and death may results due to AIDS related illnesses (Quaranta *et al*., 2012; Weber, 2001).

1.6. Current diagnostic tools and challenges

Knowing the HIV status is critical for the well-being of every individual, the availability of highly accessible, easy to use point-of-care applicable diagnostic tools are important and cannot be over emphasized. HIV testing can be done using the serologically based methods or nucleic acid determining methods (Morris *et al*., 2010). Serological methods include the enzyme-linked immunosorbent assay (ELISA) (Urio *et al*., 2015; Cilliers *et al*., 2019), the hemagglutination assays (Vasudevachari *et al*., 1989) amongst others, whereas nucleic acid-based methods include the PCR methods (both qualitative and quantitative) (Lee *et al*., 2012). The p24 antigen testing method is also circumstantially used for early detection of HIV.

ELISA and other antigen-antibody based methods are sensitized when the antibodies for the viral infection are present, usually after seroconversion. Therefore, depending on time taken for everyone to produce antibodies, diagnosis cannot be pronounced, or false negatives may be produced (Chin *et al*., 2007). The p24 antigen testing investigates the presence of p24 antigen before the antibodies are formed, thus making it suitable for early diagnosis. Unfortunately, such methods may be inconclusive, lacking sensitivity in the presence of antibodies however minute. Therefore, confirmatory tests such as the western blot are needed and further follow-up tests are required. The use of PCR methods to detect the availability of the viral nucleic acids is and has been another alternative to p24 antigen testing for early diagnosis. However, PCR-based methods are labour intensive and can only be handled by qualified personnel in laboratory settings. Inadequacies and shortcomings of HIV diagnostic tools remain a serious issue, hence the need for highly specific and sensitive tools. Fortunately, apta-agents (aptamers) have been studied over the years and considering their intriguing properties, they are promising future agents that can be applied as alternative rapid diagnostic tools. Aptamers lack immunogenicity and are highly sensitive; hence they are suitable biological agents to be employed as point-of-care diagnostic agents. Their characteristics and expansive applications are discussed in chapter 2.

1.7. Aptamer technology

1.7.1. Aptamer generation by SELEX

Systematic evolution of ligands by exponential enrichment is the process by which aptamers are selected (Wang *et al*., 2018). *In vitro* selection of aptamers involves incubation of DNA/RNA with the target molecule and separation (removal of unbound sequences) and a library, designed and flanked by primers on both ends, prior to PCR (for DNA) and reverse transcriptase PCR (RNA) amplification. Apart from the conventional SELEX method described by Tuerk and Gold (1990), other aptamer selection methods include affinity

chromatography, magnetic beads, microfluidic-based SELEX and capillary electrophoresis (CE-SELEX) and so forth.

1.7.1. 1. Affinity chromatography and Magnetic bead-based SELEX

Aptamer selection, using affinity chromatography, involves immobilization of the affinity tagged molecule to the beads (glutathione S-transferase (GST) agarose or histidine (Ni-NTA) beads) as they are packed into the column. This method purifies the target (in protein expression) and allows for separation of the non-specific binders (in SELEX) by washing, while eluting only those constituents bound to the target. The shortcoming of this technique is that it requires the tagging of the target usually by GST or histidine (HIS), and any target without the tag cannot be utilized (Song *et al*., 2012). Magnetic bead-based SELEX is the most common and rapid method used to immobilize the target and separate the unbound from the target specific oligonucleotides by a magnetic separator. The disadvantage of this method is that distribution of the target on the surface of the bead is not always even, and as a result may change target conformation or leave open spaces. This potentiates the binding of non-specific oligonucleotides to the bead surface, affecting the enrichment of the library (Yuce *et al*., 2015). Despite the occurrence of non-specific binding, other methods such as functionalization of the beads with chemical functional groups (e.g. amines, streptavidin and carboxylic acid) or coating their surface with affinity tags have been introduced to improve specificity of the ligands (Song *et al*., 2012; Yuce *et al*., 2015). Moreover, negative selection and stringency may also be introduced to recuperate the affinity of the ligands and enrich the oligos (Dembowski and Bowser, 2018).

1.7.1. 2. Microfluidic-based SELEX and CE-SELEX

Microfluidic-based SELEX is labour-free, uses a chip system onto which the target molecule is immobilized and involves heating and cooling like PCR (Yuce *et al*., 2015). This method has been shown to select aptamers with very high affinity at a small scale through just 3-6 rounds of selection. This is due to the high stringency introduced as the target is reduced and

washing by fluidics occurs (Ozer *et al*., 2014). Although this method is automated, contamination has been reported as a hindrance. However, improved microfluidic platforms to prevent contamination have been proposed (Ozer *et al*., 2014). Capillary electrophoresis-based SELEX is more advantageous than the above-mentioned methods of aptamer selection, since this method does not involve any immobilization of the target but free incubation of the target and the ligand. It can separate smaller molecules, even ions, and selection takes only about 2-4 rounds at high speed (Song *et al*., 2012; Yuce *et al*., 2015).

1.7.2. Different types of aptamers

Aptamers may be composed of short sequences of peptides, inorganic polymers or nucleic acids. These agents are known to bind with high affinity and specificity to their targets, making them good potential agents for applications ranging from bio-sensing to therapeutics (Tombelli *et al*., 2015).

1.7.3. Peptide aptamers

Aptamers made from short peptide regions are called peptide aptamers. Selection of these peptide regions may take place either *in vitro* or *in vivo* and thereafter are combined with scaffolding proteins (Hoppe-Seyler *et al*., 2004). These peptide regions can bind to their target protein with high affinity; hence they may aid in studying intracellular protein-protein interactions that may be involved in disease progression. This property of peptide aptamers may enable their application as biologically regulating agents, acting as inhibitors of the disease propagating protein interactions (Li *et al*., 2011; Hoppe- Seyler *et al*., 2001). Despite the number of described peptide aptamer advantages, several limiting factors concerning these aptamers have also been denoted; such as stability, size and immunogenicity being the main concerns. Proteins and their components are known to denature and lose their functionality in varying environmental conditions and it is for this reason that they are rendered unstable.

1.7.4. Nucleic acid- based aptamers

In medicine, early diagnosis is critical for the control and treatment of diseases. However, this requires diagnostic agents which are highly specific to their targets. Nucleic acid-based aptamers (DNA or RNA) have been recognised for their high affinity and specificity to their targets and hence, promising agents for application in diagnostics as well as therapeutics. Aptamers synthesized from either DNA or RNA pools are selected chemically through the SELEX process *in vitro*. Although synthesized *in vitro,* their size and specific character make them good agents for *in vivo* applications, ranging from bio imaging to diagnostics (Famulok *et al*., 2000). The aptamer susceptibility to nuclease degradation and their hasty excretion has solely been their documented short-comings. However, due to research advances, these problems can be solved by chemical modifications of the sugar backbones or of the nucleic acid bases (Kratschmer and Levy, 2017). These modifications work to stabilize the nucleic acids so they can resist degradation and delay the time in which they are excreted in *in vivo* applications, allowing for their use in a wide range of applications (Blind and Blank, 2015; Kusser, 2000).

1.7.5. Aptamers over antibodies

Most tools that are used for the diagnosis of several diseases are antibody (mono- or polyclonal) based (Lakhim *et al*., 2013). However, recent studies have demonstrated the superiority of aptamers over antibodies (Sun and Zu, 2015), due to their easy and inexpensive synthesis from non-animal sources, stability, high sensitivity and specificity when binding to targeted agents (Kong and Byun, 2013). Their small size enables them to be applied in several applications, ranging from bio-sensing to diagnostics. Although there may be a downfall concerning aptamers, due to their degradation by nucleases in the blood and their short life (quick renal excretion) when used for therapeutic purposes, their flexible structures allow them to be modified to escape nuclease degradation as well as to stay a little longer before being excreted. Such properties make aptamers effective for the diagnosis of viruses ranging

from HIV to Hepatitis C Virus (HCV) (Lakhim *et al*., 2013). On the other hand, antibody applications have limitations. The manufacturing process of these agents demands expensive synthesis and down-streaming processes (Sun and Zu, 2015), and since they are immunogenic, when used for diagnostics, diagnosis may be delayed, requiring a waiting period such as that required during the early phase of HIV infection. This renders aptamers invincible as they are non-immunogenic agents (Sun and Zu, 2015; Lakhim *et al*., 2013) and may provide immediate diagnosis regardless of the infection phase. This is critically important for the control of diseases.

1.8. Problem statement

The widely used rapid HIV diagnostic tools are immunologically based and depend on antigen-antibody interaction; hence they cannot diagnose HIV infection until after seroconversion, leading to a window period. This period elevates the risk of HIV transmission. PCR methods can also be used for the screening of viral RNA but are not readily available for use in clinical settings. HIV-p24 antigen testing tools often lack sensitivity to detect p24 protein, while it is in small quantities before it subsides after seroconversion. Therefore, there is a need for an alternative method that will detect the infection even during the window period and be easily employed in non-clinical settings. The up-regulation of HIV-p24 during this period makes it a significant biomarker for diagnostics. Additionally, interaction studies between HIV-p24 and its derivative p24 aptamer will be of great importance in improving diagnostics and in the development of a biologically-based method that will not only be available for use as a point-of-care diagnostic tool, but also to detect the virus during the window period, hence mitigating the spread of the virus.

1.9. Aim of the study

The aim of the study is to biophysically characterize the interaction between HIV-p24 protein and the p24 derived aptamer.

1.10. Objectives

- Recombinantly express GST-HIV-p24 in *E. coli* cell and purify the protein using affinity chromatography
- Determine protein functional groups by FTIR
- Synthesize and interact the aptamer with the target HIV-p24 protein
- Characterize the interaction between the aptamer and the HIV-p24 protein using molecular docking tool (Patch-Dock)

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CHAPTER

TWO

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CHAPTER TWO: REVIEW ARTICLE

pharmaceuticals

MDPI

Review

Molecular Application of Aptamers in the Diagnosis and Treatment of Cancer and Communicable Diseases

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Abstract: Cancer and infectious diseases such as Ebola, HIV, tuberculosis, Zika, hepatitis, measles and human schistosomiasis are serious global health hazards. The increasing annual morbidities and mortalities of these diseases have been blamed on drug resistance and the inefficacy of available diagnostic tools, particularly those which are immunologically-based. Antibody-based tools rely solely on antibody production for diagnosis and for this reason they are the major cause of diagnostic delays. Unfortunately, the control of these diseases depends on early detection and administration of effective treatment therefore any diagnostic delay is a huge challenge to curbing these diseases. Hence, there is a need for alternative diagnostic tools, discovery and development of novel therapeutic agents. Studies have demonstrated that aptamers could potentially offer one of the best solutions to these problems. Aptamers are short sequences of either DNA or RNA molecules, which are identified in vitro through a SELEX process. They are sensitive and bind specifically to target molecules. Their promising features suggest they may serve as better diagnostic agents and can be used as drug carriers for therapeutic purposes. In this article, we review the applications of aptamers in the theranostics of cancer and some infectious diseases.

Keywords: aptamers; cancer; diagnostics; therapeutics; infectious diseases; SELEX

1. Introduction

Infectious diseases such as Ebola, Zika, human immunodeficiency virus (HIV), tuberculosis, hepatitis, measles and human schistosomiasis as well as non-communicable diseases such as cancer are not only considered as life-threatening concerns worldwide, but they have also been declared as major causes of high mortalities and morbidities globally [1–3]. Although the human immune system, particularly the innate immune system, has devised means to combat host invasion and other antimicrobial actions through interferons and cytokines [4], the structural complexity and the mechanisms by which these etiologic agents replicate, assists them in impeding the actions of the innate immune defence system [5]. Hence, early diagnosis of these diseases enable prompt administration of treatment which in turn boosts immunity and prevent further viral progression, thus saving the lives of the infected. Meanwhile, existing challenges linked with these viruses include genetic evolution and resistance to treatment [6]; this requires extra channels to follow in alleviatingtheir devastating effects. Available serologically-based diagnostic tools may not be that sensitive to detect these evolving viruses and parasites immediately after infection [7]. Nonetheless, studies have shown that aptamers are potentially good candidates for resolving issues pertaining to drug resistance, non-specific drugs and diagnostic tools with low sensitivity. Aptamers, which are short oligonucleotide sequences selected in vitro via the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process, are highly specific agents which bind to their targets with high affinity [8–10]. Their specificity and affinity-binding properties make them important for use in different applications [11,12]. Although aptamers possess similar binding affinities for their targets as antibodies, varying between nanomolar and picomolar (expressed as dissociation constants, i.e., Kd) [13–15], antibodies are still not only immunogenic but is also associated with high production costs. On the other hand aptamers show great supremacy due to their lack of immunogenicity [10,16,17] and synthesis which is done chemically at lower costs, their small sizes makes them versatile and enables them to pass through blood-brain barriers which cannot be achieved with antibodies [18,19]. Table 1, however, illustrates the strengths and limitations of aptamer technology. Due to the above mentioned advantages, aptamers have been applied in the diagnosis and treatment of various diseases such as HIV/AIDS (acquired immune deficiency syndrome), tuberculosis, hepatitis, Ebola, cancer, measles, Zika and schistosomiasis [20]. These diseases pose serious threats to human health because they have the ability to escalate and lead to fatalities if not diagnosed and treated early on. Therefore, the diverse characteristics of aptamers allows for their use in various applications ranging from disease diagnostics to bio-sensing and therapeutics [9,21,22]. Therefore, this review covers the importance of the molecular application of aptamers over other biological agents such as antibodies that are used in the diagnosis and treatment of the above-mentioned communicable and non-communicable diseases, and the role aptamers play in studies directed towards finding alternative solutions for the near future.

2. The Synthesis of Aptamers

Aptamers have been widely explored and acknowledged for their structural versatility in the diagnosis and treatment of various diseases [23,24]. These short DNA or RNA sequences are selected through the SELEX process [24–26], and different SELEX methods have been described in literature and applied in several studies. Some of these include affinity chromatography and magnetic bead-based SELEX, nitrocellulose membrane filtration-based SELEX, as well as capillary electrophoresis-based SELEX [23,27,28]. The primary selection or conventional SELEX method of sequences often involves incubation of the selected oligonucleotide library with a specific target; these are usually proteins for therapeutic or diagnostic studies. Several rounds of amplification by polymerase chain reaction (PCR) then follow to screen for the most enriched pool, which can be identified by nucleotide sequencing, followed by functional aptamer synthesis [24,28–30]. The small sizes of aptamers, their ability to assume peculiar secondary structures and their strong affinity for their targets, qualify them for a variety of applications in various fields [31]. Their structural flexibility allows for post-SELEX modifications, which include substitution of the 2^t-OH group of ribose with either a 2^t-amino, 2^t-fluoride or 2^t-*O*-methylene [14], to enhance their stability properties and increase their half-life in

bio logical fluids [12,13,32]. Such characteristics make aptamers more suitable agents for employment in theranostics over antibodies [16]. Since aptamer selection is carried out in vitro, their production is quick and cost effective, unlike antibodies, which require in vivo production [33] with time consuming and expensive down-streaming processes [34]. Aptamers have the advantage of being heat stable [35] meaning that they have the ability to denature and renature, while antibodies on the other hand, denature at high temperatures and lose their three-dimensional structures as well as their functionalities in the process due to the fact that they are proteins in nature [36]. Above all, unlike antibodies, aptamers are very sensitive and capable of distinguishing between isoforms of the same target hence they are regarded as good candidates for diagnostics and can be applied in therapeutics [37]. Furthermore, aptamer technology is continually advancing, that is SELEX processes are improving to ensure aptamer yields with minimal labour, making the process efficient and worthwhile [38].

Table 1. Strengths and limitations of aptamer technology.

Strengths	Limitations
Aptamers' entire selection process can be carried out in a test tube	Unmodified RNA aptamers are susceptible to nuclease degradation in in vivo applications [24]
They have high binding affinities, selectivity and specificity to their targets making them important for diagnostics and targeted therapy	Unmodified oligonucleotides have a short half-life, i.e., they quickly undergo renal filtration [24]
They are thermostable [39]	
Can be structurally modified to enhance specificity and stability, therefore increasing aptamer half-life in in vivo applications [39–41]	
They are non-toxic and elicit little or no immunogenic response [41]	

3. Application of Aptamers in Cancer

Genetic mutations that occur during cell division predispose cells to cancer [42] and such may be caused by several external or even internal factors such as genes (hereditary), radiation, chemicals, smoking and old age. Under normal circumstances anticancer signalling pathways such as apoptosis eradicate cells with such mutations. However, the imbalances between cell growth and apoptosis can lead to carcinogenesis [43]. Once sensitized cancerous cells may divide and form aggregates in a particular part of the body, leading to the formation of malignant tumours, which if not detected or treated early, may further metastasize and become more difficult to control [44,45]. These cells may later migrate to other parts of the body, continue to multiply and successively impair the normal functioning of the affected area or organ [46]. Early diagnosis allows for early administration of therapeutic drugs thereby controlling the disease and giving affected individuals a better chance at survival [47].

3.1. Diagnosis

Genetic mutations in cancer cells can manifest in morphological, physiological and molecular changes, which may result in changes in the proteome. Differences in the cell surface proteome of healthy and cancer cells can be exploited targets (biomarkers) for diagnosis [48]. Conventional diagnostic methods and magnetic resonance imaging (MRI) involves tracking morphological changes of tumour cells to primarily diagnose cancers [49]. Conquering the ailment requires detection at a molecular level since conventional methods fail to detect cancer targets at this level [47,48]. Aptamers selected by the cell-SELEX process have shown great sensitivity in detecting cancerous cells as well as targeting cancer related proteins at an early stage regardless of their low expression levels, hence they are considered to be the best candidates for diagnosis at a molecular level [47]. Mechanisms by which DNA and RNA aptamers fold into outstanding nanostructures has piloted the development of a number of non-enzymatic isothermal circuits, to be used as molecular recognition tools, comprising of entropy-driven catalysis, hybridization chain reaction as well as catalytic hairpin assembly (CHA). RNA-based circuits have been noted for their sensitivity and this characteristic feature makes them potential agents for application in diagnosis and therapy, however, in vivo application of these circuits has been limited due to the lack of reporting (imaging) partners. Mudiyanselage and co-workers [50], devised a genetically-encoded catalytic hairpin assembly (CHARGE) method to design RNA-based CHA circuits, using split Broccoli as a reporter for sensitive RNA imaging in live cells. They focused on the CHA, which consists of two complementary nucleic acid hairpins designed in such a way that unprompted hybridization is kinetically delayed by inserting complementary regions within the hairpin stems [50]. The components of the CHA circuit comprised of two complementary RNA hairpins (H1 + H2), a target RNA (C) and a DFHBI-IT dye (a fluorescent dye). The H1 and H2 hairpins were modified with non-fluorescent Broc and Coli respectively, while the target C was used to catalyse and initiate H1 and H2 recombination which then activates DFHBI-IT thus enabling fluorescent signals to be recovered. To validate the fluorescing ability and sensitivity of these circuits, in vitro and in vivo studies were conducted, these demonstrated that CHA was able to detect RNA at very low concentrations with high sensitivity. These findings suggested a great potential of RNA-based circuits for use as molecular recognition tools and RNA imaging in live cells [50]. Additionally, the sensitivity of these circuits could be of paramount importance in the early diagnosis of cancer.

