GENERATION AND CHARACTERIZATION OF RNA APTAMER AGAINST rHuEPO-α BY SELEX TECHNOLOGY

CITARTAN MARIMUTHU

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by

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LIST OF ABBREVIATIONS AND SYMBOLS

A	Adenine
AFM	Atomic Force Microscopy
AMD	Age-related macular degeneration
APS	Ammonium persulfate
ATP	Adenosine 5'-triphosphate
bcl-xl	B-cell lymphoma-extra large
Bis	N, N'-methylene bisacrylamide
Bp	Base pair(s)
BSA	Bovine serum albumin
C	Cytosine
°C	Degrees Celsius
$CaCl_2.6H_2O$	Calcium Chloride Hexahydrate
cDNA	Complementary DNA
CE	Capillary electrophoresis
CERA	Continuous Erythropoietin Receptor Activator
CHO	Chinese hamster ovary
CM5	Carboxymethyl-dextran
C-terminal	Carboxy-terminal
CTP	Cytidine 5'-triphosphate
CXCR4	C-X-C chemokine receptor type 4
CXCL12	C-X-C motif chemokine 12
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
ddH_2O	Double-distilled water
dGTP	Deoxyguanosine triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic Acid
ENU	Ethylnitrosourea
ELAA	Enzyme-Linked Aptamer Assay
ELISA	Enzyme-Linked Immunosorbent Assay
EPO-R	EPO receptor
et al.	and others
EtBr	3, 8-diamino-5-Ethyl-6-phenyl phenanthridinium Bromide
g	Gravitational acceleration
g	Gram
G	Guanine
GTP	Guanosine 5'-triphosphate
HCl	Hydrochloric acid
HCV	Hepatitis C virus
HEGL	Hexaethylene glycol

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
Hr	Hour(s)
HRP	Horseradish peroxidise
IEF	Isoelectric focusing
Ig	Immunoglobulin
IgE	Immunoglobulin E
IPTG	Isopropyl-β-D-thiogalactopyranoside
JAK2	Janus 2 Kinase
KCl	Potassium chloride
K_d	Dissociation constant
kDa	Kilodalton
KH_2PO_4	Monopotassium phosphate
K_2HPO_4	Dipotassium phosphate
LB	Luria Bertani medium
M	Mol/Liter, molar
MCF-7	Michigan Cancer Foundation - 7
Mg^{2+}	Magnesium ion
$MgCl_2.6H_2O$	Magnesium Chloride Hexahydrate
Min	Minute(s)
mL	Milliliter
mM	Millimolar
Na^+	Sodium ion
NaCl	Sodium chloride
NaOAc. $3H_2O$	Sodium acetate trihydrate
NaOH	Sodium hydroxide
NECEEM	Non-equilibrium capillary electrophoresis of equilibrium mixtures
ng	Nanogram
NHS	N-Hydroxysuccinimide
N-linked	Nitrogen atom-linked
nM	Nanomolar
nt	Nucleotide(s)
N-terminal	Amino-terminal
OH	Hydroxyl
O-linked	Oxygen atom-linked
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
poly A_{24}	24-mer of poly adenosine
PrPC	A prion in the common form
PrPSc	A prion in the Scrapie form
PSMA	Prostate-specific membrane antigen
PVDF	Polyvinylidene difluoride
rHuEPO	Recombinant human EPO
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
RT	Room temperature
RT-PCR	Reverse transcription-PCR

s	Second(s)
SCLC	Small cell lung cancer
SDS	Sodium dodecyl sulfate
SELEX	Systematic Evolution of Ligands by Exponential Enrichment
siRNA	Small interfering RNA
ssDNA	Single-stranded DNA
STAT5	Signal Transducer and Activator of Transcription 5
T	Thymine
TAE	Tris–Acetic Acid–EDTA
TBE	Tris-Boric Acid-EDTA
TBS-T	Tris Buffered Saline with Tween 20
TEMED	N,N,N',N'-Tetramethylethylenediamine
TMB	3,3',5,5'-Tetramethylbenzidine
Tris	Tris-(Hydroxymethyl)-Aminomethane
tRNA	Transfer RNA
U	Units of enzymatic activity
UTP	Uridine 5'-triphosphate
u.v.	Ultraviolet
V	Volt (s)
VEGF	Vesicular Endothelial Growth Factor
v/v	Volume per volume
w/v	Weight per volume
X–gal	5'-Bromo-4'-Chloro-3'-Indolyl-β-D-galactoside
µg	Microgram
µL	Micro liter
µM	Micro molar
2'-F	2'-fluoro
2'-NH_2	2'-amino
^{32}P	Phosphorus-32
$\gamma\ ^{32}P$	Gamma Phosphorus-32
WADA	World Anti-Doping Agency

PENGHASILAN DAN PENCIRIAN RNA APTAMER TERHADAP rHuEPO-α MENGGUNAKAN TEKNOLOGI SELEX

ABSTRAK

'Systematic Evolution of Ligands by Exponential Enrichment (SELEX)' merupakan kaedah penghasilan aptamer, iaitu jujukan DNA atau RNA tunggal yang mampu bergabung dengan pelbagai molekul sasaran. Dalam kajian ini, RNA aptamer telah dijana terhadap rekombinasi EPO manusia alfa (rHuEPO-α) melalui kaedah 'SELEX'. Selepas 11 kitaran SELEX, pengklonan dan analisa jujukan telah mengenalpasti satu jujukan yang merupakan RNA aptamer, dinamakan sebagai REPORA-6, dengan nilai penceraian berterusan sebanyak 25±1 nM. Nilai penceraian berterusan REPORA-6 terhadap 'deglycosylated' rHuEPO-α ialah 24.6±2 nM dan aptamer ini mampu menjadi 'universal probe' terhadap pelbagai rekombinasi EPO manusia. RNA aptamer REPORA-6 dan antibodi monoklonal anti-EPO mengikat pada tempat yang berbeza di permukaan rHuEPO-α, membolehkan perkembangan 'Sandwich Enzyme-Linked Aptamer Assay (ELAA)'. Had pengesanan ELAA ialah 0.29 nM dan ini menunjukkan bahawa REPORA-6 mampu diaplikasikan untuk ujian diagnostik rHuEPO. Aptamer REPORA-6 diaplikasikan dalam satu ujian mengikat HuEPO, satu kaedah yang mempunyai potensi yang baik berbanding kaedah pH yang memberi kesan kepada sasaran. RNA aptamer REPORA-6 didapati berpotensi untuk menggantikan fungsi antibodi pertama/kedua di dalam 'isoelectric-focusing (IEF)-double immunoblotting'. Analisis 'gel shift' menunjukkan aptamer REPORA-6 mempunyai potensi terapeutik kerana ia dapat mengekang interaksi antara EPO dan EPO-R, salah satu pemangkin angiogenesis sel kanser. RNA aptamer REPORA-6 stabil terhadap tindakan nuclease dan dapat dipangkas hingga ke 58-nt melalui

