FORENSIC PATHOLOGY DIAGNOSTIC: ISOLATION OF SMALL NON-CODING RNA APTAMERS AGAINST SOLUBLE CELL SURFACE GLYCOPROTEIN CD54

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviation		Description
%	=	Percentage
°C	=	Degree Celsius (temperature)
µg	=	Microgram
µL	=	Microliter
µm	=	Micrometer
A	=	Adenosine
A_{260}	=	Absorption at λ= 260 nm
A_{280}	=	Absorption at λ= 280 nm
APS	=	Ammonium persulfate
ATP	=	Adenosine triphosphate
Bis	=	N, N'-Methylenebisacrylamide
bp	=	Base pair
C	=	Cytosine
cDNA	=	Complementary DNA
CTP	=	Cytidine triphosphate
C-terminal	=	Carboxy-terminal
CD54	=	Cluster of Differentiation 54
dH_2O	=	Distilled water
ddH_2O	=	Double-distilled water
DMSO	=	Dimethyl sulfoxide

DNA	=	Deoxyribonucleic acid
DNase	=	Deoxyribonuclease
dNTP	=	Deoxyribonucleotide triphosphate
ds	=	Double stranded
DTT	=	Dithiothreitol
EDTA	=	Ethylenediaminetetraacetic Acid
ELONA	=	Enzyme linked oligonucleotide assay
et al.	=	and others
g	=	*Gravitational acceleration*
g	=	Gram
G	=	Guanosine
GTP	=	Guanosine triphosphate
ICAM-1	=	Intercellular Adhesion Molecule 1
kDa	=	Kilodalton
K_d	=	Dissociation constant
L	=	Liter
M	=	Molar or Molarity
MBU	=	Molecular Biology Unit
mg	=	Milligram
mL	=	Milliliter
Min	=	Minute(s)
MW	=	Molecular weight
mM	=	Millimolar

nm	=	Nanomolar
ng	=	Nanogram
nmole	=	Nanomole
OD	=	Optical density
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate buffered saline
PCR	=	Polymerase Chain Reaction
pmol	=	Picomole
RNA	=	Ribonucleic acid
RNAse	=	Ribonuclease
rpm	=	Rotations per minute
RT	=	Room temperature
RT-PCR	=	Reverse transcription PCR
s	=	Second(s)
SDS	=	Sodium Dodecyl Sulfate
SELEX	=	Systematic Evolution of Ligands by Exponential Enrichment
ss	=	Single stranded
T	=	Thymine
TBE	=	Tris Borate EDTA
TEMED	=	Tetramethylethylenediamine
Tris	=	Tris (hydroxymethyl) aminomethane
U	=	Uracil (RNA)

U	=	Units of enzymatic activity
UTP	=	Uridine triphosphate
UV	=	Ultraviolet
V	=	Volt (s)
v/v	=	Volume per volume
w/v	=	Weight per volume

ABSTRAK

Salah satu tanggungjawab utama bagi pakar patologi forensik, adalah untuk menilai vitaliti luka. Secara umumnya, mereka mengaplikasi pengetahuan analisa secara "immunohistochemical" bukannya pengetahuan molekul dalam mengenalpasti vitaliti luka. Pada masa kini, antibodi monoklonal telah digunakan secara meluas untuk diagnosis vitaliti luka dengan mengesan antigen CD54 yang bertanggungjawab dalam tindak balas imun dan keradangan. Walau bagaimanapun, kaedah ini terhad disebabkan oleh proses penghasilan antibodi monoklonal yang rumit serta memerlukan kos yang tinggi. Isu ini dapat diatasi dengan pengahasilan aptamer terhadap permukaan sel "*Glycoprotein CD54*". Dalam kajian ini, penghasilan RNA aptamer telah berjaya dihasilkan terhadap permukaan sel larut "*Glycoprotein CD54*" dengan menggunakan teknologi SELEX. Penghasilan aptamer bermula dari "*library optimization*", dalam pemilihan "*in vitro*" dan analisa pengikatan aptamer dengan sasaran. Aktiviti pengikatan antara aptamer dengan protein sasaran telah dinilai menggunakan kaedah "*Enzyme Linked Oligonucleotide Assay*" (ELONA). Aktiviti pengikatan maksimum boleh dilihat dari aptamer yang diasilkan daripada kitaran 11 dan dengan menunjukkan nilai keserapan tertinggi secara purata 0.362 nm. Aptamer yang dihasilkan daripada kajian ini, mempunyai potensi tinggi untuk digunakan bagi analisa vitaliti luka serta boleh menggantikan penggunaan antibodi monoklonal dalam proses "*immunohistochemical*".