In 2010, Zhang et al. [48] reported a selection of aptamers specific to CCRF-CEM acute leukaemia cells. These aptamers were then conjugated to gold nanoparticles (AuNPs) and used in a colourimetric assay to detect cancer cells. Changes in the optical properties on the AuNPs in the presence of the biomarker resulted in detectable colour changes. Zhang and co-workers [48] further documented three other aptamers that were conjugated to quantum dots (QD), TTA1, AS1411 and MUC-1, producing different emission spectra in wavelengths ranging from 605, 655 and 705 nm respectively. When these aptamers were incubated with normal and diseased cells, QD-TTA1 showed selective visualization, which is highly dependent on the cell type. A clear spectrum was observed in C6 cells whilst in PC3, HeLa and NPA cell lines, a weak fluorescence spectrum was produced at the same 605 nm

wavelength [48]. On the other hand, QD-AS1411 produced strong fluorescence on the cell membranes of all the cell lines at 655 nm wavelength. MUC-1 produced high fluorescence in HeLa cells at 705 nm and no fluorescence was produced by the QD-aptamers conjugated with normal cells that were used as negative control. Such results and behaviour showed aptamers have great selectivity and very high sensitivity, which are important factors to consider as far as early diagnosis is concerned [48].

Later studies by Wu et al. [51] reported the use of aptamers as sensors (aptasensors) that are constructed using fluorescence. Fluorescence based aptasensors are of great interest in disease diagnosis: they can be categorized into fluorescently labelled or label-free aptasensors. In many examples, the aptamer secondary structure switches induced by target recognition are suitably translated into a detectable fluorescent signal, producing an enhancement ("signal-on") or a quenching ("signal-off") effect, which directly reflect the extent of the binding with the target. For example, Shigdar and colleagues [52] chemically formulated a new small RNA fluorescent labelled aptamer (19 base DY-647) from a 40 base RNA aptamer isolated from a random oligonucleotide library, that has an affinity for epithelial cell adhesion molecule (EpCAM), which is upregulated in various cancer cells such as breast, gastric and colorectal cancer. The interaction of this RNA aptamer with EpCAM and subsequent internalization into the colorectal cancer cells was investigated using flow cytometry and confocal microscopy and the results showed successful binding and receptor-mediated endocytosis of this DY-647 labelled aptamer, suggesting that this aptamer can be potentially applied in the advancement of targeted therapeutics and molecular imaging agents for cancer diagnosis [52].

In a recent review Musumeci and co-workers presented an updated overview on fluorescent aptamers and aptamer-based fluorescence strategies discussing important cancer biomarkers such as PDGF, VEGF, angiogenin and mucin. They particularly focused on thrombin, which, even if marginally involved in cancer-related pathologies, is typically exploited as a proof-of-concept in sensing strategies' validation. Indeed, thrombin is well known for its role in thrombosis as well as maintaining homeostasis of coagulation-related processes. Under normal health conditions, thrombin is found in very low concentrations in the blood. However, increased blood concentrations (pM to μM) of thrombin as a result of prothrombin proteolysis has been coexistent with diseases such as Alzheimer and cancer. Hence, thrombin is an important disease biomarker and a great target for diagnosis. The most studied aptamers of thrombin are the 15-mer and the 29-mer thrombin binding aptamers, able to non-competitively bind to different sites within the same protein, TBA_{15} binds to exosite I (the fibrinogen binding site) whereas TBA_{29} binds to exosite II which is the heparin binding site [32,41,53].

Wu and co-workers [51] conferred that aptamers amplified in line with Forster resonance energy transfer (FRET) and chemilluminescence resonance energy transfer (CRET) show high signal output and sensitivity which are properties that makes them important for applications intended to detect cancer biomarkers such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), tenascin-C, nuclear factor kappa-light-chain enhancer (NFκB) of activated B cells and prostate specific membrane antigen (PMSA). Hence, they are potential early cancer detectors and early diagnosis is imperative for the control and treatment of the disease [51].

3.2. Therapy

Among other cancer therapies, chemotherapy is administered as the primary treatment of cancer [54,55], however, treatment is dependent on the stage and the type of cancer and may alternatively be radiation, surgery or immunotherapy [56]. Excessive up-regulation of cancer genes such as the human epidermal growth receptor-2 (HER-2) causes disease (cancer) aggression that leads to certain chemotherapeutic resistance [26,57]. HER-2 is highly expressed in breast, ovarian and gastric cancers. Its ability to form heterodimers with other members of the epidermal growth receptor family, leads to HER-2 mediated signalling pathways that promote cell proliferation whilst inhibiting apoptosis, hence it is important in cancer cell survival [58] and for this reason it has been an important target for research towards cancer therapy [59]. Trastuzumab, an anti-HER-2 antibody has been used for the inhibition of HER-2 expression by binding to its juxta-membrane domain, leading to its down-regulation [60]. Trastuzumab also prevents heterodimerization between HER-2 and HER-3 resulting in restricted cell growth [61,62]. The use of trastuzumab in combination with chemotherapy has shown great efficacy in cancer treatment [63]. Despite the advantages that antibodies exhibit, their

low accessibility and sensitivity along with high production costs and side effects (cardiotoxicity) have been reported [64].

RNA-based aptamers have recently emerged as promising gene and drug delivery agents for cancer therapy. This can be attributed to the many attractive properties that the aptamers possess, including, non-toxicity, large production capacity, and structural flexibility and amenability [10,17]. Early methods that have been used to design these delivery systems involved assembly of RNA with cargoes of interest, which unfortunately required the synthesis of long RNA molecules together with the cargo sequences. Recently, Wang and co-workers reported a new strategy known as 'aptablocks', which requires conjugation of the RNA complementary strand and the cargo, thereby forming an aptamer-stick and a cargo-stick. This method ensures appropriately-synthesized agents with reduced lengths, and sticky ends which enables reuse of the aptamers as universal delivery agents for various cargoes [65]. To validate the aptablocks method, the researchers developed an RNA aptamer tP19 conjugated to NGF siRNA. The tP19 is a pancreatic cancer-specific aptamer, while NGF siRNA is a known inhibitor of pancreatic cancer progression. In vitro testing of these conjugates in MCF-7 cell lines demonstrated the expected functionality of the aptamer-drug delivery agents [65].

The use of RNA-based aptamers as therapeutic agents or in targeted drug delivery systems, however, comes with its own hurdles, which includes the susceptibility to nuclease degradation. Fortunately, over the years several modifications have been implemented to overcome this. These include the incorporation of hydrophilic/hydrophobic polymers such as poly (ethylene glycol) (PEG) and poly (lactic acid) (PLA) with "stealth" or stabilisation properties to enhance the stability of aptamers to resist nuclease degradation as well as to delay renal excretion, thereby making RNA aptamers suitable for both in vitro and in vivo applications. Numerous in vitro and in vivo studies have been done to demonstrate the candidacy of aptamers and RNA aptamers in cancer therapy. For example, Gijs and co-workers [57] identified two aptamers (HEA2_1 and HEA2_3) that are specific to HER-2 by whole-cell SELEX using human breast cancer cells (SKBR3). Surface Plasmon Resonance Imaging (SPRi) showed that these aptamers have a fast association rate with a relatively slow dissociation rate. This makes them high affinity agents for HER-2 meaning that they can be used in the place of antibodies such as trastuzumab, performing the same function but with higher efficiency in drug delivery [57]. Reyes-Reyes and co-workers [66] demonstrated the effect of AS1411 aptamer (a multifunctional aptamer with G-quadruplex, which also serves as an anti-proliferative agent) [67] on cancer cells as it binds to nucleolin protein which is highly expressed and found mostly on the surface of cancer cells. Nucleolin is implicated in disease progression [68] and in cancer and it is known to limit Rac 1 (Ras-related C3 botulinum toxin substrate 1) signalling, thereby inhibiting cell death and promoting cell proliferation. However, they demonstrated that targeting nucleolin on cancer cell lines (DUI45, MDA-MB-468, A549 and LNCaP) with AS1411 limits nucleolin expression and promotes Rac 1 activation which in turn induces cell death through methuosis, hence preventing the proliferative activity of cancer cells [66]. AS1411 has been found to also function as a co-delivery system of doxorubicin (Dox) and shRNA to AGS adenocarcinoma cells. It is also noteworthy to mention that AS1411 showed great response in phase II clinical trials in renal cell carcinoma patients [14,32].

A study by Lin and colleagues has demonstrated that suitably modified aptamers are able to penetrate the blood brain barrier (BBB) and also overcome the reticuloendothelial system, hence, proving as optimal candidates in chemotherapy [69]. Other studies have shown that the use of aptamers in chemotherapy, in conjunction with therapeutic agents increases drug efficacy and selectivity to target only the diseased cells thus ameliorating drug side-effects [70–72]. For example, Pi and co-workers developed a DNA/RNA hybrid nanoparticle consisting of a phi29 pRNA three-way junction (3WJ) and an Endo-28 DNA thioaptamer embedded in its core [40]. The Endo-28 DNA aptamer target annexin A2, expressed in ovarian tumours, and is also found on the extracellular surface of endothelial cells. The pRNA-3WJ-scaffold is well-known for its exceedingly thermostable property. In a hybrid, it is known to facilitate proper folding of its binding nucleic acids without interfering with their target specificity. The endo-28-3WJ hybrid was tested for specificity against several cancer cell lines, with IGROV-1 and SKOV3 serving as annexin A2 positive cells and HEK293T cells used as negative control [40]. After incubation of Endo-28-3WJ with the cell lines and the elimination of non-specific binding by washes, flow cytometry was then used to determine whether Endo-28-3WJ nanoparticles were bound to the cells. The results showed high affinity of the nanoparticles to annexin A2 positive cell lines, with about 71.2% of IGROV-1, 51.7% of SKOV3 and only 17.3% of HEK293T cells binding

endo-28-3WJ nanoparticles. These findings proved that pRNA does not interfere with the specificity of endo-28-3WJ to annexin A2. Additionally, pRNA-3WJ nanoparticles are non-immunogenic and their size enables them to pass through diverse biological barriers, hence they can penetrate cells, and this makes them ideal for in vivo targeted cancer therapy [40]. In this study, the authors further conjugated doxorubicin, an ovarian cancer drug, to the Endo-28-3WJ nanoparticle. Free Dox, Endo-28-3WJ sph1/Dox and Scr-3WJ-sph1/Dox (negative control), was tested against SKOV3 (annexin A2 positive) and HEK293T (annexin A2 negative control). SKOV3 cells treated with free Dox and Endo-28-3WJ sph1/Dox showed robust Dox fluorescent signal and the Dox conjugate is shown to be highly cytotoxic to SKOV3 cells and less effective on HEK293T cells after assessing cell viability using the MTT assay. These findings evidenced that Endo-28-3WJ sph1/Dox does not only target and penetrate cancer cells but can also deliver and release the cancer drug (doxorubicin) in specific cells without affecting healthy cells [40].

Binzel and co-workers [73] conducted an in vivo study using mice with prostate cancer to evaluate the antitumor activity of RNA nanoparticles constructed using the pRNA 3WJ core conjugated onto an RNA aptamer specific to prostrate membrane antigen (PMSA) overexpressed by prostate cancers. Results revealed successful and significant knockdown of miR17 and miR12 oncogenes, while increasing the expression of PTEN, a tumour suppressor. Ultimately, it was observed that tumour growth was repressed with very high efficacy at low doses [73]. Similarly, Pi and co-workers also conjugated three ligands with targeting abilities, (folate, a PSMA-binding 2^t-fluoro (2^t-F) modified RNA aptamer called A9g and the EGFR specific 2^tF-RNA aptamer) to the 3WJ core and displayed these on extracellular vesicle (EV) surfaces in a bid to specifically target colorectal, prostate and breast cancer cells respectively. EVs are currently undergoing robust investigation as therapeutic carriers mainly due to their role in intercellular communication but however lack specificity. Results displayed successful delivery of siRNA and miRNA to cells and repressed tumour growth in three mouse models [74]. In 2014, Sanna and co-workers [75] reported on the formulation and development of the first polymeric aptamer targeted nanoparticles called BIND-014, which are currently in use clinically for chemotherapy. The nanoparticles were constructed using the SELEX technique and they comprised of docetaxel, a well-known chemotherapeutic drug as a payload, poly (ethylene glycol) PEG and PLGA, as amphiphilic co-polymeric core with stealth/stabilizing and buffering properties, and an RNA A10 aptamer as a targeting ligand, since it has a high binding affinity to PSMA overexpressed by prostate cancer cells [75]. The antitumor activity of these nanoparticles was demonstrated by Hrkach et al. [76] in rats bearing PSMA-expressing cancer cells and PSMA-negative cells where targeted (BIND-014), non-targeted and free docetaxel were used. The findings showed that BIND-014 was 10-folds more efficient in delivering docetaxel to PSMA-expressing cancer cells when compared to non-targeted nanoparticles and free drug. This indicated the significant role of aptamers in the formulation of specific drug delivery systems for prostate cancer therapy. Shu et al. [77] demonstrated the abilities of RNA-based nanotechnology in mice, using RNA nanoparticles with an 8-nt sequence corresponding to the seed region of the MicroRNA MiR-21, incorporated into pRNA-3WJ core and an epidermal growth factor receptor (EGFR) targeting aptamer which facilitates RNA nanoparticles' internalization into cancer cells. MicroRNAs are responsible for regulating gene expression and cancer cell life cycle. Results demonstrated successful internalization of the highly stable RNA nanoparticles into cancer cells without accumulation in normal cells; the nanoparticles bound and inhibited MiR-21, which in turn reduced triple negative breast cancer (TNBC) progression [77]. These findings showed great potential of RNA-based nanotechnology application in improving cancer therapy.

Furthermore, the aptamers' agonistic or antagonistic characters have made them to be superiorly applicable in cancer therapy [53,71]. Recently, Zhou and colleagues highlighted the screening of a single stranded FOXM1-specific DNA aptamer, to target and inhibit FOXM1 proteins. This inhibition was found to subjugate cancer cell proliferation [71]. FOXM1 controls a system of proliferation-related genes, its upregulation has been shown to initiate oncogenesis, as well as cause chemotherapeutic resistance in human breast cancer [78]. Platella and co-workers in their review summarized different G4-based aptamers as drugs with antiviral, anticancer, anticoagulant activity or with potential activity in other diseases (skeletal, prion and tuberculosis diseases). Among several anticancer G4-aptamers they also described the principal features of two anticancer G4-aptamers (T40214 and HJ24) [14]. T40214 is a G-rich oligodeoxynucleotide selected to target and inhibit the Signal transducer and activator of transcription 3 (STAT3) protein functions in different cancers [79]. STAT3 protein

facilitates oncogenic signalling by mediating the overexpression of anti-apoptotic proteins Bcl-xL and Mcl-1, which in turn promote cell proliferation in cancers [14,79,80]. HJ24 is a DNA G-quadruplex aptamer which binds to Shp2 protein. Shp2 is a member of tyrosine phosphatases responsible for cell differentiation and oncogenesis, therefore is an important target for therapy. HJ24 binds and strongly inhibits Shp2 activity thus decreasing cancer progression.

4. Aptamer Application in the Diagnosis of Infectious Diseases

4.1. EBOLA

Ebola virus (EBOV) causes haemorrhagic fevers and is one of the most dangerous and deadly viral diseases [81]. The EBOV viral genome encodes seven viral proteins namely; nucleoprotein (NP), polymerase cofactor (VP35), VP40, glycoprotein GP, transcription activator (VP30), VP24 and RNA-dependent RNA polymerase L [82]. The negative stranded nature of this virus and the presence of small numbers of open reading frames in its sequence enable EBOV proteins to participate and act at different stages of the viral cycle thus promoting its propagation [81,83]. The Ebola virus's VP35 protein enables the virus to escape host immunity by inhibiting the incitation of interferon regulatory factor 3 (IRF-3) production resulting from RIG-I (retinoic acid-inducible gene I) signalling. IRF-3 activation regulates the expression of interferons in response to viral infection [84,85]. Through its C-terminal domain (interferon inhibitory domain IID), VP35 binds to dsRNA and prevents its recognition by protein kinase R (PKR) [83,86], this in turn inhibits interferon production [87,88]. This precedes the rapid invasion of host cells and causes the accumulation of viral protein inclusions in the host cells' cytoplasm [89]. This increases viral loads and results in devastating effects such as organ failure, disoriented fluid distribution as well as coagulation problems which lead to both internal and external haemorrhages in some cases [90].

EBOV threatens the lives of the infected and also places those taking care of the patients at risk [91]. EBOV is easily transmitted through aerosols or direct contact with any body fluids [92] either from an infected human or animal and hence precautions must be taken when handling infected patients as well as the corpses of those who have died as a result of the virus. Early diagnosis of Ebola remains a hurdle [93], not only due to its short incubation period inside the host, but also because of the widespread symptoms infected individuals exhibit, which are comparable and similar to symptoms caused by other viruses which are not so deadly. Sensitive diagnostic tools that make use of aptamers to detect the virus or viral proteins may be used to address this problem. In 2015, Wandtke and co-workers reported the identification of aptamers that contained conserved sequences rich in "GGGUGG" and "GAGGG" sequences in the loop region, a property that appear to be critical for the interaction between the aptamer and the antiviral protein. This information sequentially sets grounds for the design of molecular diagnostic tools capable of detecting Ebola, thus rendering aptamers better agents for use as diagnostic tools [94].

4.2. Human Immunodeficiency Virus

A competent immune system is critical to engage unwanted cell invaders such as HIV but this pathogen targets $CD4^+$ T-cells, leukocytes, macrophages and dendritic cells [95], which perform key roles in immune defence. Viral invasion of these cells, subsequently leads to the uncontrolled replication of the virus which exhausts and cripples the immune system while simultaneously leaving the infected vulnerable to opportunistic diseases such as those caused by *Mycobacterium* spp.; this infection then later progresses to Acquired Immunodeficiency Syndrome (AIDS) [96]. However, early diagnosis is important even in asymptomatic individuals because it will bring about early drug administration which will impair the multiplication and assembly mechanisms of the virus, thus preventing the spread of the virus and delay its effects and allowing infected individuals to live an adequate standard of life, but also provide them a better chance at survival [97]. The viral proteins are critical targets for diagnostic and therapeutic purposes. Among other antigen detecting diagnostic tools such as the enzyme-linked immunosorbent assay (ELISA) and p24 antigen assays, studies by Tombelli et al. [9] demonstrated the successful binding

of an aptamer-based sensor to Tat HIV protein, one of the gene expression regulatory elements that is essential to viral replication. The aptamer-based sensor was able to distinguish between Tat and Rev which proves that aptamers could be reliable diagnostic tools due to their design and the nature of their specificity [9].

4.3. Tuberculosis

Mycobacterium tuberculosis (*M.tb*) is the primary causative agent for tuberculosis (TB) and in 2015, approximately 10.4 million new cases and 1.8 million deaths were reported [98]. The infection begins with the penetration of the respiratory tract by the *Mycobacterium* spp. followed by the targeting of the alveolar macrophages, which are responsible for phagocytic immune defence mechanisms. Meanwhile, more immune defence agents including CD_4^+ T-lymphocytes and TγδS-lymphocytes producing specifically gamma interferons (IFN-γ), interleukins, tumour necrosis factor alpha (TNF-α) and macrophage colony-stimulating factor as well as other inflammatory inducing factors are produced to circumvent intracellular bacilli propagation. These defence agents stimulate macrophages and cytotoxic cells' function to inhibit the growth of foreign organisms [99], thus restricting the bacilli within an infected cell and preventing its replication. However, evolution of *M.tb* has enabled the bacilli to overcome restraining immune effects by blocking reactive oxygen species (ROS) intermediates, triggering anti-inflammatory responses and further reducing phagocytic cell acidification [100]. Nonetheless, early diagnosis is the best at controlling the transmission of the bacterium as well as in allowing early administration of treatment. The skin reaction TB test has been used for a long time, and however, may turn out to be unreliable since it depends on the immune integrity in order to produce such reactions. On the other hand, other methods such as the sputum test, which are considered more reliable, have a long turn-around time. Diagnostic strategies aimed at detecting bacterial proteins such as MPT64, ESAT-6 and CFP-10 [101] can produce more reliable rapid diagnostic tests since these proteins are abundantly expressed during the early stages of infection, even in asymptomatic individuals. Recent studies conducted by Sypabekova et al. [102] demonstrated the identification of a MPT64 specific DNA aptamers. Surface plasmon resonance (SPR) demonstrated a strong binding affinity with a sensitivity and specificity of 91 and 90%, respectively. Such results provide evidence that aptamer-based detection methods could significantly improve the diagnosis of TB, provided they are approved for use in the market [102].