pemangkasan rasional dan analisa pemetaan. RNA aptamer yang dipangkas, (REPORA-6b) mampu memiliki kecekapan transfeksi yang tinggi dan tidak mudah terdedah kepada sistem imuniti serta mengekalkan nilai penceraian berterusan terhadap rHuEPO-α (22±2 nM).

GENERATION AND CHARACTERIZATION OF RNA APTAMER AGAINST rHuEPO-α BY SELEX TECHNOLOGY

ABSTRACT

Systematic Evolution of Ligands by Exponential Enrichment (SELEX) enables the isolation of aptamer (ssDNA or RNA that possesses high binding affinity and specificity against virtually any target molecules). In this study, a RNA aptamer against recombinant human EPO-alpha (rHuEPO-α) was successfully isolated by SELEX technology. After 11 cycles of SELEX, cloning and sequence analysis showed an appearance of a single major clone constituting the putative RNA aptamer, termed REPORA-6, with a dissociation constant of 25±1 nM. Dissociation constant of REPORA-6 against deglycosylated rHuEPO-α was 24.6±2 nM. REPORA-6 could possibly act as 'universal probe' against all rHuEPOs. REPORA-6 and anti-EPO monoclonal antibody bind at different binding sites on the surface of rHuEPO-α, facilitating the design of Sandwich Enzyme-Linked Aptamer Assay (ELAA). The detection limit achieved was 0.29 nM, suggesting that this assay has the potential to be an aptamer-based diagnostic (aptanostic) test for detection of rHuEPO. REPORA-6 was used in aptamer-based capture assay, which is a better method than pH-based elution of the target protein that can incur irreversible degradation on the protein. REPORA-6 could be also be used as a potential agent for replacing both primary/secondary antibodies in isoelectric-focusing (IEF)-double immunoblotting. Gel shift assay revealed that REPORA-6 has therapeutic potential through its ability to block the interaction between EPO and EPO receptor (EPO-R); one of the interactions of which mediates tumor growth/angiogenesis. REPORA-6 is resistant against nuclease degradation action and can be truncated via rational

truncation approach (aided by mapping analyses) down to 58-nt. This miniaturized aptamer (REPORA-6b) has better transfection efficiency and is less susceptible to the immune system, maintaining its binding affinity against the target (22±2 nM).

CHAPTER 1
INTRODUCTION

1.1 Discovery of Aptamer

A functional RNA molecule must possess the genotype and phenotype that enables the survival of the molecule within its population when genetic selection is applied. The genotype in this case indicates the sequence of the RNA while the phenotype represents the functional property associated with the RNA motif (Gilbert, 1986). In early investigations by Sol Spiegelman and colleagues, Darwinian evolution experiment in the test tube was performed using RNA bacteriophage Qβ that produces copies of the viral genome (Spiegelman et al., 1965). Several generations of the Qβ replicase-mediated RNA replication was carried out, in which serial dilution was done in each generation. In the end, superior variants were isolated from the initial huge population of sequences. The variants produced were the sequences retained following survival of selective pressure, which is the speed of the Qβ replicase's replication complexed with mutation rate of the Qβ replicase. Termed 'evolution in the test tube', the variants produced are devoid of the sequences unnecessary for binding against the Qβ polymerase. This was the first ever experiment that attested the self-replicating property of the RNA that have the phenotype and genotype to 'reproduce' and to evolve *in vitro* in the presence of the selective pressure. This groundbreaking work pioneered the effort to look further into the other phenotypic capability of the RNA sequence, such as the ability in recognizing target with high binding affinity and specificity. Furthermore, the discovery of nucleic acid-binding protein that plays important roles in cellular processes (such as transcription) infers the ability of these nucleic acids to bind

virtually any target molecules to perform specific functions and roles (Dignam et al., 1983, Murti et al., 1988). These wide ranges of DNA/RNA-protein interactions inspired the effort to develop artificial functional nucleic acids that can bind target molecules. Later in the 1990s, RNA motif that can bind to specific target was 'fished out' from a huge initial pool of nucleic acids. In 1990, Ellington and Szostak isolated RNA motifs that can bind to various organic dyes (Ellington and Szostak, 1990). In another independent work, Craig Tuerk and Larry Gold (1990) generated a RNA motif of 8-nucleotides long that was able to bind T4 DNA polymerase. The resulting high-affinity molecules obtained were termed aptamers (Ellington and Szostak, 1990).

