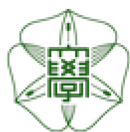
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（核への選択的薬物送達システム確立のための **protein SELEX** 法に基づくヌクレオポリン **358** 標的 **RNA** アプタマーの探索）

A thesis submitted by

Garima Shrivastava

In partial fulfillments of the requirements for the Degree of Doctor of Philosophy in Pharmaceutical Sciences

The Graduate School Of Life Sciences

(Biomedical and Pharmaceutical Sciences Course)

Laboratory of Innovative Nanomedicines

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Sapporo, Japan

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Supervisor

Prof. Dr. Hideyoshi Harashima

Laboratory for Molecular Design of Pharmaceutics

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Declaration

The thesis work described here has been done as a full time Ph.D. candidate under supervision of Prof. Dr. Hideyoshi Harashima, Faculty of Advanced Pharmaceutical Sciences, Laboratory of Innovative Nanomedicine, Hokkaido University, Japan. The study presented here is purely innovative and has not been published or reported anywhere earlier.

Garima Shrivastava

March 2014

Dedicated

To my father

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Abbreviations

μl	Microliter
Nup358	Nucleoporin358
NPC	Nuclear Pore Complex
BSA	Bovine Serum Albumin
PCR	Polymerase Chain Reaction
Kd	Dissociation Constant
kDa	Kilo Dalton
ml	Millilitre
FBS	Fetal Bovine Serum
PBS	Phosphate Buffer Saline
HEPES	(4-(2-hydroxyethyl)-1- piperazineethane sulphonic acid)
HBSS	Hank's Balanced Salt Solution
DMEM	Dulbecco's Modified Eagle Medium
NPs	Nanoparticles
LPs	Liposomes
DSPE	1,2-distearoyl-sn-glycero-3-phosphoethanolamine
PEG	Polyethylene Glycol
EPC	Egg Phosphatidyl Choline
Chol	Cholesterol
GFP	Green Fluorescent Protein
CA	Carbonic Anhydrase
CAI	Carbonic anhydrase inhibitor
DDS	Drug Delivery System
MEND	Multi Envelope type Nano Device
FDA	Food Drug Administration
ADA	Adenosine Deaminase
HSV	Herpes Simplex Virus
AIDS	Acquired Immuno Deficiency Syndrome
pDNA	plasmid DNA
FG	phenylalanine glycine

NLSs	Nuclear Localization Signal
MDa	Mega Dalton
NTD	N-Terminal Domain
CTD	C-Terminal Domain
EDTA	Ethylene-diamine-tetra-acetate
EMSA	Electro-mobility-shift assay
CLSM	Confocal Laser Scanning Microscopy
WGA	Wheat Germ Agglutinin

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Project Theme

The purpose of gene therapy is either the treatment or replacement of a defective human gene or the introduction of a new gene that encodes a therapeutic protein after nuclear entry. For this it requires both delivery of the therapeutic DNA into human cells followed by its subsequent expression. With research it is widely recognized that the transfer of exogenous DNA into non-dividing cells is a complex process, which involves the passage through numerous biological barriers. Those barriers include the extracellular matrix, the cell membrane, the intracellular environment and also the nuclear envelope. Drug Delivery Systems (DDSs) is a field of nanotechnology, these are therapeutic methods to control pharmacokinetics, pharmacodynamics, drug efficacy and non-specific toxicity of drugs. It is a wide field that is applicable on variety of organelles of a cell. Gene delivery for treatment of genetic disorders is one of the most difficult tasks in present drug delivery challenges scenario. Nuclear-targeted drug delivery system can solve this problem but still it is a field with most limited success. Numbers of physical and chemical approaches developed, are in routine practice to deliver nucleic acids into the cell but drug remains inside the cytoplasm, although ultimate target of drug delivery in any case is to attain effective drug action inside nucleus. Ligand receptor interactions have advantage of being highly selective for binding to site of interest. NLS based nuclear targeting has been reported but the success is limited and uneven. The present study is designed to develop an active targeted artificial ligand based drug delivery system towards nucleus, which can specifically target the drug loading nanocarrier to nucleus. Nup358 protein is a fiber like extensions made up of nucleoporin358 protein exposed in cytoplasmic phase of the nucleus. This protein acts as first docking site for import cargoes entering inside nucleus. Nucleic acid aptamers are single stranded DNA or RNA molecules that bind with very high affinity and specificity to their target. Increasing success in field of aptamer technology, gave us hypothesis to develop an artificial ligand that can promote specific binding of nanocarrier to the nucleus, for that we used Nup358 protein to develop a RNA aptamer ligand that can bind specifically to nucleus.