4.4. Zika Virus

Zika virus (ZIKV, ZIKAV), which is associated with birth defects such as microcephaly and other abnormalities in infants born to mothers infected during pregnancy, caused sporadic outbreaks in recent years [103]. Zika virus is an arthropod-borne virus that belongs to the *Flaviviridae* family and is primarily transmitted through mosquito bites by the *Aedes* spp. [104,105]. However, cases of mother-to-child, blood and sexual transmission have been reported [103,106]. ZIKV is related to viruses such as yellow fever (YFV), dengue (DENV) and West Nile (WNV) viruses [5,104]. Following its discovery in the Zika forest of Uganda where it was isolated from a sentinel rhesus monkey in 1947 [107], the virus has since then been reported in South East Asian countries while a recent outbreak that began in May 2015 until late 2016 was reported in the Americas', particularly in Brazil. The escalation of ZIKV infections is associated with movement of people either due to urbanisation or tourism, which then contribute to the emergence and spread of the virus. Genetic changes in the viral genome are the basis for the difficulties leading to delays in diagnosis. Analytical studies carried out using a sequencing tool known as recombination development project (RDP), used in monitoring molecular evolution of the virus has shown that the virus has undergone at least 13 genomic recombinant episodes with observatory genetic breakpoints occurring only on the E (coding for the envelope) and NS5 which is the last non-structural protein of the seven genomic regions [108]. Viral evolution results in the emergence of several viral strains thus making vaccine development and viral detection using serological methods very difficult [109]. Due to the fact that *Flaviviridae* viruses other than ZIKA virus also produce similar symptoms, a distinct detection method is required for

ZIKA, of which serologically-based tools do not qualify. Fortunately, studies have shown that aptamers are good for this purpose since they are able to detect and distinguish between similar viruses (dengue virus, West Nile virus and yellow fever). This proves them as the most reliable diagnostic agents, which could potentially offer an immediate response as soon as an outbreak begins, therefore allowing for the best control of infections [104].

4.5. Hepatitis Virus

Among other infectious diseases, hepatitis is responsible for the many of cases of liver inflammation, fibrosis and cirrhosis cases worldwide. The disease is divided into five types; namely A, B, C, D and E [110]. According to the World Health Organization (WHO), hepatitis A (HBA) and E (HEV) are transmitted through contaminated food and water as a result of poor sanitation or sanitary practices [111]. The detection of these viruses involves the use of microbial and plaque assays, serologically-based methods as well as PCR methods [112]. However, plaque assays exhibit low sensitivity since it requires high viral loads in a sample, which also imply that detection during the early stages on infection is difficult. Serologically-based methods on the other hand, rely on the manifestation of an immune response of which antibodies are not produced until the virus escalates. Although PCR methods are sensitive and definite they can only be performed in a laboratory setting and can therefore not be considered as rapid and low cost diagnostic methods. Type D (HDV) causes dual infection and occurs in patients infected with type B (HBV); a combination of the two may ultimately lead to life threatening consequences if not treated. Type B and C (HCV) are the most prevalent, with an approximately 96% mortality rate [113]. According to the Center for Disease Control (CDC), type A and B are transmitted through contact with infected blood, which includes exposure due to receiving infected transfused blood, as well as through unprotected sexual activity. Mother-to-child transmission has also been reported in type B [114] and has been noted for its distinct mechanisms of replication which involves the engagement of protein-primed transcription of an RNA intermediate, the pre-genomic RNA (pgRNA) occurring within viral capsids. The virus's pgRNA packaging, together with reverse transcriptase (RT) (also known as the P protein), is important for the assembly of viral capsids [115].

Worldwide, chronic hepatitis is mainly caused by hepatitis C. Its positively-stranded RNA encodes for one polyprotein which requires post-translational processing as well as the involvement of proteases to cleave it into its functional components which comprise of both structural and non-structural proteins. The core protein C and the two envelope proteins (E1 and E2) comprise the structural proteins whereas NS2 (Protease), NS3 (Serine protease, helicase), NS4A (Serine protease cofactor), NS4B and NS5A proteins and NS5B, an RNA-dependent polymerase and an enzyme that is critically important for HCV replication [116], make up the non-structural proteins. The mechanisms by which the viruses (HBV and HCV) replicate themselves and the elements of these mechanisms are important targets for diagnostics. There is an increasing need for rapid diagnostic tools that are specific enough to detect infection by tracking the viral components even in asymptomatic patients [117]. Aptamers are the best candidates for this since they present high specificity and affinity for their targets. Mirian and co-workers [117] reported the advantages of using aptamers as tools for the development of biosensors capable of detecting the hepatitis B virus and described the identification of an RNA aptamers (HBs-A22), which was used for the detection of HB surface antigen (HBsAg) that is expressed on the surface of infected hepatoma cells (HepG2.2.15). Furthermore, Mirian et al. [117] documented that fluorescein isothiocyanate (FITC) tagged HBs-A22 and fluorescence microscopy was used to demonstrate binding to HBsAg expressing cells. A cyanine3 tagged RNA aptamer was used to detect HCV core-protein using a chip-based assay. An aptamer selected against HCV E2 glycoprotein was used in an enzyme linked apto-sorbent assay (ELASA) was used in qualitative and quantitative analysis of viral particles. These studies showed the extensive and potential use of aptamers as tools for the early detection of viruses even during the window period [117].

4.6. Rubella Virus

Rubella virus (RV) is etiologically responsible for causing German measles. This virus consists of

a positive sense RNA genome, three structural proteins and two non-structural proteins namely the capsid and the glycoproteins E1 and E2; with P60 and P150 respectively, all of which are involved in the virus life cycle and replication and are important targets for vaccine and drug development [118]. Rubella is a highly transmissible virus, the only one from the genus *Rubivirus* of the *Togaviridae* family [22]. Its mode of transmission is mainly by inhalation of air droplets produced when an infected person coughs or sneezes [119]. Mother-to-child transmission has also been reported and may lead to deleterious effects such as miscarriages, foetal defects known as congenital rubella syndrome (CRS) [120,121], which may cause heart defects, thyroid dysfunction, loss of hearing and other conditions including autism. Rubella symptoms appear from at least 2 to 3 weeks after infection and these include; mild fever, red rashes, joint pains, headaches and enlarged neck lymph nodes. Moreover, the symptoms alone cannot be used for definite diagnosis; hence, confirmation by laboratory tests, which detect antibodies (i.e., serological assays), or the detection of viral RNA (using PCR) is required. In 1997, Revello and colleagues employed reverse transcription (RT)-PCR in the detection of prenatal RV. Among other specimens tested, it was concluded that the amniotic fluid (AF) was more suitable for the detection of prenatal RV. Regardless of the sensitivity of RT-PCR, it was also found that false negative results might be obtained when the test is done immediately after maternal viral infection [121]. Hence, Mori et al. [122] develop a novel reverse transcription loop-mediated amplification (RT-LAMP) method for the detection of RV. Once tested using RT-PCR on isolates sensitive to the Takashi vaccine strain, RT-LAMP was found to be as sensitive as RT-PCR since it detected infection with more or less the same specificity as the well-known RT-PCR. Nevertheless, PCR based methods are performed only in the laboratory by experts and for this reason it is not a readily available method and cannot always be employed in clinical settings. Moreover, despite the use of serologic assays, viral detection may be delayed because antibodies are not produced until after a certain period, also known as the window period or seroconversion. Therefore, the need for sensitive, specific and easily applicable tools such as those that are aptamer-based is currently mounting.

4.7. Human Schistosomiasis

Schistosomiasis (commonly known as bilharzia) is a disease that is caused by parasitic blood flukes or trematodes called schistosomes. *Schistosoma* species, namely *Schistosoma haematobium*, *S. mansoni* and *S. japonicum* are the main causes of human schistosomiasis [123]. Disease transmission to the human host is by means of direct contact with cercariae that are released by snails found in open water sources. Once within the human host, cercariae changes into schistosomulae which resists immune responses and enters the blood stream where the parasite lays its eggs and then migrate to different parts of the body, mainly in the urogenital area, liver, lungs, heart, kidneys and intestines [124]. Eggs hatched from adult worms are then excreted through faeces and urine and enter open water sources, were they then hatch into miracidia and quickly penetrate host snails to become sporocysts that develop into cercariae via asexual reproduction [125]. Early diagnosis of the disease is important for the control of disease escalation which may lead to deleterious effects that predispose infected persons to cancer and lead to the damage of major body organs [126]. However, low intensity of the infection may escape detection by diagnostic tests such as the Kato-Katz smear methods resulting in false negative results [123,127]. Serologically-based methods can also be used for the detection of Schistosoma. However, these diagnostics tests cannot differentiate between infections with trematodes different *Schistosoma* species or other helminths such as cestodes and nematodes, and therefore have low specificity for the detection of helminth infection. This highlights the need for extremely sensitive and specific agents such as aptamers for the detection of schistosomes [123]. Long and co-workers studied two DNA aptamers; namely LC6 and LC15. These were tested against *S. japonicum*, *S. kipo*, *Fasciopsis buskis* and *Enterobus* eggs and it was shown that both aptamers have strong affinity only for *S. japonicum* eggs. The tissue imaging results clearly showed that LC15 could strongly bind to *S. japonicum* eggs in the liver [123].

5. Applications of Aptamers for Viral Therapy

Despite the fact that there has been a huge interest in aptamers and that countless scientific studies have been published on the topic, the full potential of aptamers in diagnostics and therapeutics have not yet come to fruition. The approval of Macugen® (pegatnib sodium) an anti-vascular endothelial growth factor (VEGF) aptamer by the Food and Drug Administration in 2004 [15] for the treatment of age-related macular degeneration is a great milestone and demonstrates the potential of aptamers [14,37,53,128,129]. Since then, many more useful studies have been piloted in the search for solutions for the treatment of HIV, TB, ZIKA, Ebola, cancer, hepatitis and measles. The use of nanostructures in targeting biomolecules for diagnosis and therapy is increasing; in vivo RNA nanotechnology leads the way in this regard [76,130,131]. This is due to the fact that RNA and proteins are enzymatically synthesized by transcription and translation of analogous gene sequences and the occurrence of RNA nanostructures seen to be interacting with proteins has been found in nature involving ribosomes, spliceosome, RNase P [130].

Schwarz-Schilling et al. [130], has shown that the interaction of RNA with proteins can be mediated by aptamer motifs which have been used in identifying RNA molecules with fluorescent proteins or enzymes. Developing RNA-protein nanostructures involves co-expression of RNA and functional constituents (aptamer motifs, proteins or enzymes) in one reaction [130]. However, this co-expression of multiple aptamers specific for various biomolecules can be a major challenge, since the binding affinities and expression levels of the proteins may vary. Schwarz-Schilling et al. [130], used a 40 nt pRNA 3WJ as the core motif of an RNA-protein nanostructure, which incorporated four aptamers (TAR, PP7, streptavidin and malachite green); two of the aptamers bind specifically to fluorescent proteins (Tat and PCP), one to streptavidin and the other to malachite green (MG). With the aid of fluorescence from the MG aptamer and Förster resonance energy transfer (FRET) between the fluorescing protein molecules, the authors were able to study the binding parameters and the stability of the complex nanostructure. The TAR is a 28 nt aptamer selected to bind tat (a transcriptional activator) from bovine immunodeficiency virus (BIV), the PP7 is a 25 nt long aptamer that binds to the amino acid sequence of the peptide phage PP7 coat protein. Added to this, Schwarz-Schilling and co-workers investigated RNA stability in a cell-free expression system using crude cell extract (previously used for the assembly of protein filaments or virus particles) from *E. coli*. After studying the aptamers at different positions, it was concluded that the RNA was stable and suitable for use as a scaffold. The co-expression and localization of the RNA-protein complex was investigated in vitro in a one-pot reaction using streptavidin beads and the expression of the RNA-protein structure was investigated inside the bacteria. This study clearly indicated that in vitro 'cell-free' expression is preferably the best route, since it does not require extra measures required for optimizing in vivo RNA-protein expression and provides better expression under controlled conditions. These results are evidence to the advancing RNA-based technology, which not only ensure high efficacy of expression, but a better yield under controlled conditions in vitro. This is a great improvement, which is important in the use of RNA aptamer for both diagnostics and therapy [130].

5.1. Ebola and Zika

In vitro studies by Binning and co-workers [83] identified two aptamers (1G8-14 and 2F11-14), which bind specifically to the interferon inhibitory domain (VP35IID) of VP35 EBOLA protein. Such findings certify that these aptamers may be applied as inhibitors of the dsRNA binding to VP35 and therefore therapeutically prevent the spread of the virus. Subsequently, Wandtke et al. [94] reported that aptamers with specific binding ability to the zinc-finger functionally prevent Ebola virus replication.

5.2. Aptamers in HIV Therapy

Despite the availability of efficient treatments for both HIV and TB, the development of drug resistance is growing problem and hence, alternative treatment for HIV and TB is needed [132].

Presently, the FDA has approved anti-retroviral regimen which includes nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs). Although these are not curative agents, they do however suppress viral replication [95]. Drug resistance and the presentation of severe side effects is an apprehensive issue that signifies the need for other means of drug delivery by suitable agents such as aptamers, which are highly specific and have been considered for use as co-operative agents of chemical anti-retroviral drugs [97]. Several studies have demonstrated the effectiveness of nucleic acid-based aptamer synthesis against important HIV components such as gp120, reverse transcriptase (RT), integrase (IN) as well as long terminal repeats (LTRs) and untranslated regions (UTRs).

5.2.1. Aptamers Targeting gp120

The HIV-glycoprotein 120 (gp120) is found on the surface of the HIV virus and initiates host invasion as it binds on $CD4^+$ cell receptors, which is a critical step in HIV infection. Viral entry inhibitors like Maraviroc T-20, which have been approved by the FDA and are currently in use, can counter HIV entry. However, due to the development of drug resistance other options are being investigated. Zhou et al. [133] synthesized anti-gp120-siRNA chimera (A-1 and B-68) and tested the aptamers' affinity in comparison to a B4ot77 aptamer discovered by Dey and colleagues [134] on HIV-1BaL gp120 protein. The results demonstrated that both aptamers could tightly bind and inhibit gp120 at different regions and epitopes of the protein, hence preventing viral entry [134,135]. Additionally, this study revealed that aptamers could be used as carriers for dicer substrate siRNA, which inhibits viral replication and infectivity in cultured CEM T cells and primary blood mononuclear cells.

G-quadruplex-forming oligonucleotides have in recent years come onto the scene as potent and novel aptamers. Their three dimensional assembly and non-canonical DNA structure confers on them the ability to recognize and target proteins involved in gene expression during the life cycle of the HIV virus, which include gp120 [136]. Present on gp120 is a co-receptor binding site known as the V3 loop, which is imperative for viral entry. The phosphorothioate 8-mer d(5^tTTGGGGTT3^t) commonly known as ISIS5320 was the first G-quadruplex antiviral agent with the ability to bind the V3 region of the gp120 envelope by forming a rigid tetrameric parallel-stranded structure that inhibited viral entry and cell fusion at sub-micromolar concentrations [95,137]. Gonzalez et al. [95] reported the discovery and modifications of a 6-mer d(5^tTGGGAG3^t) known as the Hotoda sequence, which also has anti-viral properties. Modifications in the Hotoda sequence at the 5'-3' ends led to the identification of molecules which bind to CCR5- binding sites on gp120. This binding inhibits the interaction between the viral gp120 and the CD4+ receptors, thus preventing viral entry, making these molecules potential targets for use in HIV/AIDS therapy [95].

5.2.2. Aptamers Targeting Reverse Transcriptase, RNase H and Integrase

Reverse transcriptase (RT) is responsible for two functions: firstly, DNase polymerase activity, converting RNA to DNA or vice-versa. After membrane fusion and de-capsulation, RT converts viral RNA to a complementary DNA (cDNA), which can then be integrated in the cells' genome. Inhibition of RT halts viral infectivity and its progression. Therapeutic FDA approved drugs such as zidovudine, nevirapine, and stavudine [138] with this function are available but these are less effective on their own and therefore require administration in conjunction with one another. They are very effective in preventing viral replication for some time but since reverse transcriptase lacks proof reading abilities, mutations develop resulting in drug resistance. Secondly, RNase H activity in which RNase H functions in dsRNA cleavage before the action of RT. Following the RNA cleavage and reverse transcription, the viral genome is then integrated into the host cell's genome through a process catalysed by integrase (IN) [138–140].

Aptamers are superior potential solutions to drug resistance and a number of studies have demonstrated their potential therapeutic features. In 2016, Gonzalez and colleagues reported the discovery of a 37NT DNA aptamer that was found as highly specific and able bind to HIV-1 RT. When tested on the HIV-1 HXB strain, the aptamer blocked the enzyme's active site, thereby inhibiting

template binding and consequently preventing viral replication. Other sets of aptamers called ODNs 93 and 112, known to have G-rich sequences, were found to possess inhibitory properties against HIV-1 RNase H activity of HIV-1 RT in the nanomolar range in vitro. Interestingly, derivatives of ODNs 93 and 112, named ODNs 93 del and 112 del, with shorter sequences, were still found to be effective and stable since they sustained their ability to form G4-structures. The derivatives were found to inhibit HIV-1 integrase, also in the same nanomolar range [95,141].

5.2.3. Aptamers Targeting Nucleocapsid Protein (NC)

One survival mechanism exhibited by the HIV virus that has led to its extensive success is its mutating ability. This is possible through certain conserved proteins, one of which is known as the nucleocapsid protein (NC). This protein is majorly responsible for viral replication due to its chaperoning activity by means of its interaction with nucleic acids resulting in the unfolding of the secondary structure of viral RNA [142]. Inhibiting the NC protein would in turn impede viral progression and result in a non-infectious virus hence; the protein is considered a target for anti-viral therapy. It has been suggested that stabilized RNA aptamers could function as inhibitors for the viral packaging of HIV. This is based on a study where RNA aptamers with high affinity were selected and isolated from an RNA library which exhibited competitive binding with the psi RNA binding to the NC protein [143].