1.1.1 Aptamer-the potential rival to antibody

Aptamers are single-stranded DNA (ssDNA) or RNA oligonucleotides, which rely on hydrogen bonding and hydrophobic interactions rather than Watson–Crick base pairing for high affinity, specific recognition of their target (Jayasena, 1999). Between 2009-2010, there were more than 2000 publications on aptamers as reported through “SciFinder”, suggesting that aptamer research is experiencing burgeoning growth (Iliuk et al., 2011). The upsurge in the number of aptamers being generated is due to the astounding features of aptamers, which are comparable to that of antibodies (Smuc et al., 2013, Wang et al., 2011a). Aptamers are analogues of antibodies, binding target with high specificity and having dissociation constant in the low nanomolar to picomolar level (Stoltenburg et al., 2007). The smaller sizes of aptamers (usually 10,000 daltons) compared to antibodies (150,000 daltons) have made aptamers the object of intense interest in many target detection systems. The low molecular weight of aptamer has the advantages of fast tissue and tumor

penetration as well as rapid blood clearance (White et al., 2000). In comparison, antibodies can only be raised against immunogenic targets in animals, while aptamers can be generated against virtually any target molecules. Aptamers have exceptional discriminating ability; able to distinguish between closely related molecules such as caffeine and theophylline that differs by a single methyl group (Zimmermann et al., 2000). Moreover, the production of aptamer is not influenced by physiological conditions whereas antibody generation relies on *in vivo* parameters in the animal body. Aptamers can also be easily modified by additional sequences for subsequent aptamer-based applications (Yang et al., 2013). They are very stable, despite being subjected to repetitive denaturation and renaturation during the process of SELEX (Mascini, 2008). Furthermore, labeling of the aptamers with reporter groups do not alter their conformation and binding affinity (Willner and Zayats, 2007). As an alternative to antibody-based methods, aptamers can be applied to both labelled (Citartan et al., 2012a, Sassolas et al., 2011) and label-free target detection reporting methodologies (Citartan et al., 2013). The absence of hazardous side-effects and high binding specificities associated with aptamers has earned them the reputation as 'excellent candidates' for diagnostics and therapeutics (McKeague and Derosa, 2012, Rahimi et al., 2009).

1.1.2 Aptamers in Diagnostics

The intrinsic properties of aptamer suggest that it can be an excellent candidate in diagnostic applications (Germer et al., 2013, Hong and Zu, 2013). DNA aptamer was generated against the whole cell of *Francisella tularensis*, a pathogenic species of Gram-negative bacteria and the causative agent of tularemia or rabbit fever. This DNA aptamer that specifically binds *F. tularensis* sub-species *japonica* was used in

the sandwich Aptamer-Linked Immobilized Sorbent Assay towards detecting the Tularaemia causative agent (Vivekananda and Kiel, 2006). The detection limit achieved was 250 ng of antigen.

Three RNA aptamers specific against lipopolysaccharide from *Eschericia coli* O157:H7, teichoic acid from *Staphylococcus aureus* and a cell membrane protein of outer membrane protein C from *Salmonella typhimurium* were utilized in a rapid detection of the food pathogens. These aptamers functionalized with thiols were immobilized on a silver surface. Using fluorescence microscope, fluorescence emission following the formation of the bacteria-aptamer complex can rapidly monitor individual food pathogens (Maeng et al., 2010). RNA aptamer raised against core antigens of HCV was immobilized on a 96-well plate using printing technology. This aptamer-based chip assay can specifically detect the core antigens from sera of HCV infected patients (Lee et al., 2007). DNA aptamer was generated for the detection of *Campylobacter jejuni*, a causative agent of human campylobacteriosis (Dwivedi et al., 2010). The authors have proposed that this aptamer can potentially be applied in aptamer-linked immobilized sorbent assay, colorimetric analysis, dot blot assay and proximal ligation assays towards specific detection of *Campylobacter jejuni.*

1.1.3 Aptamers in Therapeutics Applications

The non-immunogenic nature and high binding affinity of aptamer suggests that they can be effective therapeutic agents (Keefe et al., 2010, Que-Gewirth and Sullenger, 2007). One way aptamer can result in this effect is by binding to the target and inhibiting the binding of other protein molecules such as receptors. For example, RNA aptamer generated against Prion proteins PrPC (normal cell-surface

glycoprotein), blocks its conversion into infectious cell-surface glycoprotein, PrPSc. This prevents the pathogenesis of prion-associated diseases, such as bovine spongiform encephalopathy (Mashima et al., 2013). Another RNA aptamer targets type IVB pilus of *Salmonella enterica* serovar *Typhi* (the point of entry into human cells) and reduces cell invasion (Pan et al., 2005). Two RNA aptamers, P-58 and P-78 were reported to interfere with the replication of Hepatitis C virus (HCV) by specifically targeting 5BSL3.2 domain residing within *cis*-acting replication element. Binding of this aptamer blocks the region at the 3'-end of the replication element, which is the coding region for viral RNA-dependent RNA polymerase (Marton et al., 2013).

Reverse transcriptase inhibition by aptamer can be a wise therapeutic strategy for the treatment of HIV, which can suppress viral replication (Whatley et al., 2013). The aptamer having the motif UCAA binds to the reverse transcriptase of HIV and inhibit its enzymatic activity *in vitro*. This impedes access of natural primer or template to reverse transcriptase, thereby halting the DNA polymerization and RNaseH activities of the enzyme. This aptamer was speculated to perform similar inhibitory reaction *in vivo*.

Some of the aptamers designed for therapeutic applications have entered clinical trials. The RNA aptamer, Pegaptanib, is undergoing testing to treat age-related macular degeneration (AMD) (Zhou and Wang, 2006). This 2′-fluoropyrimidine modified RNA aptamer reduces angiogenesis by inhibiting the activity of vascular endothelial growth factor (VEGF), a protein responsible for AMD. Pegaptanib represent the first aptamer approved by Food and Drug Administration for therapeutic application. An anti-thrombin DNA aptamer, which can prolong blood clotting time has passed Phase I clinical trial. Compared to

Heparin, this aptamer is more effective at similar clinically relevant concentrations in reducing the platelet deposition (Lee et al., 2006).

Another aptamer, that functions against nucleolin (cell surface receptor for survival and proliferation of cells) for the treatment of cancer has entered Phase I trial (Vorhies and Nemunaitis, 2007). Nimjee and co-workers generated an RNA aptamer that can block catalytic activity of factor IXa (that catalyzes the conversion of factor X to factor Xa in the blood coagulation cascade) (Nimjee et al., 2005). This aptamer is part of the anti-coagulant system known as REG1, which underwent three Phase I clinical trials (Dyke et al., 2006). RNA aptamer isolated against the C-X-C motif of chemokine 12 (CXCL12) (chemokine that binds receptor C-X-C chemokine receptor type 4 (CXCR4) for vasculogenesis, tumor growth, and metastasis) was also evaluated for clinical application. This aptamer can act as antagonist against CXCL12 by blocking its interaction with its receptor CXCR4 (Duda et al., 2011). Known as NOX-A12, the RNA aptamer has entered Phase I trial conducted on 48 healthy patients (de Nigris et al., 2012). The high potentiality of the aptamer for therapeutic purpose is the absence of side effects in treatment as was reported with nucleolin and the VEGF aptamer (Borbas et al., 2007). Other vital applications mediated by aptamers are listed in Table 1.1.