ABSTRACT

One of the primary responsibility for forensic pathologist, is to assess the wound vitality. Generally, they apply the knowledge of immunohistochemical analysis instead of molecular knowledge in identification of the wound vitality. Currently, monoclonal antibody was widely used for the diagnosis of wound vitality by detecting CD54 antigen which is responsible for mediating immune and inflammatory responses. However, this immunohistochemical analysis was practiced very limitedly due to the laborious and expensive production of monoclonal antibody. Therefore, this issue overcomes by the isolation pool of aptamers against soluble cell surface glycoprotein CD54. In this study, small pool of non-coding RNA aptamers was successfully isolated against soluble cell surface glycoprotein CD54 by using SELEX technology. The isolation of pool of RNA aptamers began from optimization of library, *in vitro* selection and analysis of binding activity with the selected target. The binding activity between the isolated aptamer and the target protein was evaluated by enzyme-linked oligonucleotide assay (ELONA). The maximum binding activity can be observed from aptamer isolated from 11^{th} cycle and it shows the highest absorbance value up to 0.362 nm in average. The aptamer isolated from this study, has the potential to be used as high affinity ligands for the capture and subsequently determination of wound vitality and eventually can be used as an alternative for monoclonal antibody in immunohistochemical process.

CHAPTER 1: INTRODUCTION

1.1. Background

Forensic pathology is a branch of a medicine which focuses on medico-legal investigations of sudden or unexpected death. Primarily, forensic pathologists play a major role in death investigation which includes establishing the cause of death, factors contributing to death which will be very helpful in the reconstruction of the circumstances lead to the death. While, post-mortem examination typically involves the careful physical examination, toxicological examination, dissection of internal organ and structures (Fraser and Williams, 2013). One of the important task for forensic pathologist during the autopsy session will be the establishment of wound vitality in order to assess the nature of the wound more precisely (Ohshima, 2000).

In many instances, forensic pathologist practicing macroscopic examination in estimating the wound vitality instead of applying molecular knowledge in the evaluation of wound aging (Holczabek and Depastas, 1979). Apart from this, forensic pathologist also applied the knowledge of immunohistochemical method to aid in identification of the wound vitality (Ohshima, 2000; Raekallio, 1976). In this scenario, CD54/ICAM-1 will be used as a biomarker in the immunohistochemical analysis for the determination of wound vitality. Since, there is moderate to strong expression of CD54/ICAM-1 in the wound surface which can be a valuable indication of the vitality of the wound or ageing the wound (Dressler *et al.*, 2000).

Generally, this immunohistochemical study which aid in the investigation of the wound vitality involves the use of monoclonal antibody (Toh *et al.*, 2014). However, there are some limitations in handling this monoclonal antibody. Since, this monoclonal antibody might be easily undergoing denaturation process, antibody screening and production considered to be laborious and expensive, cannot accommodate conjugates and inconsistency of monoclonal antibody production (Lakhin *et al.*, 2013). Thus, there is a need of new technology to replace the monoclonal antibody. Hence, the isolation of aptamer using a technique called SELEX (Systematic Evolution of Ligands by Exponential) was established (Ellington and Szostak, 1990). Aptamers are oligonucleotides either single stranded deoxyribonucleic acids (ssDNA) or ribonucleic acids (RNAs) that can bind to target molecules with high affinity and specificity (Lakhin *et al.*, 2013). Basically, this study focused on establishing RNA aptamer which is specific against soluble cell surface glycoprotein CD54/ICAM-1 as proof of concept for replacing the use of monoclonal antibody in immunohistochemical process.

1.2. Objectives of the study

General objective

The main aim of this study is to identify potential RNA aptamer that could be used as an alternative method for the estimation of the wound vitality or ageing through immunohistochemical technique.

Specific objective

To accomplish this goal, the specific objectives of the study were as follows:

1) To optimize random library amplifications.
2) To isolate pool of RNA aptamers that can bind towards soluble cell surface glycoprotein CD54 through SELEX process.

CHAPTER 2: LITERATURE REVIEW

2.1. Introduction to wound

2.1.1. Wound & Injury

The term wound or injury has been defined as "damage to any part of the body caused by the application of mechanical force" or damage to any part of the body or bodily harm caused by application of violence (Barek and Haque, 2013; Ohshima, 2000). The wound production begins when the amount of the applied force to the body or any part of the body exceeding the capability of the tissue to adapt particular force or to resist the force applied on the body (Karhunen *et al.*, 1990). Primarily, the tendency of the tissue to resist or adapt particular amount of force depends upon the velocity, mass, structure of the tissue, the exact position of the weapon pressing the body and the leverage of the force. In most of the scenario, the elucidated damage of the tissue solely depends on the degree of transfer of the kinetic energy from the relative movement of the weapon or the body (Barek and Haque, 2013).