5.3. Aptamers in Hepatitis Virus

Available treatments for HBV include adenofovir, lamivudine and entecavir. Apart from the side effects demonstrated by these drugs, mutations that occur as a result of the infidel nature of reverse transcriptase are the cause of drug resistance [144]. Drug limitations increases the significance of the search for other modes of therapy that produce no side effects such as aptamers which are non-immunogenic and are therefore better agents for therapeutic applications. In 2008, Feng and colleagues [145] selected two aptamers (S1 and S2) against reverse transcriptase P protein; they subjected the aptamers to complete DHBV genomes PCD16, which are regulated by a CMV promoter. These were then transfected into LMH cells to determine potential roles in the virus's replication cycle. S2 could strongly bind and inhibit P protein and hence prevent DNA synthesis and capsid formation. The binding of S1 on the other hand, displayed no effect on the formation of the capsid [145]. Following this study, Feng et al. [115] selected two aptamers; S6 and S9, and found that these aptamers are specific and have high affinity for Mini P (protein) in vitro. They further introduced S9 to HBV infected HepG2 cells and the observations revealed a great suppression of pgRNA and DNA synthesis. Additionally, S9 could successfully bind and inhibit P protein, thus preventing its association with pgRNA and therefore inhibiting viral replication.

Hepatitis C related drug resistance is an accumulating problem [146] and fortunately, studies have increasingly demonstrated that aptamers show great potential for use in the road to discovering specific therapeutic agents for this infirmity. Lee and co-workers [116] synthesized two aptamers; 2^t hydroxyl RNA aptamer and 2^t fluoro RNA aptamer against NS5B of HCV and tested the aptamers on whole cells. The 2-OH RNA and 2-F RNA aptamers were found to bind specifically and inhibit the replicase activity of NS5B, hence preventing viral replication. Later, Wandtke et al. [94] reported the selection of an aptamer that is highly specific to the E2 glycoprotein of HCV, a co-receptor of human CD81 observed on hepatocytes and B-lymphocytes. The ZE2 aptamer functions as a complete inhibitor of E2 and when tested on human established cell lines of hepatocellular carcinoma, it was noted for decreasing viral RNA and reducing E2 protein levels following verification by reverse transcription—qPCR (qpT-PCR) and western blot [94].

5.4. Aptamers in Rubella and Schistosomiasis

Despite the present vaccines and treatment of measles, emerging drug resistance remains the problem and the use of Veraptus therapeutic aptamers against infectious viral diseases, have been reported and serve as potential solutions to drug resistance [147]. Nevertheless, more aptamer work

still needs to be done as an alternative method for therapy.

Among other schistosomiasis therapeutic agents, praziquantel has been the most reliable drug used for the control of the disease and is effective on all three strains of the main *Schistosoma* spp. [148,149]. However, praziquantel is slowly losing its value due to drug resistance and its noted ineffectiveness against the juvenile form of the worm [150]. The exact mechanisms by which these helminths resist PZQ are still not understood and this drug resistance seriously declares the need for novel therapeutic agents such as aptamers which will be specific and highly effective in retarding disease progression and hence controlling its spread. The LC15 aptamer that was discovered by Long et al. [123] demonstrated specificity for *S. japonicum* eggs and highlighted aptamers as potential agents that could act as drug-carriers that ensure specificity and hence provide improved and alternative forms of therapeutic agents [151].

6. Conclusions and Future Perspectives

The acceptance of certain aptamers such as Macugen® for use in the pharmaceutical industry with others going through phase trials mark the aptitude of aptamers. Aptamers are highly non-immunogenic, are specific to their targets and very sensitive, thus they succeed in being able to distinguish between isoforms of the same target. Hence, to address the problems encountered by diagnostic tools, they can therefore be used as a basis for diagnostic tools that will ensure early detection of diseases without any waiting period before definite diagnosis. This will greatly improve the time of diagnosis and enable good and better disease control. Aptamer structures can be modified easily for functional enhancement and stability, and these functions suggest that they can be used in conjugation with any therapeutic agent, directing the drug to specific sites and improving drug specificity while combating drug resistance in the process. Many studies have demonstrated the potential of aptamers as shown in Table 2, but there is still a gap in the search for aptamers against Rubella virus. Taken together, aptamers are the future and their application will be enhanced even for evolving diseases since they can be synthesized within short periods and in high batches.

Table 2. Molecular Application of Aptamers in Diseases.

Aptamers	Sequence 5'	Structure	Organism	Target	Function
AS1411 (DNA)	d(GGTGGTGGTGGTTGTGGTGGTGGTGG)	G-quadruplex	Cancer	Nucleolin	Specifically detects cancer cells [14,48] Promotes cell death, prevents cell proliferation via Rac 1 activation [66] Up-regulates Galectin-14 and suppresses hepatocellular carcinoma [68]
T40214 (DNA)	d(GGGCGGGCGGGCGGGC)	G-quartet	Cancer	STAT3- protein	[14,79,152]
HJ24 (DNA)	d(AGCGTCGAATACCACACGGGGGTTTT GGTGGGGGGGGCTGGGTTGTCTTGGGGG TGGGCTAATGGAGCTCGTGGTCAT)	G-quadruplex	Cancer	Shp2	In vitro studies (IC50 = 29 nM) [14]
TTA1 (RNA)	-	-	Cancer	Domain of tenacin	Prevents angiogenesis, invasion and tumour growth [48]
MUC 1 (DNA)	GCAGTTGATCCTTTGGATACCCTGG	-	Cancer	Mucin-1	[26]
AS1411 (DNA)	d(GGTGGTGGTGGTTGTGGTGGTGGTGG)	G-quadruplex	HIV	Nucleolin	Antiviral activity (in vitro) (EC_{50} = 15.4 nM) [14]
37NT (DNA)	-	-	HIV (HXB strain)	HIV reverse transcriptase (RT)	Blocks RT active site, prevents viral replication [95]
ISIS5320	d(T*T*G*G*G*G*T*T)	G-quadruplex	HIV	HIV gp120	Prevents viral adsorption, inhibits HIV infection (in vitro) IC50 = 0.30 μM) [14]
Hotoda sequence	DBB-d(TGGGAG) and TBDPS-d(TGGGAG)	G-quadruplex	HIV	HIV gp120	In vitro (IC_{50} = 0.37 μM and 0.88 μM, resp.) [14]
Zintevir	d(G*TGGTGGGTGGGTGGG*T)	G-quadruplex	HIV	HIV Integrase	Completed phase 1 [137]
ODN93	d(GGGGGTGGGAGGAGGGTAGGCCTTAGGTTTCTGA)		HIV	HIV RNase H	Inhibits RNase H activity In vitro (IC_{50} = 0.50 μM) [14,95]
ODN 112	d(CCAGTGGCGGGTGGGTGGGTGGTGGGGGGACTTGG)		HIV	HIV RNase H	Inhibits RNase H activity In vitro (IC_{50} = 0.50 μM) [14,95]
ZE2 (DNA)	-	-	HCV	HCV-E2 glycoprotein	Inhibits E2 binding on CD81 receptors [12]
Apt.No.28 (DNA)	-	-	HBV	HBV nucleocapsid	Prevents nucleocapsid assembly and DNA synthesis [153]

NK2 (DNA)	-	-	Mycobacterium tuberculosis H37Rv strain	Membrane Proteins	Acts as an antibacterial agent, promotes cytokine production [12]
LC6 and LC15 (DNA)	-	-	Schistosomiasis	Schistosoma eggs	Binds specifically to Schistosoma eggs [121]

NALATS: Not Available in Literature as at the time of search [-].

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CHAPTER

THREE

(HIV-p24 expression, *in vitro* selection of HIV-p24 derived aptamers and characterization of the conjugates using *in silico* techniques)

CHAPTER THREE: MATERIALS AND METHODS

3.1. Introduction

HIV is world-wide epidemic. In 2017, about 36.9 million individuals were reported to be living with HIV, 1.8 million of those were children of ages 15 or less, and about 1.8 million newly infected, with reports of at least 5000 new cases per day [https://www.hiv.gov/hiv-basics/overview/data-and-trends/global-statistics (Accessed 16 May 2019)]. Moreover, only about 75% of the infected people have knowledge of their infection and 25% still need to be tested. Early diagnosis is imperative for mitigating viral transmission but unfortunately, available rapid tools are serologically based and may not detect the virus in time and moreover, other methods such as PCR methods and p24 antigen tests have limitations resulting in diagnostic delays. Literature attests that HIV-p24 protein is overexpressed during the early and last stages of viral infection (Tang *et al.*, 2010). This makes it an important biomarker and requires a sensitive point-of-care diagnostic tool. Fortunately, a study by Tuerk and Gold introduced a whole new insight on oligonucleotide (DNA/RNA) aptamers, which are selected through **s**ystematic **e**volution of **l**igands by **ex**ponential enrichment (SELEX) (Tuerk and Gold, 1990). These were found to have high affinity and specificity for their targets without eliciting immunological response and are good targets for applications including diagnostics and therapy (see Chapter 2). This section of the study presents the recombinant expression of HIV-p24 protein, selection of HIV-p24-derived aptamer and *in silico* studies of the protein~aptamer complex through bioinformatics.

3.2. Methods

3.2.1. Transformation of *E. coli* XL1-Blue cells with GST-p24 plasmid

In this study, an HIV p24 plasmid, sub-cloned into a pGEX-6P-2 vector was transformed into *E. coli* XL1 Blue competent cells (prepared using the chemical method of $CaCl_2$ and $MgCl_2$). In performing the transformation, a vial of competent cells (previously prepared) was thawed on ice, after which 2 µl of pGEX-6P-2~p24 construct and 100 µl of the competent cells were mixed into a clean, sterile microfuge tube and kept on ice for 20 minutes. The mixture was then heat shocked for 5 minutes at 37°C and then placed back on ice for 10 minutes. Thereafter, a 900 µl warm YT broth without ampicillin was added into the mixture and mixed gently by inverting the tube a few times. This was incubated at 37°C for 1 h 30 minutes. Meanwhile, agar plates with 100 µg/ml of ampicillin were prepared and thereafter, 100 µl of the incubated culture were spread onto the agar plates and incubated overnight at 37°C.

3.2.2. Expression screening

Four positive transformants were picked randomly from the overnight transformed culture plates and inoculated separately into 5 ml broth containing 100 µg/ml of ampicillin. The cultures were then incubated at 37°C for 4 hrs, after which a 2 ml measure of each culture was distributed equally into two sterile micro-centrifuge tubes. Thereafter, 2 µl of 0.5 mM isopropyl β-D-thiogalactoside (IPTG) was added to one of the tubes to induce protein expression, while the culture without IPTG served as the un-induced culture. Both the induced and the un-induced cultures were then incubated with shaking at 37°C for 2hrs. This was followed by centrifuging the cultures at the highest speed for 5 minutes. Thereafter, the supernatants were discarded and then pellets were re-suspended with 50 µl of 2X sample buffer. The contents were mixed properly, heated in a dry bath at for 5minutes and a part of the mixture (20 µl) were electrophoresed on a 16% SDS-PAGE gel. After observation,

glycerol stocks were prepared from the colony-culture with the best protein expression and stored at -80°C for future use.

3.2.3. Large scale expression

A 100 µl of the best expressing colony-culture glycerol stock (thawed) was pipetted into 100 ml of LB broth with ampicillin and incubated with shaking at 37°C overnight. The overnight culture was then scaled up to 2L with LB broth containing ampicillin and then incubated at 37°C with shaking. Periodic optical density (OD) observations were performed and when the OD was between 0.4 – 0.6, 0.5 mM IPTG was added to the culture to induce protein expression. The culture was then incubated at 25°C overnight. After incubation, the culture was transferred to 250 ml centrifuge tubes for spinning at 5000 rpm for 20 mins. The supernatants were discarded, and the pellets were re-suspended in Tris buffer containing DTT, PMSF and lysozyme at pH 7.0. Thereafter, the bacterial lysate was electrophoresed on a 16% SDS-PAGE gel to validate the expression of the target HIV-p24 protein.

3.2.4. Cell lyses and Protein extraction

To extract the recombinant protein, the total bacterial lysate previously stored at -80°C was subjected to four cycles of freeze-thaw, followed by sonication at 4°C for 5 minutes at 20% power with shaking for 3 intervals every 1 minute to lyse the cells. The bacterial lysate was centrifuged at 5000 rpm for 30 minutes. Thereafter, the supernatant (clear lysate) was transferred to a clean 50 ml Eppendorf tube and then stored at 4°C.

3.3. Protein purification by affinity chromatography

To purify the expressed GST-tagged p24 protein, a column containing glutathione-agarose beads was used. The column was first washed with 5 column volumes of 1M NaCl. Subsequently, five column volumes of equilibration buffer containing 50mM Tris and 20mM NaCl (pH 8.0) was added to sensitize the beads. The lysate was then loaded onto the column

and the flow through was collected into a clean tube. Thereafter, the column was washed with ten column volumes of equilibration buffer which was flown until the last 1 ml was collected before the column dried off. The protein was then eluted seven times using elution buffer which contained 15 mM glutathione, 1 mM DTT and 0.02% NaN_3 (pH 8). After 10 ml elution fractions were collected, the column was washed with five column volumes of 2 M NaCl. After wash 1 and 2 was collected, the rest of the salt solution flowed out freely. After all these were done, the column was then kept at 4°C with a buffer containing 0.02% NaN_3.

3.4. SDS-PAGE Gel Electrophoresis

Glass casting technique was used to perform gel electrophoresis. This was followed by the preparation of a separating buffer containing Tris (pH 8.8), 40% Bis-Acrylamide, 10% SDS, APS and TEMED. The mixture was poured into the casted glasses, while leaving space for the stacking buffer and left to solidify. After polymerization, a stacking gel made up of the same reagents as the separating gel but with 0.5M Tris buffer (pH 6.8) was prepared and then poured on top of the separating gel till overflow. Combs were immediately placed in before the stacking gel polymerized to form wells. Once the stacking gel solidified, it was left to stand for at least 20 minutes before it was put into running buffer. The Precision Plus Protein Dual Color Standards (BIO-RAD) molecular weight marker was loaded into the first well followed by the respective loading of the samples. Subsequently, an electric field was introduced, causing migration of the molecules to the oppositely charged electrode. When the samples were done running, the gel was stained with a coomasie stain for alternating 1 minute warming (in a microwave) and 10 minute cooling at room temperature for 3-5 intervals and further 20 minutes on a belly dancer (Company) to ensure proper staining before de-staining with 10% acetic acid to view the bands.

3.5. Protein quantification

The concentration of the protein was determined using NanoDrop ND2000 (ThermoFisher Scientific, USA). The process was carried out using the same elution buffer that was used to elute the protein, as well as the eluted protein. A 2 µl sample of the elution buffer was placed into the lower optical face of the NanoDrop spectrophotometer and its arm was closed; this buffer was used to blank the machine. Thereafter, the buffer was wiped off gently, followed by an addition of the same volume of the eluted protein. The arm was then closed and the protein concentration (A_{280}) was measured.

3.5.1. Characterization of expressed GST-p24

After purification, the protein concentration was estimated using NanoDrop ND2000, after which functional groups present in the protein were identified using Fourier Transform Infrared Spectroscopy (FTIR) spectroscopy. UV spectroscopy was used to determine the folding, purity and light absorbing capacity of the protein.

3.5.2. Determination of functional groups

FTIR was used to characterize the HIV-p24 protein by determining the functional groups that are present in the protein. FTIR analysis of the protein solution was carried out using Perkin Elmer FTIR spectrum two in the range of 4000-4500 cm^{-1}. Within the mentioned range, the functional groups present in the protein solution were visible.

3.6. DNA-Aptamer selection and synthesis

The aptamers in this study were selected in the laboratory of Professor Mervin Meyer, Department of Biotechnology, University of the Western Cape. *In vitro* selection of the aptamers was performed using two methods, normal (GST bead-based separation) and a modified 1-POT SELEX method by Scoville and co-workers (2017). The PCR thermocycler that was used for each method was purchased from Multigene (Labnet International Inc.).

GST-magnetic beads used in this study for separation were acquired from SIMAG and Invitrogen. The two conserved primers used were synthesized and purified using HPLC (Whitehead Scientific, Cape Town).

Following selection, the samples were sequenced and the sequences were analysed using Biomatters' Geneious software (Biomatters, Auckland, New Zealand). Thereafter, the final synthesis was done by Biomatters via Inqaba Biotech (Johannesburg, South Africa).

3.6.1. Aptamer selection

Aptamer selection (conventional, normal SELEX) began with the preparation of the DNA library to a suitable ratio of 10:1 (800 pmoles: 80 pmoles of target). An 8 µl quantity of the DNA library was diluted with 92 µl of 1X SELEX buffer (containing PBS) after which the mixture was incubated in a thermal cycler at 95ºC for 5 minutes, with a gradual decrease in temperature by 10ºC every 30 seconds, until 25ºC was reached and then held for 1 minute. After the incubation with temperature decrements, 1 µl of the target protein was added into a prepared DNA library, vortexed briefly and then incubated at 25ºC for 30 minutes at 500 rpm. Meanwhile, a 15 µl measure of glutathione magnetic beads were washed and prepared with 100 µl of the SELEX buffer for three intervals. After preparation, the mixture (DNA + target protein) was then added into the beads and further incubated with shaking at 25ºC for 30 minutes at 500 rpms. After incubation, the beads were captured using a magnetic bead separator for 1-2 minutes; the supernatant was removed and transferred into a new microfuge tube and saved for future. The captured beads were then washed 3 times with 1X SELEX buffer. Following the wash, the beads were resuspended in 25 µl of nuclease free water and this served as a template for pilot PCR. To prepare the pilot PCR reaction, a volume of 150 µl of the master mix was prepared as described in Table 3.1.

Table 3.1: PCR constituents

Initial concentration	Final concentration	Volume
2X Kapa extra HS ready mix	1X	75 µl
25 mg/ml BSA	0.5 mg/ml	3 µl
10 µM Forward primer	0,2 µM	3 µl
10 µM Reverse primer	0.2 µM	3 µl
50 mM $MgCl_2$	3Mm	9 µl
nuclease free water		57 µl

When the master mix was ready, 25 µl was aliquoted into a PCR tube as a negative control. Thereafter, 5 µl of the template previously prepared was added to the remaining master mix, mixed well and then 25 µl was aliquoted into 5 different PCR tubes labelled **5**, **10**, **15**, **20** and **25**. These numbers represented the cycle numbers. The samples were then run on PCR thermal cycler with the following conditions; initial denaturation at 95ºC for 3 min held for 1 cycle, denaturation at 95ºC for 30 sec, annealing 58ºC for 30 sec, extension at 30 sec, final elongation at 72ºC for 5 minute and hold at 4ºC. Once the PCR starts, the tube labelled **5** was removed together with the negative control, a few seconds before the 5^{th} cycle passed. The same procedure was followed for all the other tubes, corresponding to the PCR cycle numbers. The tube corresponding to the 25^{th} cycle was left to enter 4ºC held for 1 minute.

After amplification, the samples were electrophoresed on a 3% agarose gel at 110V for 35 minutes to determine which cycle produced the most distinct DNA band. The best expressing cycle was then used for a scale-up PCR of 220 µl master mix. A 30 µl measure of the master mix was aliquoted as a negative control and then 10 µl of the initially prepared template was added to the PCR mix, mixed well and a 100 µl of the mix was aliquoted into two separate

PCR tubes (representing amplicons). Thereafter, the negative control and the two amplicons were amplified, following the same conditions as those employed on the pilot PCR, but using the optimised number of cycles. After the completion of the PCR cycles, the samples were electrophoresed.

After the confirmation of positive amplification by electrophoresis, 10 µl from the amplicons was transferred to a clean PCR tube, to serve as negative control for electrophoresis after digestion. T7 enzymatic digestion was employed by adding a final concentration of 1X NE buffer 4 and 30 (3 µl) of the T7 enzyme to the amplicons. The mixture was then incubated in a thermocycler at 25ºC for 60 mins (digestion), 65ºC for 5 mins (heat inactivation) and thereafter held at 4ºC until the next step was performed.