Table 1.1 Aptamer-Based Applications

TARGET FOR APTAMER	APPLICATIONS	REFERENCES
	Aptamer-Based *In Vivo* Imaging	
Ramos cell	*In vivo* imaging of temporal distribution of Cyanine 5 (fluorophore)-tagged TD05 aptamer in Ramos tumor bearing mouse.	Shi et al., 2010
Nucleolin transmembrane protein in cancer cells	*In vivo* cancer cell imaging.	Lee et al., 2010
Prostate-specific membrane antigen (PSMA)	*In vivo* imaging of prostate cancer cells.	Min et al., 2010
Small cell lung cancer (SCLC) cells and tumors containing p68	*In vivo* imaging of SCLC cells and tumors containing p68.	Bagalkot et al., 2007, Kunii et al., 2011, Mi et al., 2010
	Aptamer-Based Drug-Delivery System	
PSMA	Anti-PSMA aptamer was conjugated to Gelonin, which is a ribosomal toxin that inhibits the process of protein synthesis, killing the cancer cells upon internalization by binding to PSMA on the cancer cells.	Chu et al., 2006a
Tenascin-C	*In vivo* tumor targeting by using fluorescently labelled aptamer that is specific for tenascin-C, an extracellular matrix protein up-regulated in glioblastoma cancer xenografts.	Hicke et al., 2006

Table 1.1 Continued

TARGET FOR APTAMER	APPLICATIONS	REFERENCES
	Aptamer-Based Drug-Delivery System	
mAb20, specific autoimmune antibody	Binding of the aptamer to the antibody averts the interaction with human insulin receptor, preventing antibody-mediated receptor internalization. Useful in the treatment of insulin resistance.	Doudna et al., 1995
PSMA	Conjugation with nanoparticle known as poly (lactic acid)-block-polyethylene glycol copolymer with a terminal carboxylic acid functional group **(**PLA-PEG-COOH) to mediate the uptake by cells expressing PSMA.	Farokhzad et al., 2004
	Aptamers in Microarray	
IgE and thrombin	RNA aptamers specific for lysozyme and ricin were conjugated with biotin and spotted onto streptavidin coated microarray slides for RNA aptamer-based microarray.	Cho et al., 2006
Interleukin-16, VEGF, and endostatin	17-plex photoaptamer array, whereby the target protein was fixed onto the aptamer in a permanent manner via irreversible cross-linking covalent bonds. Quantification of the protein bound was executed by labeling with fluorophore N-hydroxysuccinimide (NHS)-Alexa555 that targeted exposed primary amines of the protein, subsequently detected by fluorescence measurement.	Bock et al., 2004

Table 1.1 Continued

TARGET FOR APTAMER	APPLICATIONS	REFERENCES
	Aptamers-Based Ribozyme	
ATP	Fusion of the hammerhead self-cleaving ribozyme to the aptamer domain. Decrease in the catalytic activity of the ribozyme by up to 180-fold in the presence of ATP.	Tang and Breaker, 1998
Theophylline	Conjugation of the aptamer to hammerhead ribozyme. Cleavage activity of the ribozyme was increased by up to 110-fold in the presence of theophylline.	Soukup and Breaker, 1999a

1.2 Method of Aptamer Generation-Systematic Evolution of Ligands by Exponential Enrichment (SELEX)

One of the main criteria that attributes to the 'fame' of aptamer lies in the method of aptamer generation known as Systematic Evolution of Ligands by Exponential Enrichment (SELEX). The high reproducibility of SELEX is evident with the myriads of aptamers generated against various targets, such as dyes (Ellington and Szostak, 1990), ATP (Lato et al., 2002), metal ions (Kawakami et al., 2000), proteins (Calik et al., 2009, Niazi et al., 2008, Schurer et al., 2001, Sekiya et al., 2005, Stevenson et al., 2008, Wochner et al., 2008, Yoshida et al., 2008), toxin (Tang et al., 2007), whole organism (Boiziau et al., 1999, Cheng et al., 2008) and tumor initiating cell (Kim et al., 2013). In general, SELEX is a combinatorial process that comprises of four important steps: (1) preparation of random oligonucleotide library and target-nucleic acid complex formation (2) separation of the target-bound from unbound molecules (partitioning) (3) the amplification of the target-bound molecules (4) cloning and sequence analysis (Figure 1.1).

1.2.1 Preparation of Random Oligonucleotide Library and Target-Nucleic acid Complex Formation

The basic important criterion of the SELEX is the design of the random DNA oligonucleotide library (Gold et al., 1995). A typical random DNA oligonucleotide library comprises of central randomized region (25-100 nucleotides long) flanked by a constant primer binding regions of about 18-40 nucleotides long. The library is prepared by phosphoramidite-based chemical synthesis using phosphoramidite containing all the 4 bases (A, G, C and T). To ensure the production of the 'neutral'