2.1.2. Type of wound

In forensic pathology, the wounds are classified according to the manner of the mechanical force applied to the skin as shown in Table 2.1 (Barek and Haque, 2013).

Table 2.1: Types of wound according to the manner of the mechanical force applied to the skin (Barek and Haque, 2013)

Types of wound	Explanation
Abrasion	In abrasion wound, the damage will occur in the epidermis layer and some dermal papillae also will undergo the damage. This is caused by rough hard blunt objects or by drawing the tip of the pointed objects against the mucous membrane. This abrasion causes vertical pressure due to the rubbing effect. Besides, the pattern of the object might be visible at the abraded area.
Bruise	This type of wound happens due to the application of the blunt force which leads to the rupture of the blood vessels. As a consequence, the extravascular collection of blood can be observed in the dermis of the skin and subcutaneous tissue. The size of the bruises varies, for example the petechial haemorrhage of pin head size while the ecchymosis will be more than 5 mm.
Laceration	This is a condition in which the tearing and the splitting of the skin, mucous membrane and surfaces of any internal organs can be observed. It happens due to the application of the blunt force. As well, the escape of the blood from the wound to the exterior or any body cavity is visible.

Incised wound This type of wound has regular clean edges. It is caused by the application of the sharp edge of the weapon which is applied to the skin perpendicularly or obliquely.

Stab wound The puncture wound as well the penetrating wound is classified under this category. The stab wound normally greater in depth and length caused by the pointed knife or dagger. Whereas, the puncture wounds is produced by the pointed thin bodied weapon where depth of the wound is greater than its diameter. On the other hand, penetrating wound will have an entry and an exit and a tract through the tissue.

Firearm wound These types of wound are produced by the bullets or pellets from the firearms. Normally the bullets will cause perforation while the pellets will cause the penetrating wounds. In this type of wound examination, always the entry hole and exit hole of the particular ammunition will be analysed carefully. Moreover, the firearm entry wounds are always associated with burning, blackening, tattooing of the surrounding skin.

2.1.3. Wound examination

The studies of forensic pathology, proves that wound examination especially in establishing wound vitality requires experience and skills. It is considered to be a fundamental component in establishing systematic wound examination procedure. The functions of wound examination are described accordingly in Gradwohl's textbook (Camps *et al.*, 1976; Ohshima, 2000) as follows: (1) whether the particular wound is an ante mortem wound , happens at the time of death , or postmortem wound which happens after the death; (2) what is the causative factor; (3) what is the causative agent; (4) the amount of force required to produce particular wound; (5) what is the severity of injury resulted from it and whether it contributed death or disability. Normally, based on the wound vitality, properties of mechanical force and the relationship between the mechanical force and the person's death or disability are the important points to be explained.

2.1.4. The current application of clinical techniques and devices for wound examination

Macroscopic and histological examinations are the gold standard for wound examination. Apparently, nowadays the implications of modern diagnostic techniques being practiced in forensic medicine which can be apply in the forensic wound examination as follows:

2.1.4.1. Radiological examination

The radiological examination using magnetic resonance imaging has becomes part of forensic autopsy. It is very effective tool for the identification of foreign bodies, bone fractures, air embolism, foreign materials before proceed with the autopsy process. Besides, the

postmortem angiography can be performed to estimate the origin of the hemorrhage. This advance clinical diagnostic device can be very much helpful in forensic autopsy. At the same time, this technique requires a special magnetic resonance imaging (MRI) device apparently increase the cost of analysis (Ohshima, 2000; Schmidt and Kallieris, 1982).

2.1.4.2. Endoscopic examination

The usage of the endoscopic instrument can be observed in the forensic autopsy. It is very helpful in the examination of the cavities and sinuses and can be inspect without any morphological damage. This instrument particularly will be useful in the genital wounds especially among the rape victims (Slaughter and Brown, 1991).

2.1.4.3. Diphanoscopic examination

This examination will aid in the identification of the presence or absence of subcutaneous hematomas which is considered to be very hard to identify through naked eye observation. Normally, through cutaneous incision hematoma can be easily detected. However, in living individual it's not advisable to implement skin incision method. Since, this diphanoscopic procedure can be very helpful in determination subcutaneous hematoma (Horisberger and Krompecher, 1997).