After digestion, the 10 µl volume of the previously saved sample from amplicons (negative control) and the enzymatic digestion products were electrophoresed to ascertain the formation of the ssDNA. After the gel electrophoresis, the ssDNA was purified using the oligo clean and concentrator kit (Zymo Research, California, USA) following the protocol provided by the kit. After purification, 2 µl of the purified template was combined with 10 µl of 6X DNA dye and 3 µl of NaOH and electrophoresed. Thereafter, purified ssDNA was quantified using a Qubit assay and of the purified ssDNA, 2 µl was added into the ssDNA qubit working solution.

The remainder of the purified ssDNA was made up to 100 µl of 2X SELEX buffer and prepared for the next round at 95ºC for 5min with gradual 10ºC decrements for every 30 secs until 25 ºC was reached. From round 2, negative (with DNA prepared from ssDNA+ GST beads) and positive (DNA+ beads + target protein) SELEX were prepared. For the negative SELEX, after DNA preparation, beads were added and the mixture was incubated at 25ºC for 30 minutes at 500 rpm. Right after incubation, the beads were captured for about 1-2 minutes,

while the supernatant was transferred into a clean sterile 1.7 ml tube. The beads were washed 3 times with 100 µl SELEX buffer, resuspended in 25 µl of nuclease free water and then stored at -20ºC until further use. For positive SELEX, the supernatant from the negative SELEX, saved in the 1.7 ml tube was used as the positive library and 1 µl of the purified target protein was added and prepared by incubation at 25ºC for 30 minutes at 500 rpm. After the preparation, washed 15 µl beads were added to the mixture and incubated further at 25ºC for 30min at 500 rpm. This was then followed by bead capture and the removal of the supernatant into a sterile tube as the unbound. The positive beads were then washed 3 times and then resuspended in 25 µl of the PCR grade water.

Pilot PCR was then prepared following the previously described method for both negative and positive SELEX. After pilot scale, samples were electrophoresed and scale up was done, following the method described above, and then electrophoresed. The scaled-up amplicons were then digested and electrophoresed. The resulting ssDNA was purified, quantified and readied for the next round of SELEX. The above steps and conditions were used until the 4^{th} round of SELEX. From rounds 5-7, stringency was employed whereby the purified ssDNA obtained from the previous round + beads were incubated for 20 minutes as negative SELEX, instead of 30 minutes at 25ºC for 500 rpms, and for positive SELEX, 15 picomoles of the target and the unbound DNA were incubated for 20 minutes. From Round 8-9, the same conditions were applied for negative and positive SELEX. However, in these rounds, Counter-SELEX was introduced whereby the unbound ssDNA and the target protein were incubated for 10 minutes at 25ºC for 500 rpms. This was immediately followed by the addition of human serum albumin (HSA) as the counter protein and a further incubation for 10 minutes at 25ºC for 500 rpms.

3.6.1.1. Monitoring evolution

Monitoring evolution SELEX is important in determining when to stop the selection as well as in selecting aptamers with high binding affinity (Luo *et al*., 2017). Aptamer evolution was monitored by selecting rounds from normal SELEX (between 1-4), when stringency was introduced (between rounds 5-7) and when counter SELEX was introduced (from round 8-14). Rounds 3, 6, 9, 12 and 14 were selected and a non-duplicate PCR scale-up was performed. Thereafter, T7 exonuclease digestion was employed on all the selected rounds confirmed by electrophoresis and purified using the Zymoclean Kit. The samples were quantified and then diluted with nuclease-free water in reference to the lowest round concentration. Quantified, dilute ssDNA samples were used as templates for pilot PCR, prepared following the same conditions and optimal cycles for each round. The samples were electrophoresed in order to determine the round with better aptamer evolution.

3.6.2. Modified One-Pot SELEX

The modified One-Pot SELEX used in this study was adapted from the method described by Scoville *et al*., 2017. Conventional SELEX involves iterative rounds (usually 10 rounds) of aptamer selection, whereas the method postulated by Scoville and co-workers includes a total of 5 rounds only. In this study, only one round of selection was done. To perform this method, 3X 1.5 ml Eppendorf microtubes were used. A 100 µl of nuclease-free H_2O was added into one tube as control and 100 µl of the target protein (p24) was added to the remaining two tubes (labelled as tests T_1 and T_2). The test solutions and control were evaporated on a SpeedVac in a concentrator overnight without heat. The tubes were then washed twice with 200 µl of PBST containing 0.1 M $NaPO_3$, 0.15 M NaCl and 0.05% Tween-20 at pH 7.2 and transferred to clean tubes labelled wash one and two (W_1 and W_2). Washes from the targets were then quantified; thereafter T_1 and T_2 as well as the control tube were dried at room temperature. Meanwhile, the DNA library was prepared by mixing 1 µl of

a 100 µM library with 99 µl of SELEX buffer and boiled at 95ºC for 5 minutes and held at 25ºC. The prepared 100 µl DNA was added to the uncoated tube (which previously contained Nuclease-free H_2O) and incubated at 25ºC for 15 minutes. Thereafter, 100 µl was distributed equally to the target coated tubes and incubated for 1 hour at 25ºC. Meanwhile, 50 µl of SELEX buffer was added to the uncoated tube (negative control) and incubated for 1 hour at 25ºC. After an hour, DNA from the coated tubes and SELEX buffer from the uncoated tube were transferred to sterile tubes and the coated were quantified, washed twice with 200 µl PBST. The washes were discarded, and the tubes were dried completely in a SpeedVac. Thereafter, a 170 µl PCR mix was prepared, after which 20 µl of the PCR mix was aliquoted to a clean PCR tube as control for the reaction. Then, 150 µl of the remaining PCR mix was distributed equally to T_1, T_2 and W tubes and boiled at 95ºC for 5 minutes, vortexed and centrifugated. The mixtures were transferred to PCR tubes to begin the PCR reaction, using the same conditions described in conventional SELEX.

3.6.3. Oligonucleotide sequencing and synthesis

Random rounds with promising evolution were selected for sequencing. Next Generation Sequencing (NGS) was used to sequence the samples at Inqaba Biotech (Johannesburg, South Africa). Thereafter, analysis of the sequences was done using the Biomatters Geneious software (Biomatters, Auckland, New Zealand) obtained online at https://www.geneious.com/academic/.

M-fold web server, available at (http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form) was then used to predict the folded structures of DNA- aptamers, as well to determine structures with high stability as this is an important characteristic. Finally, best aptamers structures were selected, after which synthesis of functional aptamers was done at Inqaba Biotech (Johannesburg, South Africa).

3.7. Protein-aptamer interactions Studies

Several methods such as isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), electrophoretic mobility shift assay (EMSA) and microscale thermophoresis (MST) can be used for the characterization of biomolecular interactions (Asmari *et al*., 2018). Studying biomolecular interactions such as protein-protein or protein-nucleic acids is important in understanding biological systems and pave ways for efficient drug delivery and disease diagnosis (Rainard *et al*., 2018, Asmari *et al*., 2018). HIV-p24 protein-Aptamer (Oligo 1 and 2) interaction was characterized using *in silico* PatchDock.

3.7.1. Homology modelling of the HIV capsid and Aptamers

Proteins are long sequences of building blocks called amino acid residues with unique sequences (Alberts *et al*., 2002). Proteins fold into a specific 3-D shape due to these sequences and the prediction of the protein 3-D structure remains a key research hitch in structural bioinformatics. Various algorithms and techniques have been set to remedy these hitches ranging from comparative modelling (homology modelling), threading (fold recognition methods) and ab-initio method (Zhang, 2002; Lee *et al*., 2017). However, several databases such as PSIPRED, HHpred, PORTER, Jpred, Phyre2 and I-TASSER amongst others, have been employed in predicting the function and generating 3-D models of a given query sequence. In this study, I-TASSER which is an example of composite method for modelling was used. Homology model predicting the three-dimensional structure (3-D) of the p24 protein was achieved using I-TASSER http://zhanglab.ccmb.med.umich.edu/I-TASSER/ [Accessed 24 January 2019]. I-TASSER generates five models with a confidence score (C-score) that ranges between -5 to +2, hence the model with the highest C-score was selected for this study in order to get accurate results. The cartoon view of the modelled structure analysing secondary structural elements was visualised using PyMOL Molecular Graphics (2003). In order to ascertain the reliability of the forecasted model, RAMPAGE

Ramachandran plot was used http://mordred.bioc.cam.ac.uk/~rapper/rampage.php [Accessed 24 January 2019]. Subsequently, the aptamers' 3D structures were generated using Discovery Studio visualization tool (DSV) (2017) http://www.3dsbiovia.com/products/collaborative-science/biovia-discovery-studio/ [Accessed 20 February 2019].

3.7.2. Molecular Docking

Molecular docking can be employed in modelling the interaction between a protein and small molecules at the atomic level, allowing for the characterization of the activity of the small molecules in the binding site of proteins (McConkey *et al*., 2002). Hence, the binding of aptamers to p24 protein was assessed by performing molecular docking using PatchDock an online tool available at https://bioinfo3d.cs.tau.ac.il/PatchDock/ [Accessed 22 February 2019]. This is a geometry-based molecular docking algorithm designed to ascertain docking transformations that yields good molecular shape complementarity (Schneidman-Duhovny *et al*., 2005). PatchDock works with high efficiency because of its swift transformational search, powered by local features complementing rather than using six-dimensional transformation space search by brute force (Schneidman-Duhovny *et al*., 2005). This was achieved by considering the diagnostic candidate (aptamers) as ligand and the p24 protein as receptor.

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CHAPTER

FOUR

(Results and Discussion)

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1. Sequence retrieval and homology modelling of HIV capsid (p24)

The DNA sequence shown in Fig. 4.1A was retrieved from the National Centre for Biotechnology Information (NCBI) online database https://www.ncbi.nlm.nih.gov/ [Accessed 20 June 2016] and was translated into the amino acid sequence as shown in Fig.4.1B using the translate tool of the **Ex**pert **P**rotein **A**nalysis **Sy**stem (ExPASy) https://web.expasy.org/translate/ [Accessed 25 June 2016]. The amino acid sequence is important in determining the amino acid composition of the target protein by abundance as depicted in Table 4.1, which shows that HIV-p24 has low abundance of aromatic amino acids and high abundance of polar amino acids such as lysine. This shows that although the protein has poor light absorption, it is highly stable and soluble. High lysine dominance contributes to the stability of the protein and aids in solubility, which is achieved when positively charged amino acids form hydrogen bonds with water molecules (Warwick *et al*., 2013). This information is crucial in ensuring that the protein biophysical characterization methods to be employed are well-suited and accurate for the particular protein. Furthermore, the amino acid sequence is used for modelling the 3D structure of proteins, showing their folding states and secondary structure components such as helices, coils and beta sheets. This was done using I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER) as shown in Fig.4.1C which displayed that the protein consists of 6 alpha helices and one 3_{10} helix as the secondary structural element found in the protein. Alpha helices tend to fold and unfold; the function of the 3_{10} helix is to mediate the folding of the alpha helix thereby contributing to protein stability (Armen *et al*., 2003). The 3-D modelled structure of the p24 protein was generated using the I-TASSER database, which employs a confidence score (C-score) ranging between 2 to -5 to evaluate the quality of the predicted model. Hence, the modelled structure in this study had a C-score of -3.36, which is indicative of good confidence and correct folds. The

confidence score signifies that the predicted model can be employed for drug design and structure-based virtual screening, as well as other molecular and biotechnology investigations (Vangrevelinghe *et al*., 2003; Kopp and Schwede, 2004). Subsequently, the model was validated using the RAMPAGE server (Lovell *et al*., 2002). RAMPAGE is a user-friendly program designed to ascertain the stereochemical quality of a modelled protein structure. It was shown that 75.5% of the amino acid residues were in the most favoured region, which signifies the quality of the built model and another 16.3% residues in allowed region.

ATGCTGCACTGTGTGCAGCGCGCGCTGATCCGCAGCCAGGAGCTGGGCGACGAGAAGATCCAGATCGTGAGCCAG
ATGGTGGAGCTGGTGGAGAACCGCACGCGGCAGGTGGACAGCCACGTGGAGCTGTTCGAGGCGCAGCAGGAGCTG
GGCGACACAGTGGGCAACAGCGGCAAGGTTGGCGCGGACAGGCCCAATGGCGATGCGGTAGCGCAGTCTGACAAG
CCCAACAGCAAGCGCTCACGGCGGCAGCGCAACAACGAGAACCGTGAGAACGCGTCCAGCAACCACGACCACGAC
GACGGCGCCTCGGGCACACCCAAGGAGAAGAAGGCCAAGACCTCCAAGAAGAAGAAGCGCTCCAAGGCCAAGGCG
GAGCGAGAGGCGTCCCCTGCCGACCTCCCCATCGACCCCAACGAACCCACGTACTGTCTGTGCAACCAGGTCTCC
TATGGGGAGATGATCGGCTGCGACAACGACGAGTGCCCCATCGAGTGGTTCCACTTCTCGTGCGTGGGGCTCAAT
CATAAACCCAAGGGCAAGTGGTACTGTCCCAAGTGCCGGGGGGGAGAACGAGAAGACCATGGACAAAGCCCTGGAG
AAATCCAAAAAAGAGAGGGCTTACAACAGG**TAG**

Figure 4.1A: DNA sequence of HIV- p24

```
10         20         30         40         50         60
 MLHCVQRALI RSQELGDEKI QIVSQMVELV ENRTRQVDSH VELFEAQQEL GDTVGNSGKV

        70         80         90        100        110        120
 GADRPNGDAV AQSDKPNSKR SRRQRNNENR ENASSNHDHD DGASGTPKEK KAKTSKKKKR

       130        140        150        160        170        180
 SKAKAEREAS PADLPIDPNE PTYCLCNQVS YGEMIGCDND ECPIEWFHFS CVGLNHKPKG

       190        200        210
 KWYCPKCRGE NEKTMDKALE KSKKERAYNR
```

Figure 4.1B: Full-length of amino acid sequence of HIV-1 p24 excluding the mRNA sequence

Table 4.1: HIV-p24 amino acid abundance by percentage

Amino acids	Abundance (%)
Alanine (A)	6.7
Arginine (R)	7.1
Asparagine (N)	7.1
Aspartate (D)	6.7
Cys (C)	3.8
Glutamine (Q)	4.8
Glutamate (E)	9.5
Glycine (G)	6.2
Histidine (H)	2.9
Isoleucine (I)	2.9
Leucine (L)	4.8
Lysine (K)	**11.0**
Methionine (M)	1.9
Phenylalanine (F)	1.4
Proline (P)	4.8
Serine (S)	7.6
Threonine (T)	2.9
Tryptophan (W)	1.0
Tyrosine (Y)	1.9
Valine (V)	5.2

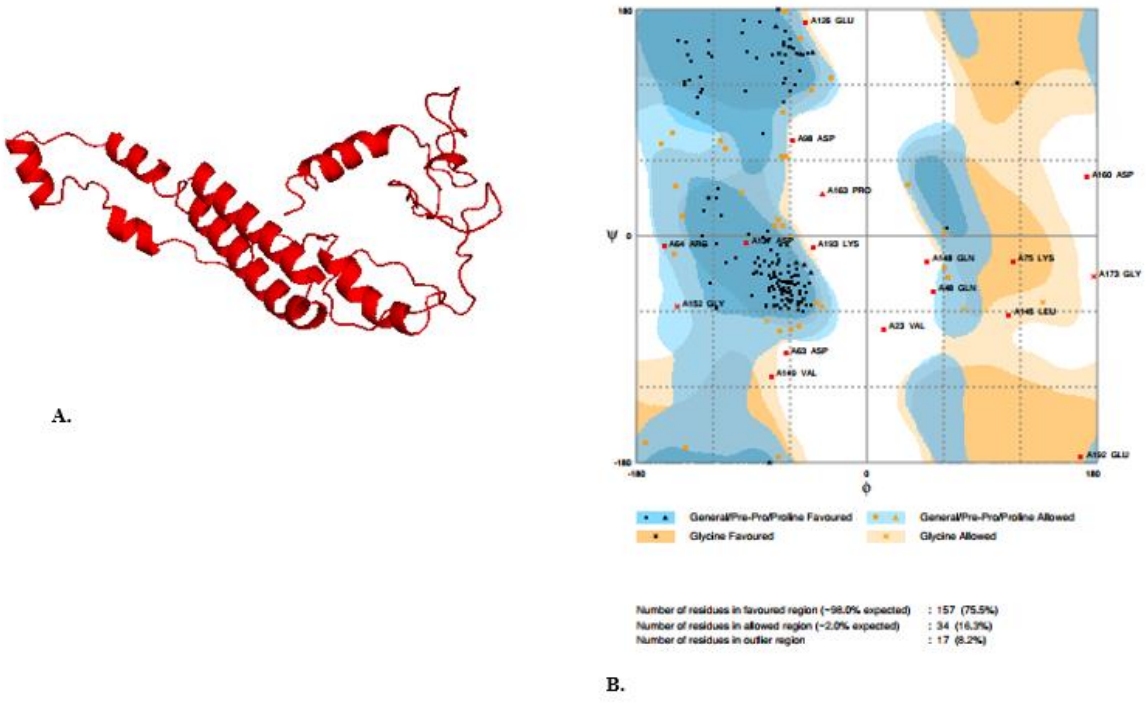

Figure 4.1C: HIV-p24 protein 3D model prediction. A shows predicted 3D model from I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) with 6 alpha helices and one 3_{10} helix. B is the Ramachandran plot from RAMPAGE validating the quality of the predicted 3D model showing 75.5% of residues in the favoured region and 16.3% of residues in the allowed and the outlier regions (Lovell *et al.*, 2003).

4.2. Transformation *E. coli XL1-Blue* cells with GST-HIV-p24

Escherichia coli cells used in this study were made competent using the chemical method with $CaCl_2$ and $MgCl_2$. HIV-p24 plasmid was transformed into both *E. coli BL21* (recommended for expression) and *XL1-Blue* cells (mostly used for plasmid mini-preps). Surprisingly, the best transformants were observed on *E. coli XL1-Blue* cells. Figure 4.2A shows the agar-plate which contained plated cells without plasmid (as control) and Figure 4.2B shows positive transformants, cells into which an ampicillin-resistant HIV-p24 plasmid was successfully transformed.

A. **B.**

Figure 4.2: Transformation of XL1-Blue cells by GST-p24 plasmid. **A** show the results of plated E. coli XL1-Blue cells without the plasmid which serves as a control (C) and **B** as a test (T) showing colonies of the cells transformed with GST-p24 plasmid.

4.3. Recombinant expression and purification GST-HIV-p24 protein

Expression screening was done by selecting several colonies and inducing them with 0.5 mM IPTG, while others were not induced. Expression screening provides information of the best expressing colonies as shown on the SDS-PAGE in Figure 4.3A. The best expressing colony was used for large-scale expression of the protein; this is done to produce the protein in large quantities. This is shown by the intense protein bands seen in Fig.4.3B, which migrates at the expected size of the target protein at approximately 50 kDa. After a large-scale expression, it is paramount that the protein is extracted from the cell suspension. The extraction allows for the release of soluble protein into the medium and separation takes place by centrifugation, thus pelleting the cell debris and leaving the protein lysate to be obtained as supernatant and ready for affinity purification. Proteins intended for biological applications are required in the purest form. In this study, the protein was purified using GST-agarose affinity chromatography, which binds the GST-tagged HIV-p24 protein, whilst allowing unbound bacterial proteins to flow through. The eluted HIV-p24 protein is shown in Figure 4.3C together with other collections which are loaded on the SDS-PAGE gel.