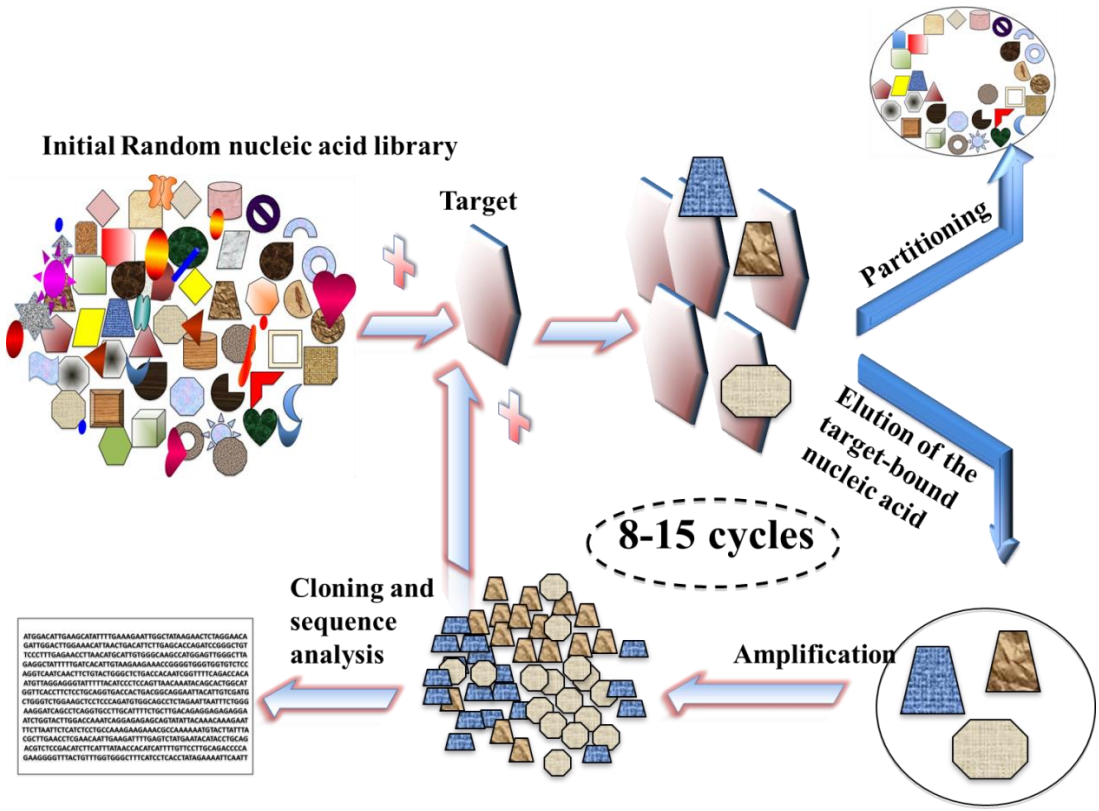


Figure 1.1 Basic Steps of SELEX (this study)

One complete step of SELEX comprise of complex formation, partitioning, and amplification. Starting with incubation with the chemically synthesized initial random nucleic acid library, partitioning is carried out to separate the target-bound molecules from the unbound molecules. The target bound molecules are eluted and amplified by PCR. After 8-15 iterative rounds, the selected molecules were cloned and sequenced for the identification of potential aptamer candidates.

library with equal representation of all the four nucleotides, the ratio of delivered phosphoramidites containing the nucleotides is adjusted according to the coupling efficiencies of these phosphoramidites (Lato et al., 1995). As each of the nucleotide positions in the central randomized region can be occupied by either of the nucleotides (dATP, dGTP, dCTP or dTTP), this leads to the different sequences of the ssDNA. For example, library with the randomized region of 35 nt will have approximately 4^{35} or 10^{21} number of different ssDNAs (Gold et al., 1995). This 'synthesized library' can be directly used for the first step of DNA SELEX. The advantage of an initial PCR amplication step is the ability to eliminate the aberrantly synthesized DNA in the library (Marshall and Ellington, 2000). However, an additional step is required to convert the PCR-amplified double-stranded DNA (dsDNA) into ssDNA by several strategies (Marimuthu et al., 2012). In contrast, for the RNA SELEX, ssDNA library is converted to dsDNA by PCR amplification before they are subjected to *in vitro* transcription to produce the RNA library (Figure 1.2). Following the oligonucleotide library preparation, binding is performed with the target of choice. Binding is carried out by incubation of the library with the target under optimum buffer conditions. The most common incubation parameter is incubation at room temperature (RT), for 10-20 min (Gopinath et al., 2012).

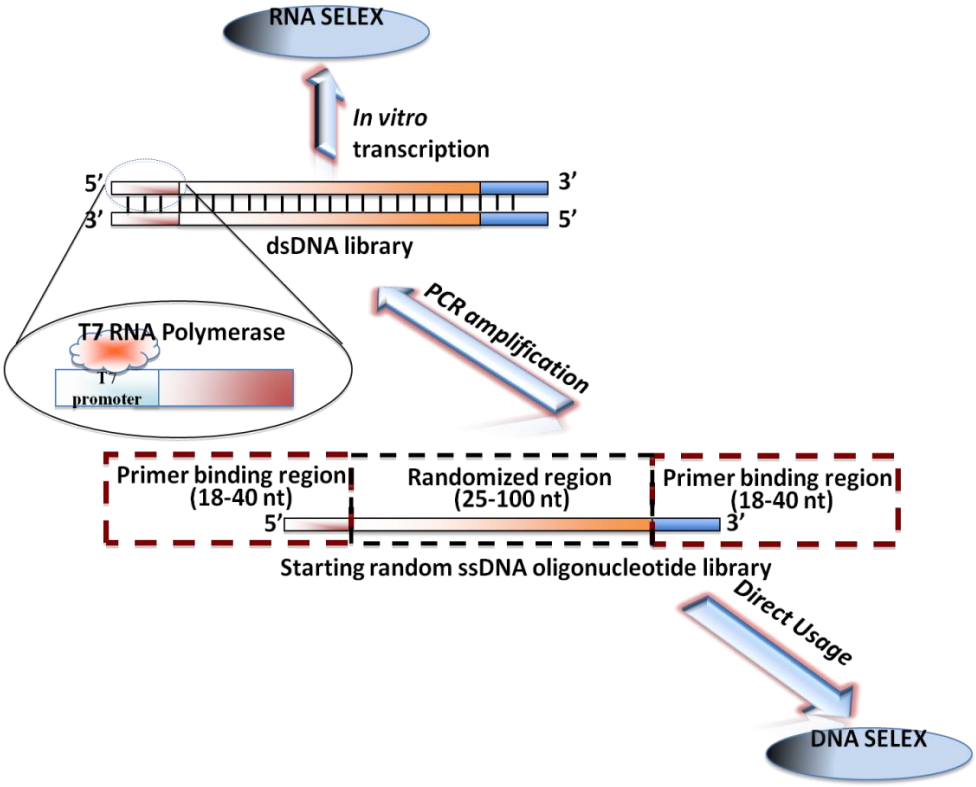


Figure 1.2 Starting random ssDNA oligonucleotide library for DNA and RNA SELEX (this study)

The random ssDNA oligonucleotide contains a central randomized region with two constant primer-binding sites. For the DNA SELEX, the ssDNA library can be used directly. PCR amplification can also be carried out followed by the conversion of dsDNA to ssDNA. However, for RNA SELEX, the ssDNA library has to be converted to dsDNA library, which is used to generate the RNA library. T7 promoter region is incorporated at the 5'-end of the sense primer to facilitate *in vitro* transcription.