2.1.5. The determination of wound vitality based immunohistochemical analysis

It is very crucial component to determine sustainability of the wound and biochemical reaction as well how long the wound has been sustained (Ohshima, 2000; Stephenson and Bialas, 1996). The age estimation of bruises is very essential when a cadaver or injured person has multiple bruises of different ages, especially in the case of child abused. In order to achieve this objective, forensic pathologist need to understand not only the wound morphology but also need to understand the pathophysiology of the wound healing process. Principally, skin wound healing process going through few stages which includes inflammatory, proliferative and maturation process. At the meantime, different kinds of biological substances as shown in Table 2.2 involved at each phase of wound healing process (Ohshima, 2000).

Betz and co-workers executed a set of immunohistochemical studies on wound age determination. They proved that, these are the immunohistochemical parameters which included in age estimation such as P-selectin, E-selectin and ICAM-1, extracellular matrices such as collagens, tenascin and laminin are very useful criterion at 1-5 days after injury (Betz, 1995; Ohshima, 2000). Besides, the fluctuation of cytokines during the wound healing process also has been considered as parameter in wound ageing (Ohshima, 2000). Cytokines is a type of glycoprotein produced by various kinds of cells which includes macrophages, neutrophils and lymphocytes which closely related with immune, inflammation endocrine systems (Kondo and Ishida, 2010; Ohshima, 2000).

Table 2.2: The immunohistochemical parameters in age estimation of human skin wounds (Betz, 1995)

Antigen	**Earliest appearance**	**Routine appearance**	**Latest appearance**
P-selectin	minutes	-	7h
fibronectin	10-20 min	>4 h	months
E-selectin	1h	-	17 days
ICAM-1/ CD54	1.5h	-	3.5 days
fibroblast proliferation	1.5 days	>6 days	-
fibroblast apoptosis	1-2 days	-	-
tenascin	2 days	>5 days	months
collagen III	2-3 days	>6 days	months
collagen IV	3 days	>6 days	months
collagen VI	3 days	>6 days	months
collagen I	5 days	>6 days	months
Myofibroblasts			
laminin	1.5 days	-	months
HSPG	1.5 days	-	months
collagen IV	4 days	-	months
α actin	5 days	-	months
basement membrane			
bm-fragments	4 days	>13days	>21days
bm complete	8 days	>21 days	-

Generally, when carrying out an autopsy session, the survival time of the deceased following particular injury is often unknown. By employing immunohistochemical technique, the vitality of the wound can be assessed to a certain level, primarily because of the limited of markers in the initial state of the wound healing process (Wyler, 1996). This adhesion molecule play a vital role in estimating wound vitality based on immunohistochemical study. The adhesion molecule includes ICAM-1, P-selectin and VCAM-1 (Betz, 1995). Since, these molecules shows a strong positive immunohistochemical reaction towards the Avidin–Biotin Complex (ABC) staining technique (Dressler *et al.*, 2000), mostly employed in detecting the wound vitality. In order to proceed with this staining method, forensic pathologists requires the use of monoclonal antibody in which the production of monoclonal antibody is laborious and expensive, not stable at high temperature and have to maintain the chain of custody. Therefore, in this study the isolation of aptamer against soluble cell surface glycoprotein CD54/ICAM-1 can be used as an alternative for monoclonal antibody in determination of wound vitality.

2.2. Introduction to Aptamer

2.2.1. Theory and history of aptamers

Aptamers are known as synthetic single stranded RNA or DNA molecules which are capable of binding to various molecules with high affinity and specificity (Kulbachinskiy, 2007). The term aptamers derived from the combination of the Latin word "aptus" (meaning to fit) and the Greek word "mers" (particle) (Aquino-Jarquin and D.Toscano-Garibay, 2011). These aptamers are primarily short RNA or single stranded oligonucleotides range from 20-80 nucleotides with 6-30 kDa molecular weights and capable of folding into three dimensional conformations (Sun and Zu, 2015). Moreover, the ssDNA and RNA aptamers tend to have different folding pattern and sequence even though they bind to the same target (Song *et al.*, 2012).

The revolution in establishing aptamers begins in 1990, when a mutation experiment was conducted by Tuerk and Gold to describe the properties of the translational regulation exerted by the phage T4 replicase over its own messenger (Tuerk and Gold, 1990). Based on their research, they randomized stretch of eight nucleotides within the regulatory loop of the mRNA and systematically exposed the resulting pool of sequences to the replicase. In later path, 4^8 potential combinations were isolated based on their very similar binding affinity. So, basically this will be the first milestone in establishing SELEX (Systemic Evolution of Ligands by Exponential Enrichment) technique for the first time in order to establish nucleic acids as flexible ligands which will be very effective in protein recognition (Aquino-Jarquin and Toscano-Garibay, 2011).

This aptamers, single stranded DNA or RNA molecules, generated by a method called SELEX which have been widely being practiced in various fields.