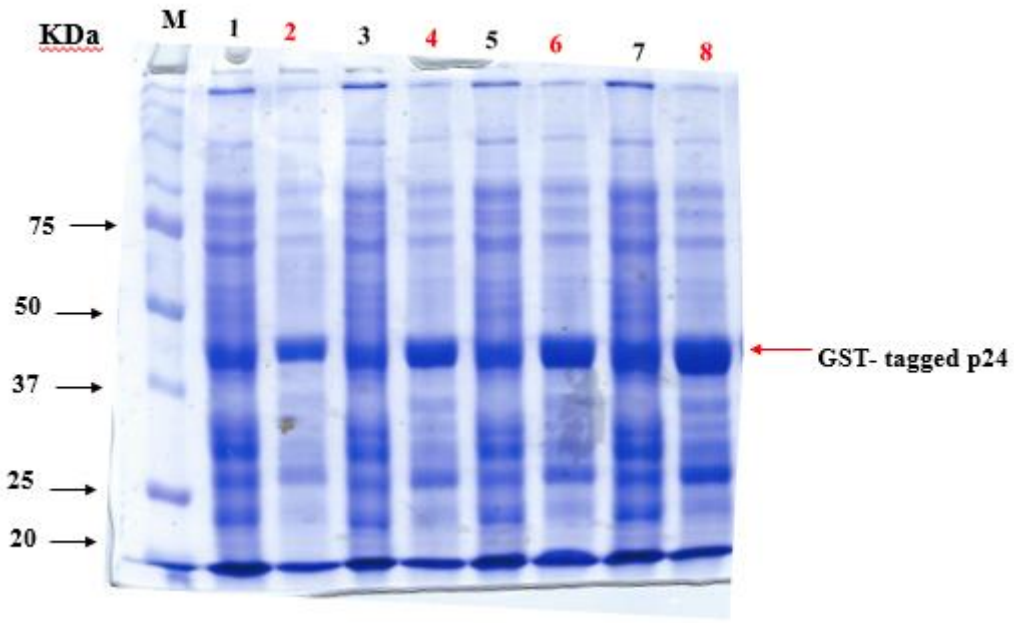

Figure 4.3A: Expression screening of E. coli XL1-Blue cells transformed with GST-p24 plasmid. **M** the molecular marker shows size in kDa. Lanes 1, 3, 5 and 7 represent un-induced colonies whereas lanes 2, 4, 6 and 8 represent colonies induced with 0.5 mM IPTG. Both the induced and the un-induced transformants expressed the protein (GST-p24) which is shown by the bands, running at approximately 50 kDa.

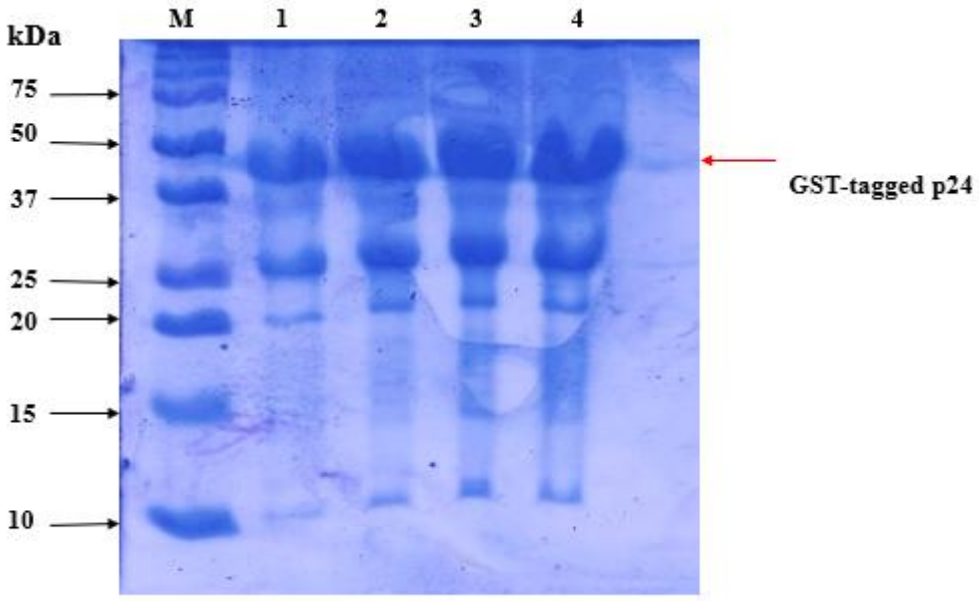

Figure 4.3B: Large scale expression. Lanes 1, 2, 3 and 4 show protein bands from bacterial lysate pipetted from 5 to 20 µl respectively and running at 50 kDa, the size of GST-tagged p24.

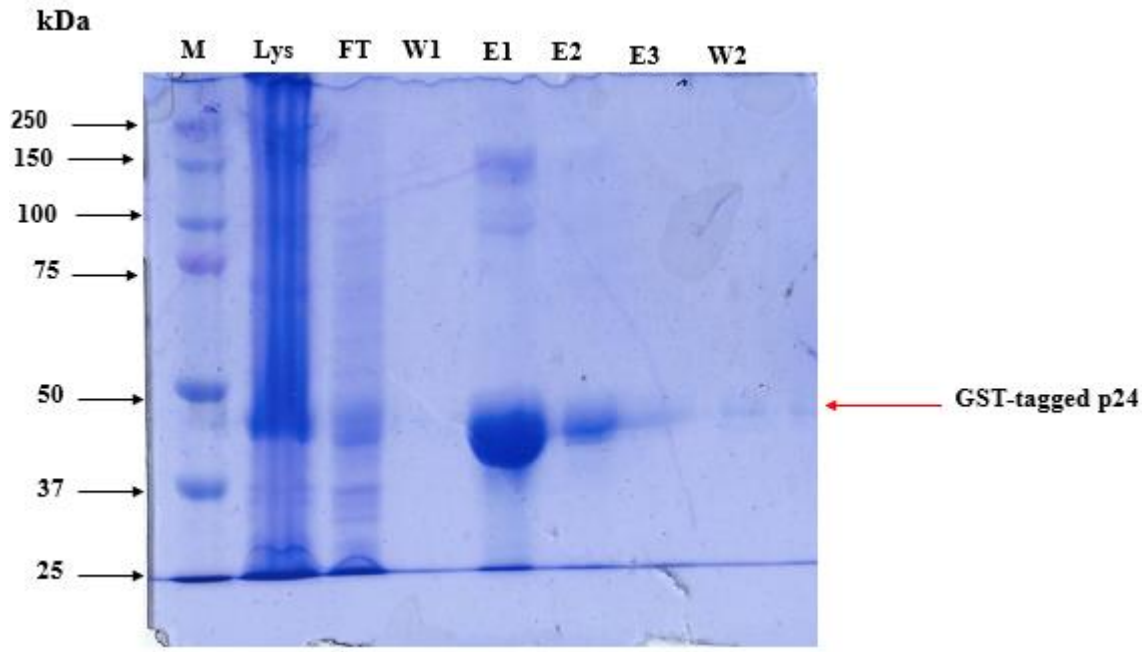

Figure 4.3C: GST-agarose affinity chromatography purification. **Lys**- stands for Lysate obtained before purification, **FT** for Flow through, **W1** for Wash 1 with equilibration buffer, **W2** for Wash with 2M NaCl. The protein bands showing at **E1**, **E2** and **E3** ran at 50kDa display the purified **GST-tagged HIV-p24 protein**.

4.4. Protein quantification by NanoDrop (ND2000)

The concentration of the HIV-p24 protein was determined by NanoDrop as shown on the spectrum depicted in Figure 4.4. The peak at 230 nm represents the visible light, whereas the one at 280 nm corresponds to that of the HIV-p24 protein absorbance, with a value of 4.26mg/ml in accordance to Beer-Lambert's law. However, this concentration is low and does not correspond to the high expression and the amount of the protein shown on the SDS-PAGE (Paynther, 1981; Desjardins *et al*., 2009). The low concentration could be due to the low extinction coefficient of the protein which is used by the NanoDrop to estimate the concentration http://www.u.arizona.edu/~gwatts/azcc/InterpretingSpec.pdf [Accessed 13 May 2019]. The contradiction of the NanoDrop results with that of the SDS-PAGE suggests that any method using light to deduce protein concentration may display misleading low concentration results, especially if the protein has been shown to have low light absorbing properties. Thus, the use of an alternative method, which does not rely on light properties such as Bradford Assay is recommended (Bradford, 1976).

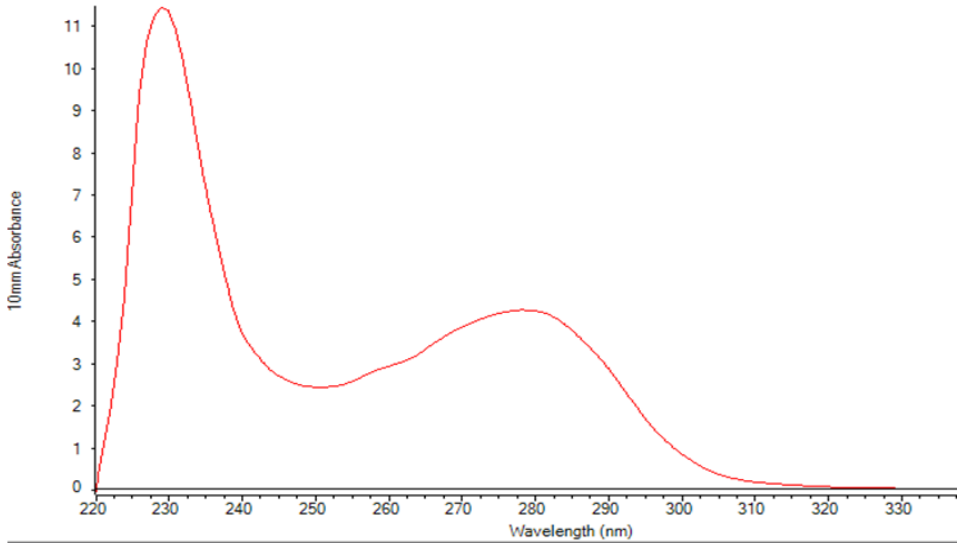

Figure 4.4: HIV- p24 quantification by Nanodrop. The peak shown at 230nm is that of the visible light and the one seen at 280 nm shows the protein absorbance value of 4.236 following Beer Lambert's law that 1Abs=1mg/ml.

4.5. Biophysical characterization of HIV-p24 by UV and FTIR

The absorption spectrum shown in Figure 4.5A projects below 300 nm, a short, near UV wavelength used for characterizing biological proteins at 280 nm wavelength. This method depends on tyrosine and tryptophan or a small amount of phenylalanine which are relatively low in this protein. Despite the low light absorbing capacity of HIV-p24, UV was sensitive enough to pick the available content of the aromatic amino acids and show the high peak observed around 280 nm, which indicates that the protein is pure and well folded (Mach *et al*., 1995). FTIR is used to estimate the functional groups present in the protein, this is essential in determining if the protein has not lost some functional groups which contribute to its chemical structure and functions (Barth, 2007). Figure 4.5B showed the spectrum of functional groups corresponding to the wavelength. The first trough represents the abundance of primary and secondary amines as well as amino acids; these amines are highly nucleophilic and provide good sites for protein labelling (Patil *et al*., 2013). The second trough represents the range of the protein that is dominated by carboxylic groups, carboxyl groups are essential in the formation of a functional secondary and tertiary structures of the protein forming via several forces of attraction such as hydrogen bonding Van der Waals as well as important for protein stability (Pace *et al*., 2014).

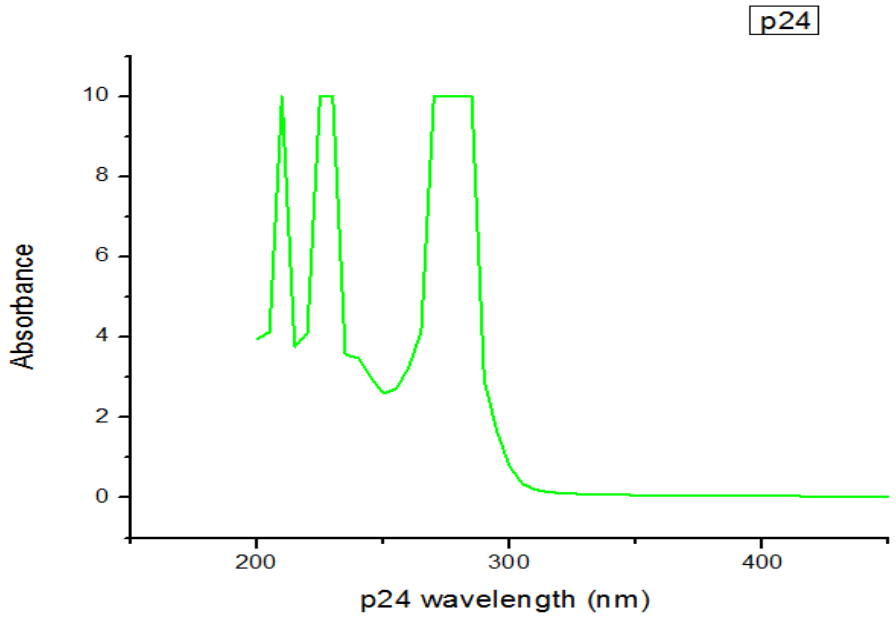

Figure 4.5A: Biophysical characterization of HIV-p24 by ultraviolet (UV/VIS) spectrum. The peaks seen between 250 and 300nm (short wavelength) shows that the expressed protein is pure and well-folded.

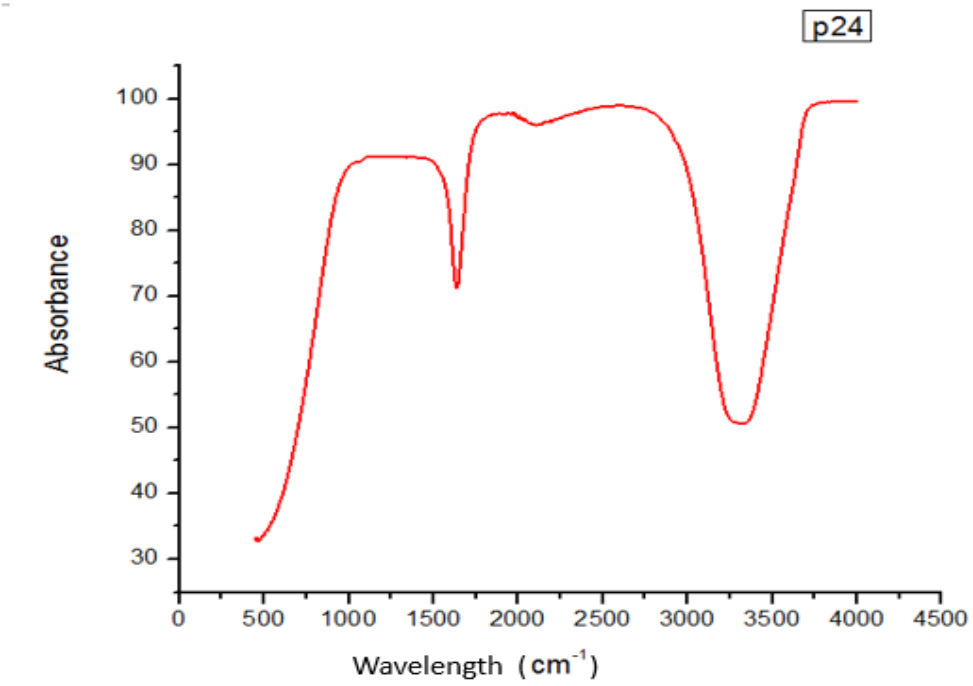

Figure 4.5B: HIV-p24 estimation of functional groups by FTIR. The first trough (1000 – 2000 cm^{-1}) shows that the protein is dominated by primary and secondary amines, which correspond to the alpha protons in the protein and the second trough (3000- 4000cm^{-1}) shows the abundance of carboxylic functional groups.

4.6. Aptamer selection

Aptamer selection experiments were carried out in iterated rounds. A total of 14 rounds for the magnetic bead-based method and only one round for the modified One-Pot SELEX method. Both methods were performed to evolve oligonucleotides with high specificity and affinity for the target HIV-p24 protein. Figure 4.6A showed round 1 of the SELEX method on agarose gel using ethidium bromide, displaying the different stages of selection ranging from the pilot scale, scale-up of dsDNA amplification to exonuclease digestion to produce ssDNA oligonucleotides and their purification using Oligoclean and concentrator (Zymo Research, Irvine, USA). Figure 4.6 B - F showed Rounds 3, 6, 9, 12 and 14 pilot scale selected for monitoring evolution based on the band intensity and evolution of their pilot scale upon introduction of positive SELEX, stringency and counter selection. The introduction of these steps improves aptamer enrichment by reducing the binding of non-specific oligo sequences and the introduction of similar molecules (counter SELEX) enhances HIV-p24 target recognition (Stoltenburg *et al.*, 2012). Figure 4.6G shows the pilot scale PCR viewing on agarose gel of the rounds selected for monitoring aptamer evolution. Figure 4.6H displayed the comparison of evolution and the ligands among different rounds and according to the migration of the oligonucleotide observed on the agarose gel, great evolution was seen on round 6. However, due to the visible large amount of non-specific binding observed in the negative SELEX, round 9 was then chosen as the best. Figure 4.6I showed the amplification of one round from the modified One-Pot SELEX.

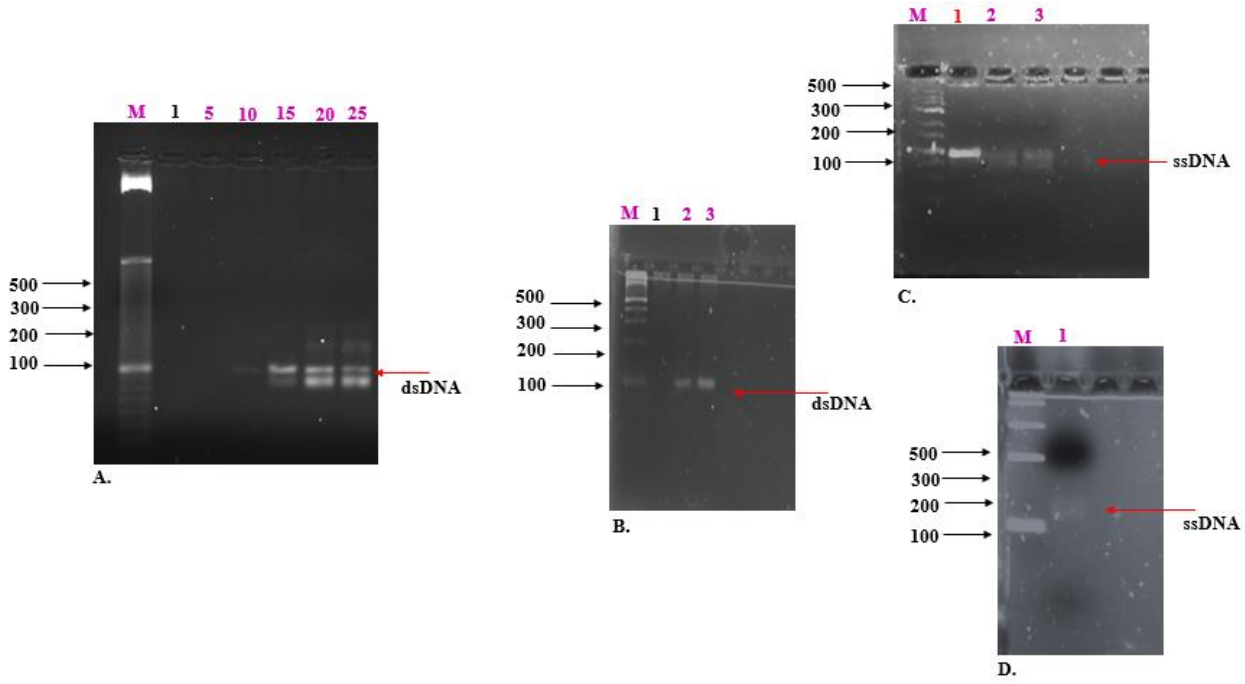

Figure 4.6A: Round 1. A) Pilot PCR, **M** marks the size of DNA in base pairs (bp), lane 2 (Labelled **1**) represents the negative control (PCR mix without template) whereas lanes 3 to 7 corresponds to PCR cycle numbers ranging from 5 to 25 in that order. Amplified bands (dsDNA) are shown running at approximately 100 bp in size which is the size of the DNA library. **B) Scale-up**. Lane 2 (Labelled **1**) represents the negative control and lanes 3 and 4 (Labelled **2** and **3** respectively) show dsDNA amplicons running at the expected size of the library. **C) T7 exonuclease digestion**. Lane 2 represents the positive control (from undigested scale up amplicons) and lanes 3 and 4 show smeared bands representing ssDNA. **D) ZymoResearch Oligoclean purification**. Lane 2 shows the smeared ssDNA band of the purified product obtained (as template for the next round).