1.2.2 Partitioning

One of the most crucial steps in any SELEX experiment is to separate the target-bound oligonucleotides from unbound oligonucleotides after the nucleic acid-target complex formation (Stoltenburg et al., 2007). Numerous methods have been adopted to enable the separation of these bound from unbound species. One of the most common methods of separation is the usage of nitrocellulose filter membrane (Zhou et al., 2011). Nitrocellulose filter allows the retention of target protein that bind to the nucleic acid sequences with high affinity. By virtue of vacuum suction, the unbound nucleic acid sequences will pass through the filter. The retained nucleic acid sequences can then be eluted and amplified (Rahimi et al., 2009). Using this partitioning method, several aptamers were selected against a wide range of targets including T4 DNA Polymerase (Tuerk and Gold, 1990), bovine factor IX (Gopinath et al., 2006a) and human influenza virus (Gopinath et al., 2006b).

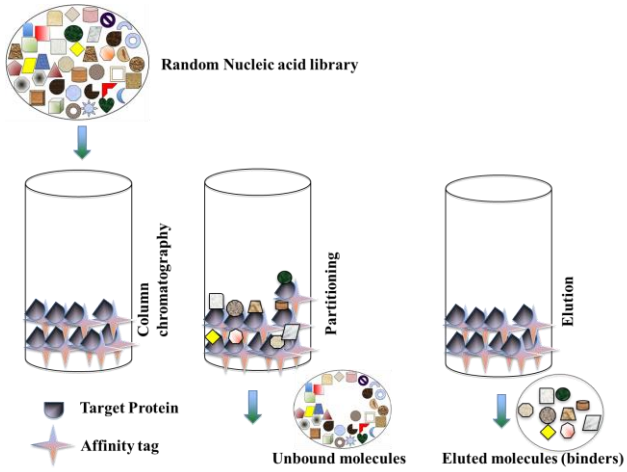
Another method involves the usage of affinity tags that facilitate immobilization of proteins in the affinity chromatography column (Gopinath, 2007). Affinity tags include cyanogen bromide sepharose, N-hydroxysuccinimide (NHS), glutathione S-transferase tagged at the N-terminal or C-terminal of the target protein facilitate protein immobilization (Nilsson et al., 1997). Nucleic acid ligands that have affinity against the target protein remain bound despite washing with excess washing buffer (Figure 1.3 [A]). The retained nucleic acids (of the nucleic acid-protein complex) is then eluted by strong denaturant such as 7 M urea (Moore et al., 2011). Another simple separation technique is by microtiter plate-based partitioning method. The input nucleic acid molecules are incubated in wells coated with target protein for a certain period of time. Following washing steps, the bound molecules are eluted (Wochner and Glokler, 2007, Zhang et al., 2003).

Surface plasmon resonance-based Biacore system has made vital contribution in SELEX by providing an efficient platform for partitioning (Misono and Kumar, 2005). In this method, the randomized RNA library was injected to the target molecule immobilized on the surface of biacore chip. After the removal of the unbound molecules, target-bound nucleic acids are collected into a fraction tray. Using Biacore-mediated partitioning, the amount of non-specific binders and the number of SELEX cycles were drastically minimized (Figure 1.3 [B]). Misono and Kumar (2000) have generated RNA aptamer against human influenza A/Panama/2007/1999 (H3N2) virus using Biacore-sensing plate as the platform to separate target-bound nucleic acids from unbound nucleic acids.

Missailidis et al. (2005) performed SELEX by passive absorption of the target protein (antibodies) on the walls of the PCR tubes. Separation of the target-bound from unbound nucleic acids was performed by simple pipetting of the solution out of the tubes following incubation of the nucleic acid library with the target protein. PCR amplification was carried out directly inside the PCR tubes without any prior elution step as the denaturation during the PCR amplification releases the antibody-bound sequences (single-pot strategy) (Missailidis et al., 2005).

Recently, Capillary Electrophoresis (CE) is another favourite scheme of partitioning in SELEX (Ashley and Li, 2013). The high resolving capacity is accounted for by the differential migration of the target-bound sequences and the unbound sequences in the capillary. This method greatly reduces the time spent on SELEX cycles, from several days to only a few hours. Using CE, DNA aptamer was

(A)



(B)

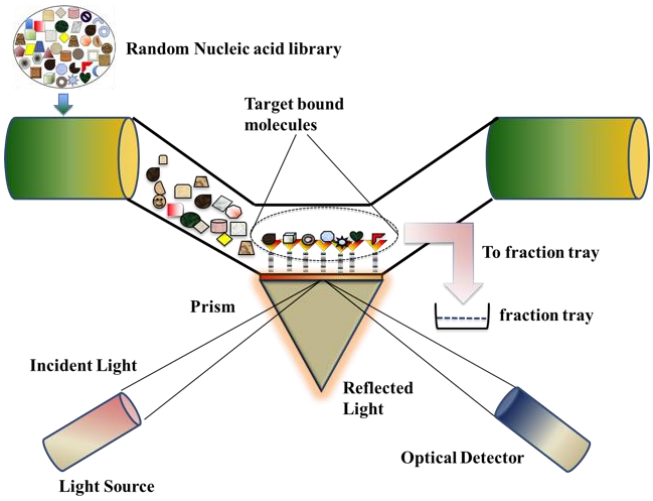


Figure 1.3 Partitioning Step of SELEX (this study)

(A) The application of the affinity tag enables immobilization of the target protein in affinity column chromatography. Following incubation of the target-bound resin with the nucleic acid library, unbound sequences are removed from the column by washing steps. The target-bound molecules are eluted and amplified (this study).