2.2.2. Aptamer characteristics

2.2.2.1. Structural features of aptamers

Aptamers possess unique characteristic in which they can form a very promising secondary structure (Kulbachinskiy, 2007). These aptamers are single stranded nucleic acids of either made up of DNA or RNA which is synthetic in nature tend to fold two or three dimensional structure as well bind to the target molecules with high affinity and specificity (Tuerk and Gold, 1990; Ye *et al.*, 2012). In many instances, it has been proven that the unpaired sites in the oligonucleotide pool are very crucial for a definite interaction with targets. Meanwhile, the sites with stable secondary structure are aid in the appropriate spatial arrangement of the recognition elements (Kulbachinskiy, 2007; Patel *et al.*, 1997; Ringquist *et al.*, 1995).

Primarily the aptamers with a very specific and complex three dimensional structure characteristic such as stems, loops, hairpin, bulges, pseudoknots, triplexes and quadruplexes tend to bind to various targets (Wang, 2009). These specific three dimensional structures allow aptamers to form specific complexes with enormous of targets (Ye *et al.*, 2012). Meanwhile, the hairpin, pseudoknots and quadruplexes are the most frequent structural elements. The hairpin structure can be observed in both RNA and DNA aptamers. While the pseudoknot structure is formed as a result of interactions of the sequence located in the hairpin loop and it is very exclusive for RNA aptamers (Ringquist *et al.*, 1995). Besides, the quadruplex is formed by four guanine nucleotides where each guanine base in the guanine

nucleotides forms hydrogen bonds with two adjacent bases. This structure shows very tremendous stability compared to simpler structural motifs (Jing *et al.*, 1997; Kulbachinskiy, 2007).

2.2.2.2. Affinity and specificity

One of the important considerations while producing aptamers, is to make sure that it can interact with their targets with high affinity. Normally, the proteins are considered to be best target for aptamers selection since the dissociation constants (K_d) for aptamers complexes range from nanomolar to subnanomolar. Besides, the larger area of the aptamers contact can be closely linked to the binding affinity of the proteins to the target (Eaton *et al.*, 1995; Kulbachinskiy, 2007). While the aptamers specificity to particular target can be modified during the selection process. The SELEX cycle will be an essential tool in identifying sequences that specifically bind to the protein target (Kulbachinskiy, 2007).

2.2.3. Aptamer versus antibody

In recent times, the usage of monoclonal antibodies can be observed in order to capture ligands or antigen from pathogen in a wide variety of medical, biological and diagnostic applications. However, there are limited drawbacks associated with antibodies. On the other hand, aptamers possess unique merits which make them more enticing diagnostic agent in certain instance even transcend antibodies as shown in Table 2.3. Thus, it can be a promising material for diverse areas, not only act as an alternative to antibodies, but as the important medium for medical and analytical equipment.

Table 2.3: Properties of aptamers and antibody (Jayasena, 1999; Nimjee et al., 2005)

	Aptamers	**Antibodies**
Size	Made up of small molecules, the molecular weight range from 8-12 kDa	Made up of large molecules, the molecular weight around 150 kDa
Selection procedure	The selection process is an *in vitro* chemical process which can target any protein.	It requires a biological system, where difficult to raise antibodies to toxins
Immunogenicity	No evidence of immunogenicity	Significant immunogenicity
Working condition	Can select for ligands under a variety of conditions for *in vitro* diagnostics	Limited to physiologic conditions for optimizing antibodies for diagnostics
Activity	Uniform activity regardless of batch to batch variation	Activity of antibodies varies batch to batch (polyclonal antibody)
Kinetic parameters	Kinetic parameters such as on/off rates can be changed on demand	Kinetic parameters of Ab-Ag interactions are difficult to be changed on demand

Shelf life	Unlimited shelf life	Very limited shelf life
Temperature	Return to original conformation after temperature insult	Temperature causes irreversible denaturation
Storage	Stable to long term storage and do not require refrigeration	Sensitive to temperature and require refrigeration
Modification	Wide variety of chemical modification can be introduce to enhance the functionality	Very limited modifications of the molecule
Labelling	The reporter molecules can be attached to aptamers at precise locations not involved in binding	The process of labeling antibodies can cause loss in affinity
Reverse activity	Can develop aptamer-specific antidote to reverse the inhibitory activity of the drug	No rational method to reverse molecules
Cross-reactive compound	Can utilize toggle strategy in order to isolate cross-reactive compounds which facilitate preclinical studies	No method for isolating cross-reactive compound

2.3. *In vitro* selection process

2.3.1. SELEX TECHNIQUE

The application of the SELEX technique being well established and have gained great attention for the past 25 years (Ellington and Szostak, 1990). Primarily this SELEX technique is a combinative chemistry procedure in which it allows rapid selection from a large initial library of oligonucleotides, high binding affinity and specificity (Tuerk and Gold, 1990). The SELEX technique plays an essential role in order to infer the interactions between proteins with dsDNA. Moreover, the SELEX technique was performed in many circumstances in order to identify dsDNA sequences are the strongest binders to the protein of interest (Djordjevic, 2007).