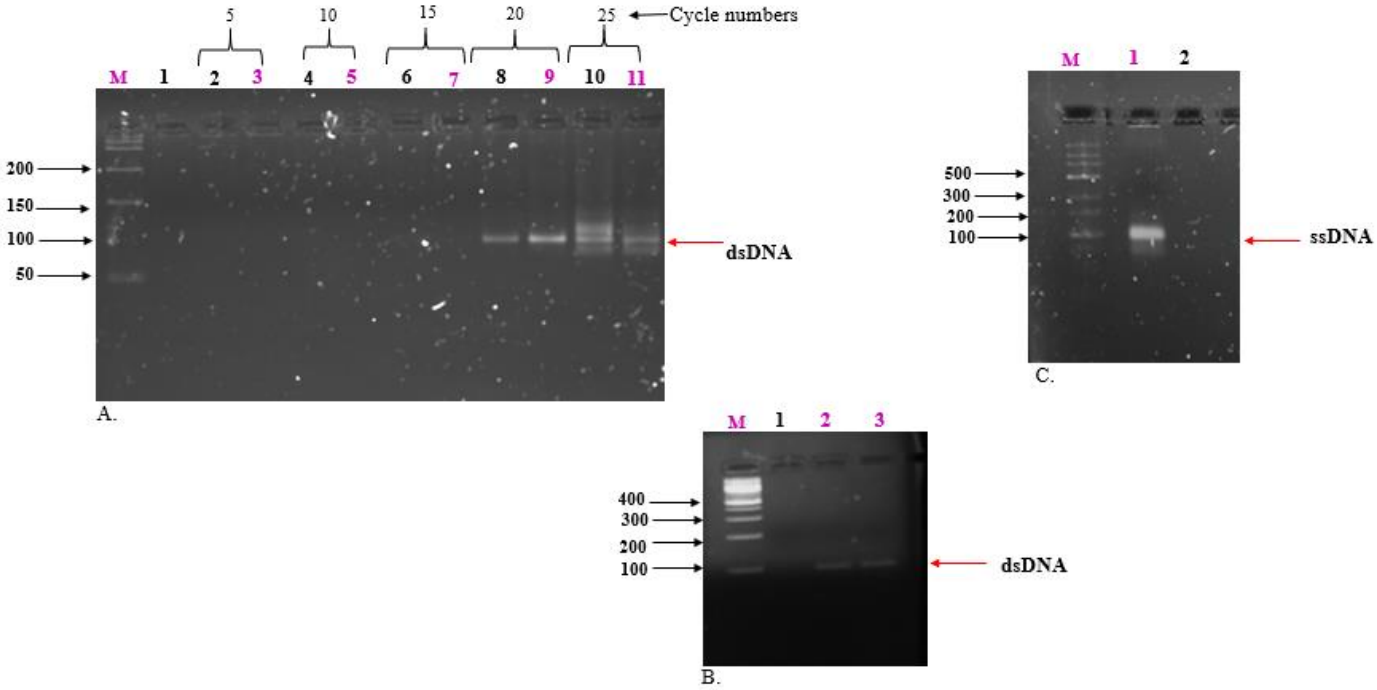

Figure 4.6B: Round 3. **A) Pilot PCR**. Lane 2 (Labelled **1**) represents the negative control (PCR mix without template), even numbered lanes represent negative SELEX (for sequences bound to separation GST- magnetic beads without HIV-p24) and the odd numbered lanes represent positive SELEX (ssDNA+ GST-beads+ target protein) amplified with respect to their corresponding cycle numbers. **B) Scale up**. Lane 2 (Labelled **1**) represents the negative control and lanes 3 and 4 (Labelled **2** and **3** respectively) show dsDNA amplicons running at the expected size of the library. **C) ssDNA PCR verification after purification**. Lane 2 shows dsDNA band of the amplified purified product and lane 3 represents the negative control (ssDNA).

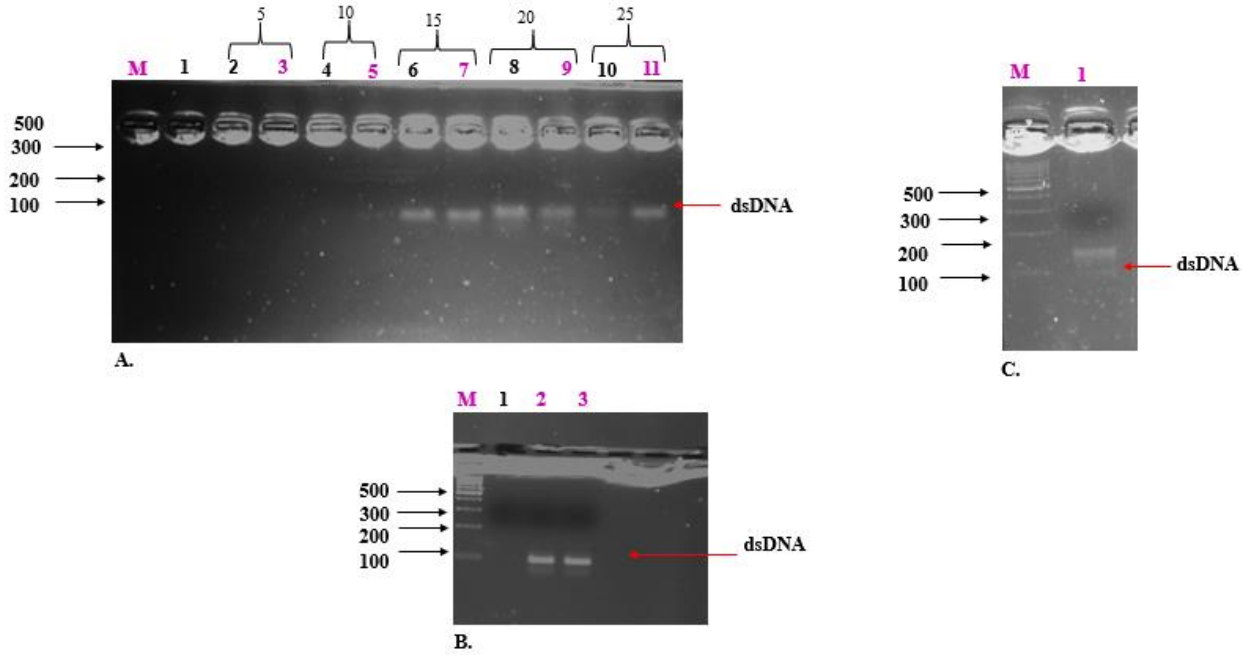

Figure 4.6C: Round 6. A) Pilot PCR. Lane 2 (Labelled **1**) represents the negative control (PCR mix without template), even numbered lanes represent negative SELEX (for sequences bound to separation GST- magnetic beads without HIV-p24) and the odd numbered lanes represent positive SELEX (ssDNA+ GST-beads+ target protein) amplified with respect to their corresponding cycle numbers. **B) Scale up**. Lane 2 (Labelled **1**) represents the negative control and lanes 3 and 4 (Labelled **2** and **3** respectively) show dsDNA amplicons running at the expected size of the library. **C) ssDNA PCR verification after purification**. Lane 2 shows dsDNA band of the amplified purified product and lane 3 represents the negative control (ssDNA).

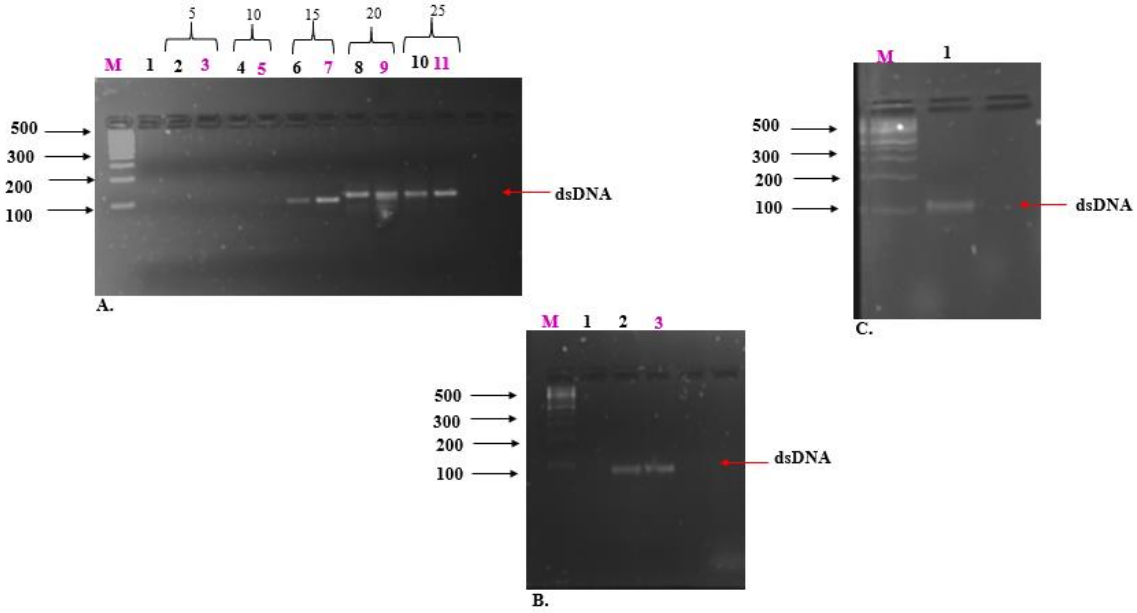

Figure 4.6D: Round 9. A) Pilot PCR. Lane 2 (Labelled **1**) represents the negative control (PCR mix without template), even numbered lanes represent negative SELEX (for sequences bound to separation GST- magnetic beads without HIV-p24) and the odd numbered lanes represent positive SELEX (ssDNA+ GST-beads+ target protein) amplified with respect to their corresponding cycle numbers. **B) Scale up**. Lane 2 (Labelled **1**) represents the negative control and lanes 3 and 4 (Labelled **2** and **3** respectively) show dsDNA amplicons running at the expected size of the library. **C) ssDNA PCR verification after purification**. Lane 2 shows dsDNA band of the amplified purified product and lane 3 represents the negative control (ssDNA).

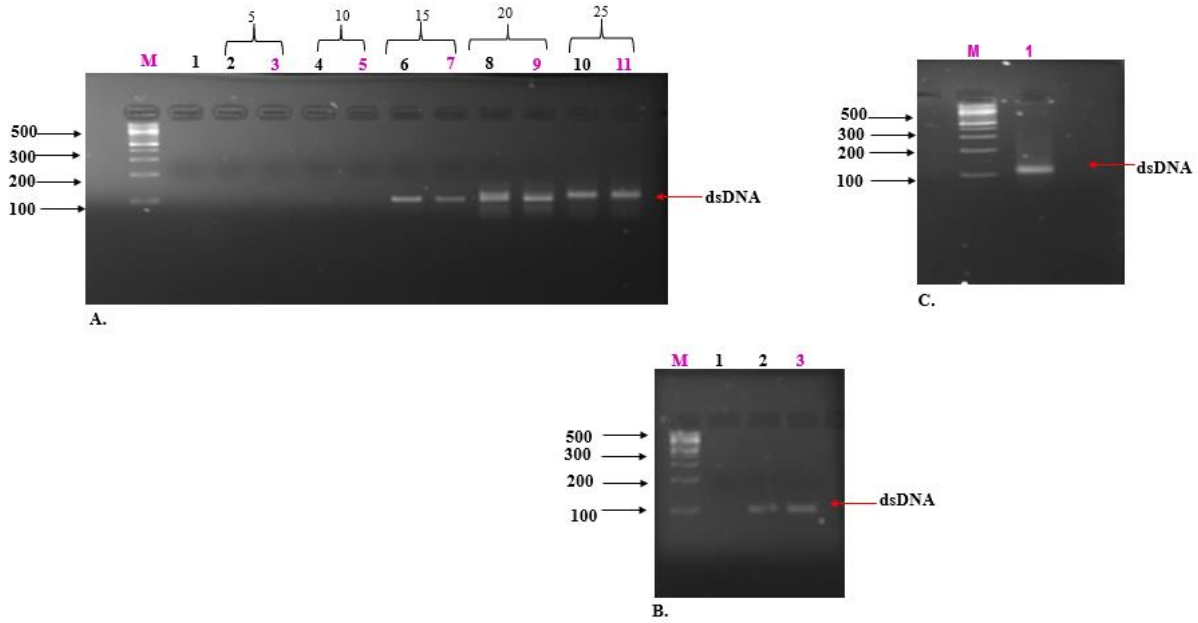

Figure 4.6E: Round 12. A) Pilot PCR. Lane 2 (Labelled **1**) represents the negative control (PCR mix without template), even numbered lanes represent negative SELEX (for sequences bound to separation GST- magnetic beads without HIV-p24) and the odd numbered lanes represent positive SELEX (ssDNA+ GST-beads+ target protein) amplified with respect to their corresponding cycle numbers. **B) Scale up**. Lane 2 (Labelled **1**) represents the negative control and lanes 3 and 4 (Labelled **2** and **3** respectively) show dsDNA amplicons running at the expected size of the library. **C) ssDNA PCR verification after purification**. Lane 2 shows dsDNA band of the amplified purified product and lane 3 represents the negative control (ssDNA).

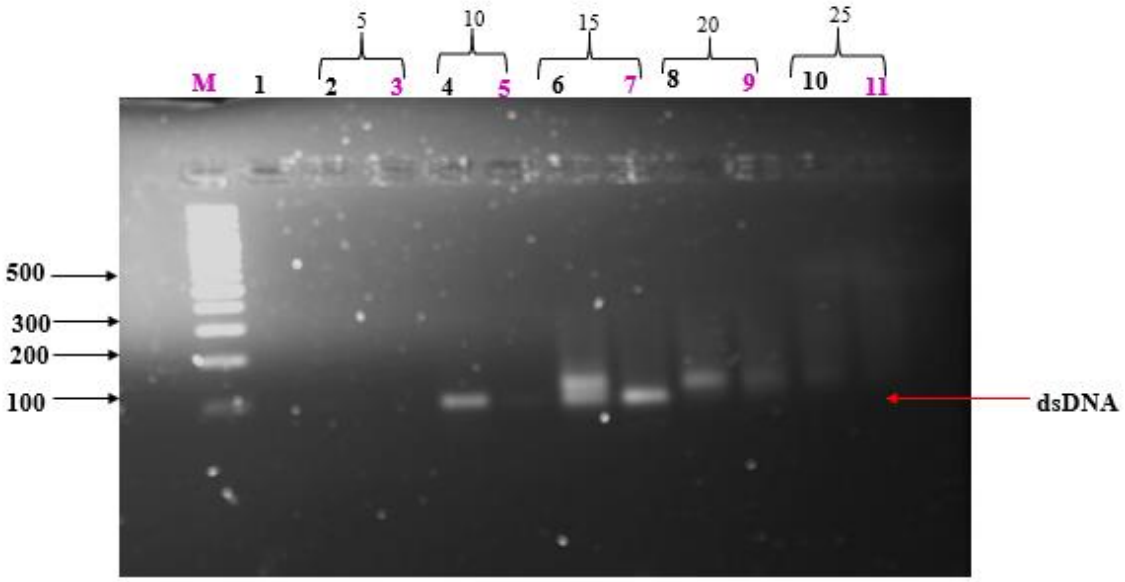

Figure 4.6F: Round 14. **A) Pilot PCR**. Lane 2 (Labelled **1**) represents the negative control (PCR mix without template), even numbered lanes represent negative SELEX (for sequences bound to separation GST- magnetic beads without HIV-p24) and the odd numbered lanes represent positive SELEX (ssDNA+ GST-beads+ target protein) amplified with respect to their corresponding cycle numbers.