(B) Biacore-assisted partitioning involves the use of the Biacore chip (such as carboxymethyl-dextran (CM5) chip) for the immobilization of the target. Similarly, after the removal of the unbound nucleic acids, the target-bound nucleic acids are collected inside the fraction tray during the dissociation step and is then PCR amplified (this study).

generated against Neuropeptide Y within 2-4 cycles of SELEX (Mendonsa and Bowser, 2004).

1.2.3 Amplification

Subsequent to the partitioning step, the target-bound sequences are eluted using denaturants such as urea (Gopinath et al., 2012). Elution with urea or any other denaturants has the probability of eluting not only nucleic acids that form complexes with the target protein, but also those molecules that bind to the matrix non-specifically. Since the interaction between the target and the bound sequences are non-covalent, the use of other elution methods including affinity elution with excess amount of target (Geiger et al., 1996) or excess amount of competitor (Bridonneau et al., 1999) is possible. This method assures the elution of only target-bound molecules. As the resulting eluted target-bound sequences are present in very minute amount, PCR amplification is necessary to increase the copy number of these sequences to a sufficient amount applicable in the consequent cycles of SELEX (Duan et al., 2013, Han and Lee, 2013). This particular step of SELEX (after the PCR amplification) is different for RNA and DNA SELEX, which will be delineated in the next section.

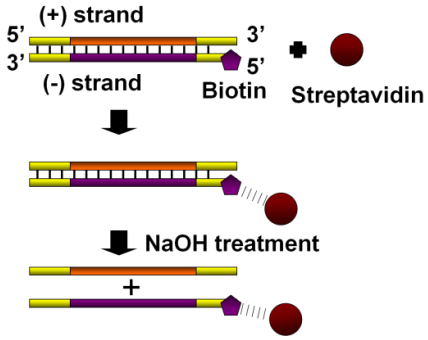
1.2.3.1 DNA SELEX

Following optimal PCR amplification, efficient generation of ssDNA from dsDNA PCR product is the key point of a successful DNA aptamer generation (Svobodova et al., 2012). This is because ssDNA can form diverse structural conformations to enable binding to target molecules as opposed to dsDNA that adopts only double-helix conformation. Structural conformations of ssDNA comprises of unpaired nucleotide and regions that form stable secondary structures such as hairpin structures, pseudo-knots (Schneider et al., 1995) and quadruplex structures (formed by planes of two neighboring G-quartets) (Jing et al., 1997).

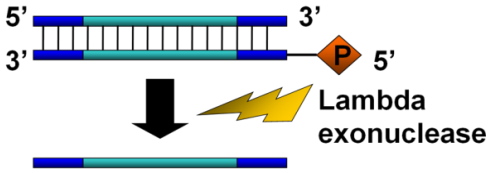
As described below, there are different methods of converting dsDNA into ssDNA, before utilizing these molecules as the input in the subsequent cycle of SELEX. These methods are asymmetric PCR (Citartan et al., 2012d), biotin-streptavidin separation (Figure 1.4 [A]), lambda exonuclease digestions (Citartan et al., 2011) (Figure 1.4 [B]) and size separations on denaturing polyacrylamide gel electrophoresis (Marimuthu et al., 2012) (Figure 1.4 [C]).

ssDNA preparation in DNA SELEX is slightly cumbersome as 'scaling up' is necessary for maximum production of the ssDNA (Marimuthu et al., 2012). Moreover, the residual amount of dsDNA in the reaction mixture can be ‘fatal’ to the SELEX process, which otherwise requires careful optimization.

(A)



(B)



(C)

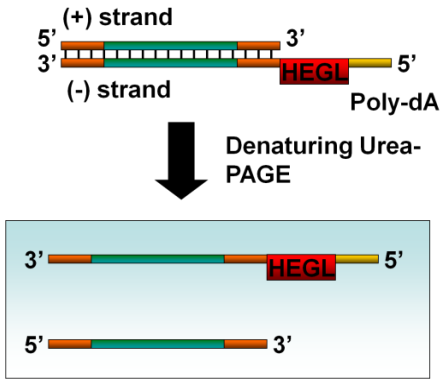


Figure 1.4 Methods of converting dsDNA into ssDNA

(A) ssDNA generation from biotinylated PCR product using streptavidin (adopted from Marimuthu et al., 2012).

(B) Lambda exonuclease digestion on the phosphorylated PCR product (adopted from Citartan et al., 2011).

(C) Separation of the amplicons-derived-modified primers on PAGE (adopted from Marimuthu et al., 2012) .

1.2.3.2 RNA SELEX

The difference between DNA and RNA SELEX lies in the process of generating the input nucleic acid molecules to be applied in the subsequent cycle of SELEX. As explained in the previous section, in DNA SELEX, the dsDNA must be converted to ssDNA before the next cycle of SELEX. However, in RNA SELEX, the eluted target-bound RNA molecules must be converted to first strand cDNA by reverse transcription before subjecting to PCR amplification (RT-PCR). The resulting PCR product is used to generate RNA molecules by *in-vitro* transcription before subjected to the next round of SELEX. Compared to the preparation of ssDNA from dsDNA in DNA SELEX, *in vitro* transcription in RNA SELEX can produce microgram amount of RNA directly from the dsDNA template without any scaling-up. This template DNA can be removed by DNase treatment to prevent carry-over of the DNA into the subsequent SELEX cycles.

Upon generation of sufficient amount of ssDNA or RNA, subsequent cycles of SELEX can be carried out. The initial few rounds of SELEX are performed in such a way that the condition is not so stringent This prevents the loss of potential high affinity binders that are usually present at very minute amount (Nieuwlandt, 2000). The binding stringency is subsequently increased in the next cycles to allow for the isolation of high affinity binders (Zimmermann et al., 2010). This is achieved by using different target/competitor (yeast tRNA/salmon sperm DNA) ratios (Liu et al., 2012) or by a stringent washing step (Lauridsen et al., 2012).