SELEX considered being an immense methodology for developing specific aptamers. This SELEX process allows multiple rounds of amplification and enrichment, which allows formation of aptamers with high specificity and affinity towards the target from a random oligonucleotide pool (Ellington and Szostak, 1990). The SELEX process comprises of some basic techniques in order to develop target-specific aptamers as shown in Figure 2.1.

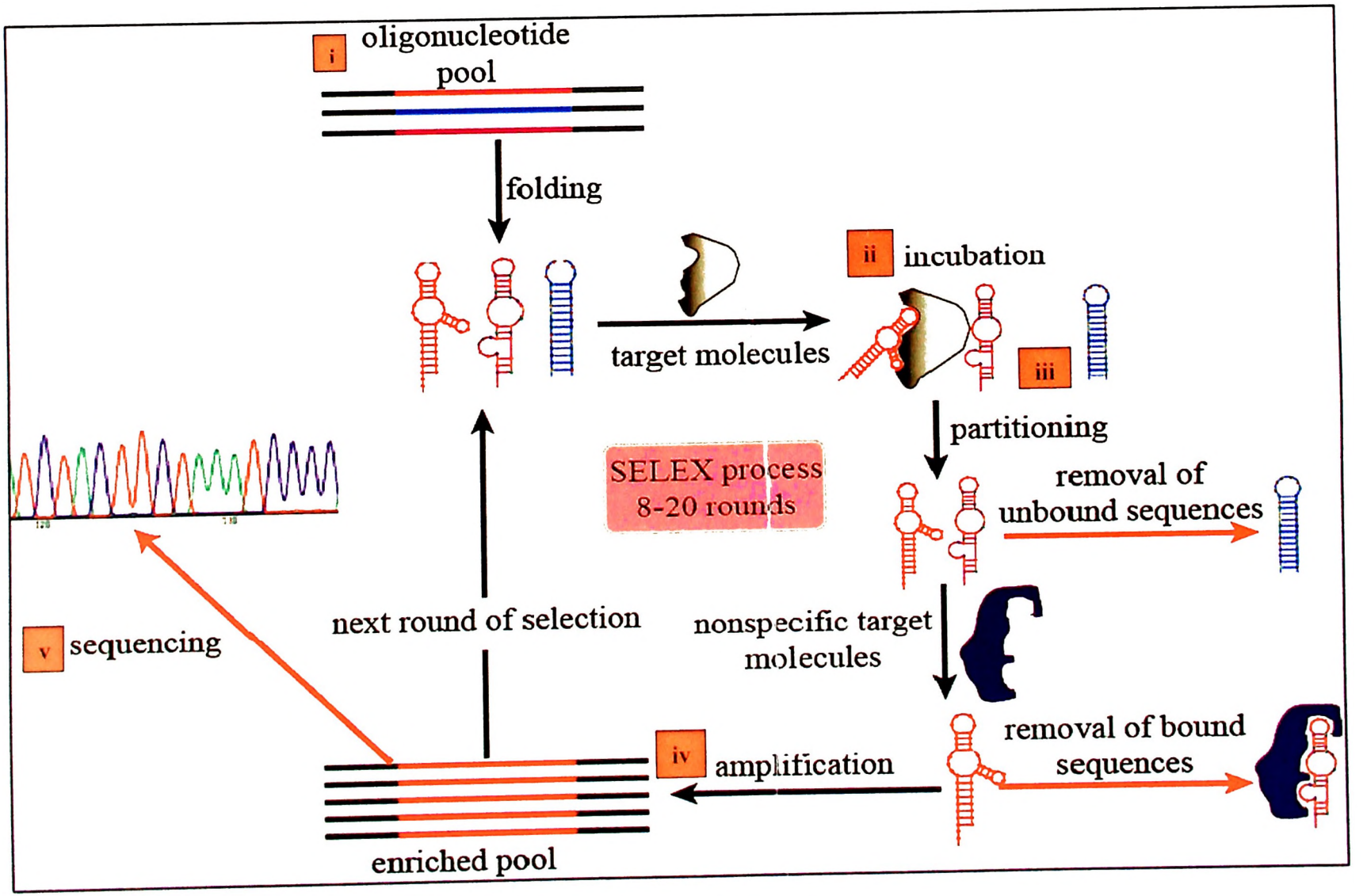

Figure 2.1: Schematic diagram of the SELEX process steps (Sun and Zu , 2015)

(i) Preparation of initial oligonucleotide pool

As the first step in the SELEX cycle, the design of the chosen oligonucleotide pool will be the primary concern. In which a single stranded DNA oligonucleotide pool consists of 10^{14}-10^{15} random sequences will be designed accordingly and chemically will be synthesized. Normally these individual sequences will have random oligonucleotides range from 30-50 nucleotide in between two conserved primer binding sites which is an essential component in generating specific aptamers with high specificity and affinity towards the target (Hall *et al.*, 2009; Sun and Zu, 2015).