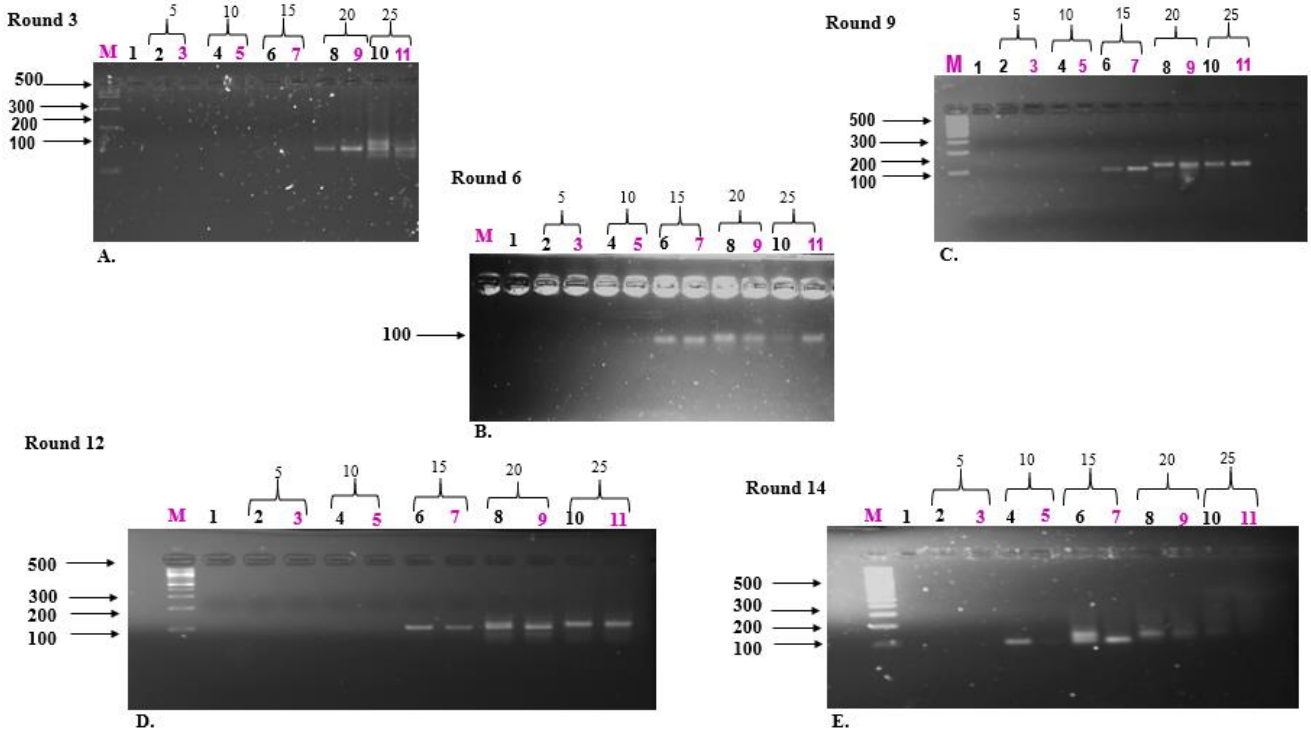

Figure 4.6G: SELEX rounds selected for monitoring evolution. Rounds (**3**, **6**, **9**, **12** and **14**) selected for monitoring aptamer evolution based on their pilot scale amplification results

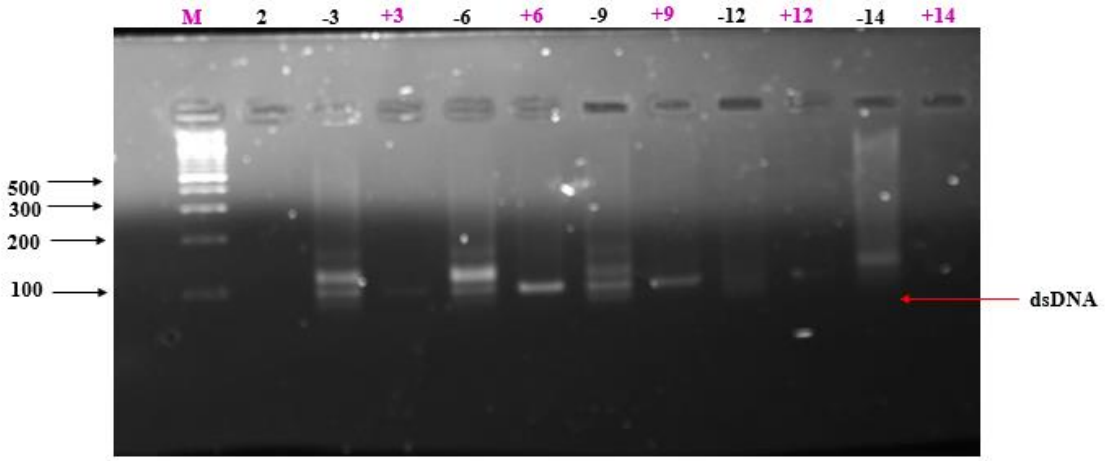

Figure 4.6H: Monitoring aptamer evolution. Lane labelled **2** represents the negative control (PCR mix without template) and lanes labelled **3**, **6**, **9**,**12** and **14** corresponds to the selected rounds, with negative representing negative SELEX and positive for positive SELEX of each round and DNA amplification is represented by bands seen at approximately 100 bp.

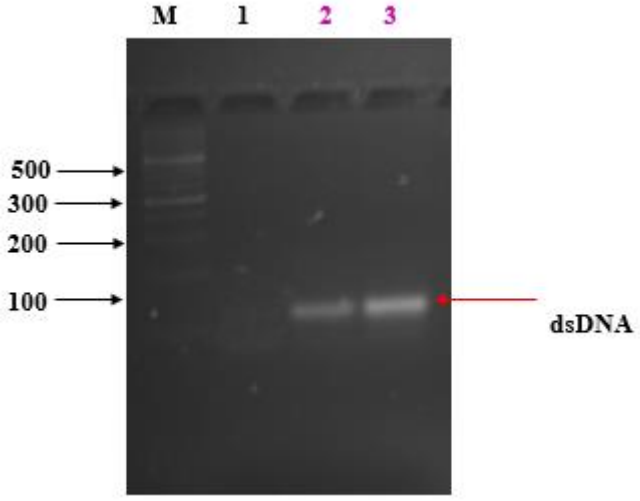

Figure 4.6I: One-Pot SELEX. Lane labelled **1** represents the negative control (PCR mix without target) and lanes labelled **2** and **3** corresponds to the scale up amplicons. One-round SELEX DNA amplification is represented by bands seen at approximately 100 bp.

4.7. Aptamer sequence analysis and protein/aptamer homology modelling

Aptamer analysis for magnetic bead-based SELEX and modified One-Pot SELEX was performed using Geneious software (Biomatters, Auckland, New Zealand). The software was used to generate a phylogenetic tree in order to determine aptamers with high affinity to the target based on the number of conserved motifs appearing in the variable region of each aptamer per clade. The phylogenetic tree in Figure 4.7A (bead-based SELEX) showed that aptamers in clade 1 consist of some conserved motifs however, clade 2 displayed remarkable motif conservation and Figure 4.7B (modified One-Pot SELEX) showed some motifs on clade 1 and on clade 2. In order to determine the best aptamer sequence to select for synthesis, M-fold server (http://unafold.rna.albany.edu/?q=mfold) was used to fold and truncate the DNA sequences to determine the most stable structure after truncation. Truncation refers to the shortening of oligonucleotides without affecting their affinities to their targets (Akitomi *et al*., 2011). From M-fold DNA folding, the structures with the lowest free energies were selected for synthesis; one from normal SELEX (Apt_9(7) after extraction) and the other from the modified One-Pot SELEX (Apt_9(2) after extraction. This selection was based on Zuker's algorithm, which states that the lower the free energy of the structure, the more stable it is (Zuker, 2003).

In order to study protein-aptamer interaction, PatchDock a molecular docking algorithm based on shape complementarity principles (https://bioinfo3d.cs.tau.ac.il/PatchDock/) was used to model the interaction between the HIV-p24 protein and the conjugate aptamers (Oligo 1 and Oligo 2) as shown in Figures 4.7 C and D. More so, Table 4.2 depicts the binding affinities of the oligos to the target protein, with oligo 1-HIV-p24 complex displaying a high binding score and area of contact but lower than that of oligo 2-HIV-p24 complex. However, this outcome is expected since the methods used in selecting these aptamers are

different. It could possibly be that oligo 1 (magnetic based SELEX) may have potentially lost some of the sequences during the repetitive rounds (Imashimizu *et al*., 2018).

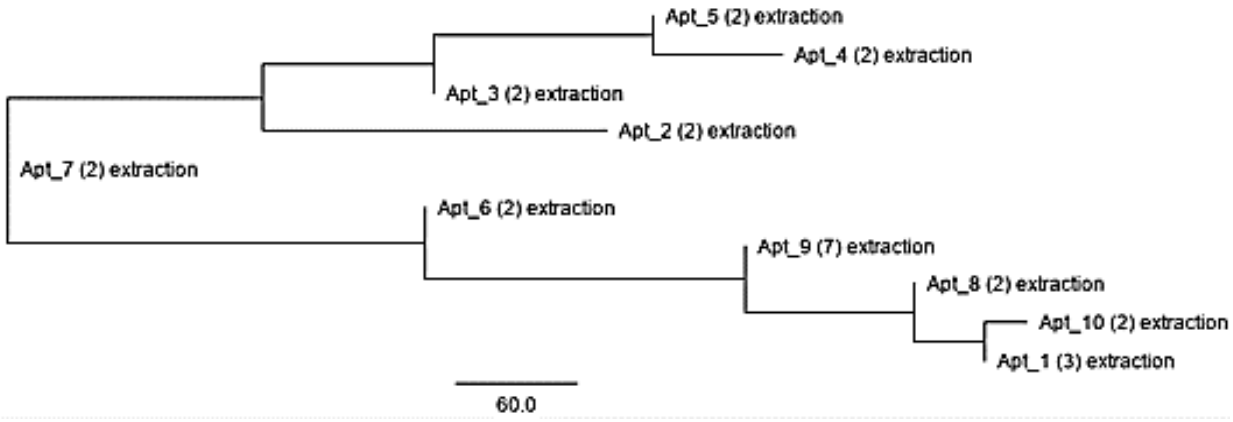

Figure 4.7A: Phylogenetic tree of round 9 (Magnetic bead-based SELEX). The tree shows relation of the aptamers based on the conserved motifs (represented by the numbers in brackets) in the variable region, with aptamers (apt) 5, 4, 3, 2 classified as clade 1 and apt_ 6, 9, 8, 10 and 1 as clade 2.

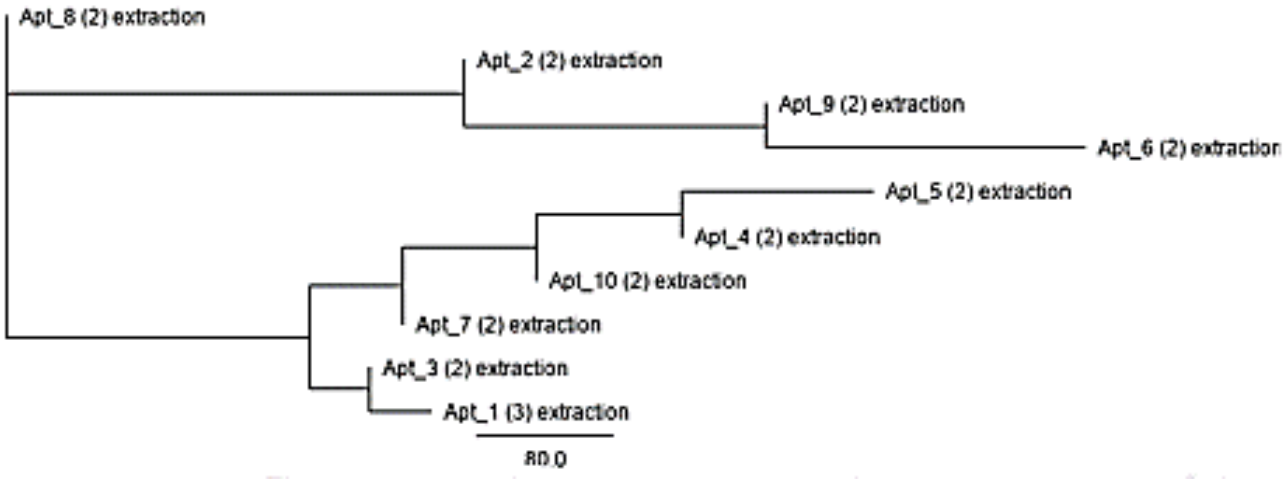

Figure 4.7B: Phylogenetic tree for the modified one-Pot SELEX. The tree shows relation of the aptamers based on the conserved motifs in the variable region, with aptamers (apt) 2, 9 and 6 classifies as clade 1 and apt_ 5, 4, 10, 7, 3 and 1 as clade 2.

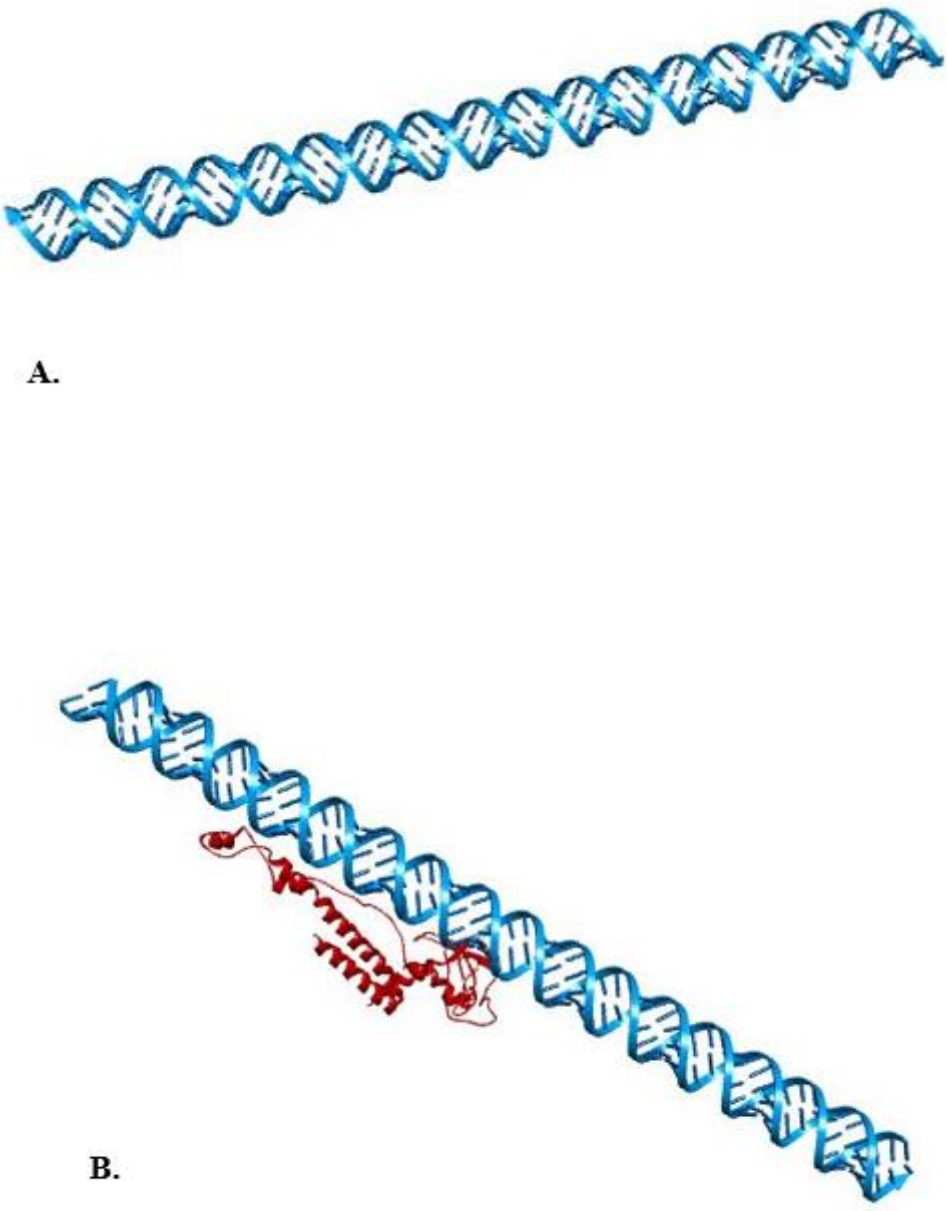

Figure 4.7C: Aptamer modelling and HIV-p24 protein-aptamer (Oligo 1) docking. A shows oligo 1 (from normal SELEX) model as generated by DSV (http://www.3dsbiovia.com/products/collaborative-science/biovia-discovery-studio/ [Accessed 20 February 2019]. **B** shows oligo 2 and HIV-p24 docking model as generated by PatchDock available online at https://bioinfo3d.cs.tau.ac.il/PatchDock [Accessed 22 February 2019].

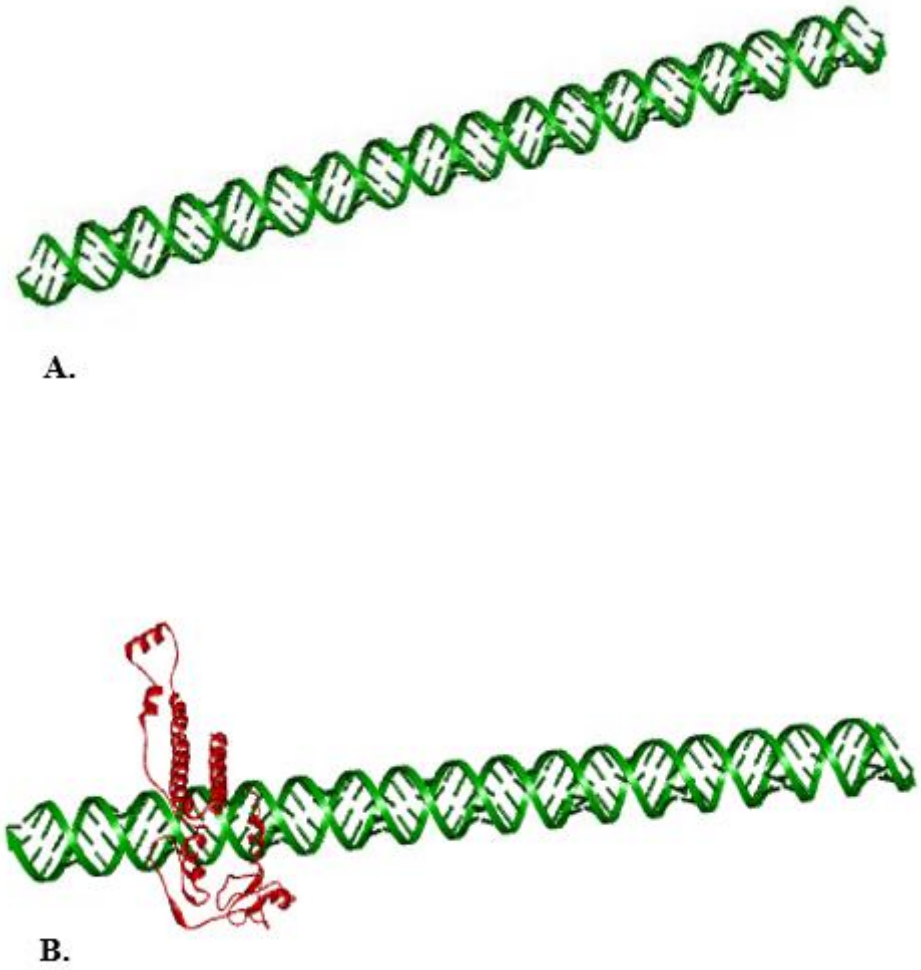

Figure 4.7D: Aptamer modelling and HIV-p24 protein-aptamer (Oligo 2) docking. A shows oligo 2 (from the modified One-Pot SELEX) model as generated by DSV http://www.3dsbiovia.com/products/collaborative-science/biovia-discovery-studio/ [Accessed 20 February 2019]. **B** shows oligo 2 and HIV-p24 docking model as generated by PatchDock available online at https://bioinfo3d.cs.tau.ac.il/PatchDock/ [Accessed 22 February 2019].

Table 4.2: Putative HIV-p24- aptamer binding properties

Oligonucleotides	Binding Score	Area of contact	Atomic contact energy
Oligo 1 docking	**15184**	**2854. 20**	**-869. 08KJ**
Oligo 2 docking	**15868**	**3651. 90**	**-1525. 54KJ**

4.8. General discussion and future studies

In the absence of a pronounced HIV cure, early diagnosis which precedes treatment is paramount for the control of viral transmission across the population. HIV capsid-p24 protein remains an important biomarker for HIV diagnosis due to its expression in the early stages of infection. This study has shown recombinant expression of soluble HIV-p24 protein and successful selection of HIV-p24 specific DNA aptamers. Studying these biological agents in conjunction is important for future fabrication of an alternative specific, point-of-care diagnostic tool, which will be easily accessible to provide early diagnosis thus, controlling the spread of the virus. *In silico* studies have shown that HIV-p24 is highly stable and soluble. UV-VIS spectrum has also shown that the protein remained folded after expression; this is an important trait for diagnostic applications. Algorithm in PatchDock has also been applied and has shown there are strong interactions between HIV-p24 protein and the DNA aptamers. From the findings in this study, the plausible conclusion is that aptamers truly bind to their targets with high affinity, which may explain successful interactions. However, biophysical characterization using tools such as microscale thermophoresis (MST) and isothermal calorimetry (ITC) are needed to further confirm and determine the thermodynamic parameters of these interactions, which may shed more insight towards the design of a point-of-care diagnostic tool for HIV.

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