1.2.4 Cloning and Sequence Analysis

After approximately 8-15 cycles of SELEX, the resulting nucleic acid pool is cloned and subjected to sequencing. Sequence analysis is imperative to identify individual sequences that have the highest binding affinity against the target (Ditzler et al., 2013, Latulippe et al., 2013, Wang et al., 2013). Prediction of binding motif can be realized by alignment of the sequences (usually 50-100 sequences). Sequences that have homologous and common motif(s) are classified into similar groups. Each group of the sequences is then checked for its binding against the target. The sequence that exhibits the most prominent binding is the potential aptamer candidate. The number of different aptamer candidates obtained is usually influenced by the complexity of the target (Conrad et al., 1995).

1.2.5 The 'Tweaking' of SELEX Strategies

With the progress of time, diverse types of SELEX procedures have been designed, which involves modification or improvement in the basic steps of SELEX (Hamm et al., 2002, Lorenz et al., 2010, Wu and Curran, 1999). Most of these modifications of the SELEX strategies result in the minimization of the number of SELEX cycles and ensure efficient separation of unbound nucleic acid sequences from target-bound sequences. For example, to maximise the separation of target-bound sequences from unbound sequences, Capillary Electrophoresis (CE)-based partitioning was employed. Also known as CE-SELEX, this enhanced SELEX procedure reduces the overall number of SELEX cycles (from 10-15 cycles to 2-4 cycles) (Mosing and Bowser, 2009).

In Atomic Force Microscopy (AFM)-SELEX, AFM was applied to measure the adhesion between the cantilever and the sample surface. This SELEX methodology was proven to isolate high affinity aptamers. As a proof-of-concept, aptamer against target protein thrombin was isolated (within 3 cycles), which has binding affinity higher (200 pM) than that of the conventional anti-thrombin aptamer (200 nM) (Miyachi et al., 2010).

Other non-SELEX method was also proposed, in which non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) was adopted for the purpose of partitioning without amplification of the target-bound molecules (Ashley et al., 2012). Similar to CE-SELEX, this modified non-SELEX method also greatly reduced the number of SELEX rounds (from the usual 10-15 to 3 rounds), without compromising the generation of aptamer with high binding affinity (Berezovski et al., 2006).

1.3 Human Erythropoietin (HuEPO)

Human Erythropoietin (HuEPO) or Erythropoietin (EPO), a glycoprotein hormone, is the most important erythropoietic growth factor responsible for erythroid differentiation, survival and proliferation (Fisher, 2003). It is initially synthesized as a polypeptide containing 193 amino acids, whereby the first 27 amino acids is the signal peptide. Before secretion, these terminal 27 amino acids are removed, resulting in a 166-amino acids polypeptide (Lai et al., 1986). Oligosaccharide side chains are added at the N-glycosylation sites of the HuEPO, at the positions 24, 38 and 83 of the amino acid asparagine. Similar glycosylation takes place at the amino acid serine located at the position 126 (Narhi et al., 1991). These oligosaccharide site chains are requisite for *in vivo* activity of the HuEPO, so as to prevent fast

degradation of the HuEPO in the liver even before it reaches the target site. The main stimulating factor for the production of HuEPO is tissue hypoxia, whereby the oxygen capacity in the blood and the artery reduces (Huang et al., 1998, Ivan et al., 2001, Jaakkola et al., 2001, Jiang et al., 1996, Maiese et al., 2004).

1.3.1 Recombinant Human EPO (rHuEPO) and Doping

With the advent of cloning technology, the gene that encodes HuEPO was cloned and expressed in several eukaryotic hosts. This gives rise to different types of recombinant human EPO (rHuEPO) including Epoetin alfa (Jelkmann, 2008), Epoetin beta (Storring et al., 1998), Epoetin omega (Pascual et al., 2004), Epoetin delta (Llop et al., 2008) and Darbepoetin alpha (Egrie and Browne, 2001). The availability of these rHuEPOs has tremendously enhanced the lives of patients with chronic kidney, which is the key causative agent of anemia due to the inadequate production of EPO in kidney. The amino acid sequences of the rHuEPOs and the HuEPO are shown in Figure 1.5.

The oxygen carrying capacity of the blood to the muscles is the major obstacle to the ability to carry out extended physical activity. During this event, oxygen is consumed very quickly, which poses a great limitation to muscular function. As red blood cells are the main vehicle for carrying oxygen, athletes resort to blood doping as a way to increase the oxygen carrying capacity of the blood (Lippi and Guidi, 2000). This act of using blood transfusion is termed blood doping and was banned in 1987 by the International Olympic Committee (IOC). Many athletes have then switched to rHuEPOs as an alternative. Following this, rHuEPO was banned by World Anti-Doping Agency (WADA) in the 1990s (Lasne et al., 2002).

There are several methods to detect the doping of rHuEPO, such as measurement of the hematologic parameters as the markers of the erythropoiesis level (Bressolle et al., 1997), chromatographic (Skibeli et al., 2001) and mass spectrometry analyses (Sasaki et al., 1988). Currently, the method accepted by WADA for rHuEPO doping is by Isoelectric Focusing (IEF)-Double Immunoblotting, which depends on antibodies (Lasne, 2001, Lasne and de Ceaurriz, 2000, Lasne et al., 2002) (Figure 1.6).

1.3.2 EPO-EPO-R interaction in Tumour Growth

Tumor growth is a multi-factorial phenomenon. One of the causative factors is mediated by the interaction between EPO and EPO Receptor (EPO-R), which leads to the phosphorylation of tyrosine residues on the surface of the EPO-R. Signal Transducer and Activator of Transcription 5 (STAT5), a SH_2 domain containing protein, interacts with these phosphorylated tyrosine residues on the surface of the EPO-R, resulting in the phosphorylation of the STAT5 protein itself. STAT5 protein forms a dimer, which goes to the nucleus and acts as the transcription factor regulating the expression of genes such as *bcl-xl*, an anti-apoptotic gene (Acs et al., 2001, Ribatti et al., 2007). Other genes that are also upregulated result in cancer cell proliferation, tumor oxygenation and angiogenesis that aids in cancer progression (Hardee et al., 2006).