(ii) Incubation

The random sequences in the initial pool which having tendency to fold into different secondary and tertiary structures are incubated with free target group under optimal conditions in order to form aptamer target complexes (Sun and Zu, 2015).

(iii) Partitioning

In this process, the unbound sequences are separated from the target bounds by applying different methods which include membrane filtration, affinity columns, magnetic beads or capillary electrophoresis (Darmostuk *et al.*, 2015). Besides, there are plenty of methods are recommended in the SELEX process to enhance separation efficiency (Lin *et al.*, 2014; Sun and Zu, 2015). This step in SELEX cycle will shorten the duration for the selection period and apparently results 4 to 8 rounds of SELEX cycle in producing the aptamers.

(iv) Amplification

The conventional PCR technique plays a major role in amplification process. At the meantime, due to the nature of the oligonucleotide pool consisting of abundance of random sequences as templates, it leads to the production of enormous by-products which is not specific to the aptamer sequences (Sun and Zu, 2015; Tolle *et al.*, 2014). Even though, the primer concentration, annealing temperature and amplification cycles are well optimized the tendency of producing random sequences might be arising during the PCR amplification and barricade for a successful SELEX process.

(v) Sequencing

The specific aptamers with high affinity and specificity always will be identified in the last round of selection by Sanger sequencing method. Based on this method, only few sequences will be identified and the limited amount of information regarding the sequences not enough to justify true binding sequences. Perhaps the next generation sequencing with high throughput able to identify the important binding sequences present in the previous rounds may be lost (Elle *et al.*, 2015). Basically during the beginning selection rounds of SELEX process, the sequencing step aid in identifying high affinity aptamers (Guo *et al.*, 2014).

2.4. Applications of aptamer in diagnostic

The evolution of the aptamers begins in early 1990s (Ellington and Szostak, 1990) itself and have attracted attention of many scientists, due to the magnificent properties (Song *et al.*, 2012). Due to their functional similarity with antibodies, aptamers have been used abundantly in various fields including as research tools (Qiao *et al.*, 2015; Sun and Zu, 2015), tissue staining (Zeng *et al.*, 2010), cell detection (Cai *et al.*, 2015), *in vitro* and *in vivo* imaging (Li *et al.*, 2015), targeted therapy and nanomedicine (Qiu *et al.*, 2014) and environment monitoring and food safety (Yang *et al.*, 2015). As a result of excellency of these aptamers, it mainly engaged in the clinical setting for the development of diagnostic assays (Qiu *et al.*, 2014). Predominantly, the vast usages of these aptamers are due to the high degree of target specificity and sensitivity (Joshi *et al.*, 2009).

One of the classic example would be the diagnostic-based aptamer for detecting mycotoxins and aflatoxins (Gold *et al.*, 2010). They employed aptamer-beacon which can be act as rapid biomarker detection. By employing this method of detecting analytes will eliminates the possibility for multiple washes and tedious incubation periods as well indirectly boost the detection sensitivity. This apta-beacons can be an exceptional medium for enhanced the patient treatment plan (Kedzierski *et al.*, 2012). In addition, another breakthrough of these aptamers will be in the field of bio-imaging. In particular, these aptamers are conjugated to fluorophore which is very constructive for magnetic resonance imaging (MRI). The aptamers can be used as imaging agent, potentially reduce the risk of being toxic, since oligonucleotides moieties are present in the human body.

CHAPTER 3: MATERIALS AND METHODS

3.1. Study design

The overall methodology used has been simplified in the flow chart below.

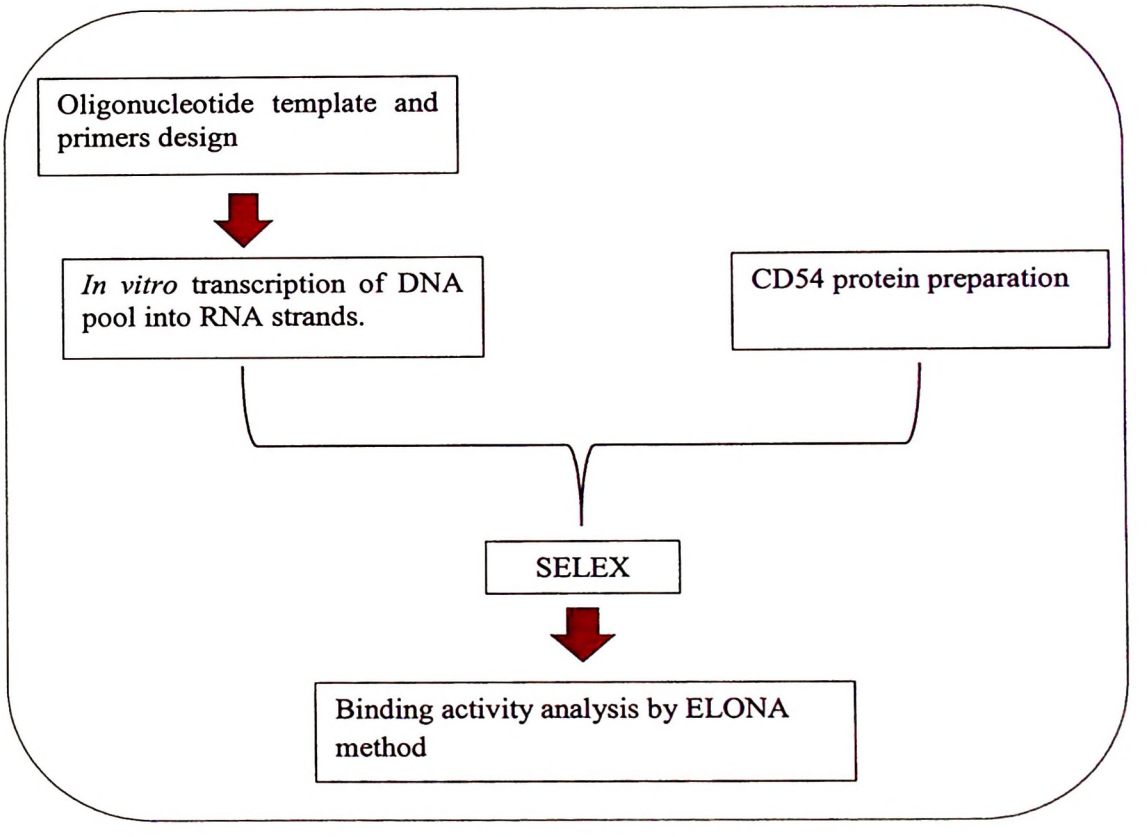

Figure 3.1: Flow chart of the study

3.2. Preparation of buffers and reagents

All chemicals and reagents that were used in this study are listed in the Appendix 1 and Appendix 2. The chemicals and reagents were used are molecular biology. All the buffers and solutions sterilized by autoclaving were performed under 121°C for 20 min.

3.3. Equipment

All the equipment were used in this study were listed in the Appendix 3.

3.4. Determination of protein purity

The Recombinant Human CD54 Fc Chimera (Catalog Number :720-IC) was purchased from R&D Systems. The lyophilized CD54 protein was reconstituted in PBS buffer (pH 7.4) and the purity of protein was analysed using SDS-PAGE under reducing condition and Native-PAGE for non-reducing condition.

3.4.1. SDS-PAGE

The protein was mixed with sample buffer added with 2-mercaptoethanol and bromophenol blue dye in 1:1 ratio. The protein mixture was heated at 100°C for 5 minutes. Then the protein sample was loaded into gel and electrophoresed at 200 volt for 40 minutes. The recipe for SDS-PAGE gels preparation as shown in appendix A2.12 and A2.13. The gel was stained with coomassie blue for 30 minutes and destained until the background became less dark.

3.4.2. Native-PAGE

The reconstituted protein was mixed with 6X loading dye and mixed well. Then the protein sample was loaded into gel and electrophoresed at 140 volt for 40 minutes. The recipe for Native-PAGE gels preparation as shown in appendix A2.9. The gel was stained with SYPRO Ruby for 3 hours and destained for 1 hour with destaining solution and the recipe shown in appendix A2.4.

3.5. Preparation of DNA pool

3.5.1. Designing Combinatorial Library for SELEX

Oligonucleotide library (combinational synthetic oligonucleotides) was purchased from Integrated DNA Technologies, USA. The oligonucleotide template consist of 40 nucleotides randomized region, flanked with primers binding sites at the 5' and 3' ends of the oligonucleotide template as shown in Table 3.1. Moreover, there are two sets of primers also were designed for the template amplification purposes using PCR. Forward primer was designed with T7 RNA Polymerase promoter sequences at the 5' end for the transcription process.