Hypothesis- Identification of Nup358 specific RNA aptamer using Protein-SELEX method to achieve a nuclear targeting delivery system using a MEND.

Chapter 1

Introduction

1.1 General Introduction

1.1.1 Gene Therapy

Gene therapy is one of the most attractive and challenging fields in medicine. It is a type of molecular medicine, which is based on the transfer of a functional gene into cells to modify a cellular dysfunction. Gene therapy was originally developed as a strategy to treat hereditary disease such as cystic fibrosis, but now gene therapy is considered for many non–life-threatening conditions such as adverse affect of a quality of life of patient (1-5). Joshua Lederberg first introduced the term gene therapy in 1963, but research on this mainly started from 1980s (6-8). Anderson et al in 1990 did the first FDA approved clinical gene therapy. In that study, a four years old girl with ADA deficiency was treated by transfection of the ADA gene into her white blood cells, which resulted in remarkable improvements in her immune system (6,9-11). Molecular biology and genomics advent has proved gene as most promising therapeutic agents as it is therapeutically applicable to

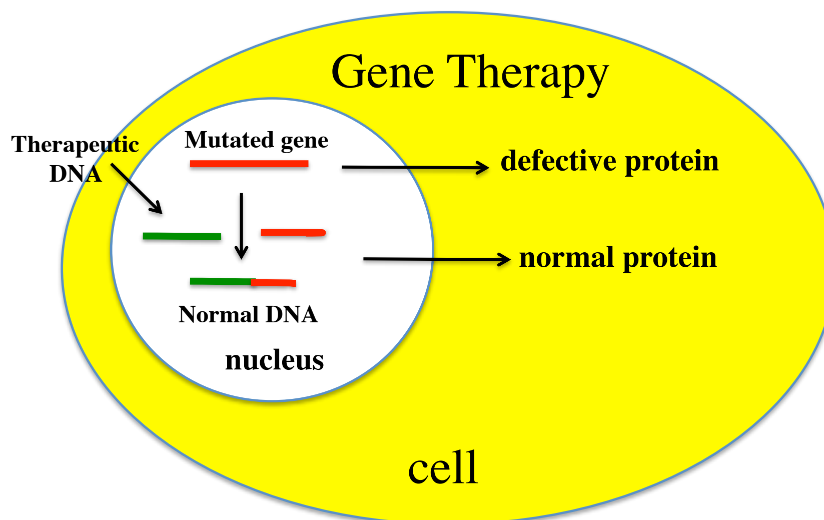

Figure 1-1. Gene therapy

different types of disease. To date gene therapy is considered for many non-life-threatening conditions. Gene therapy has made significant progress, although much

slower than it was initially predicted (12).

1.1.2 Gene therapy and drug delivery system

The purpose of gene therapy (Figure 1-1) is gene expression of the protein coded in the target cells and ultimately to treat the disease by protein expressed in the cells transfected Gene transfection is a technology, which is indispensable to research progress in molecular biology (13-21). Viral and non-viral vectors and physical methods have been developed to make gene delivery safer and more efficient (22). There are many viral vectors used in gene delivery for most fatal diseases such as AIDS (23,24), cancer (25,26) and refractory diseases (27,28). But many disadvantages over advantages make viral vectors an unpleasant option. Immunogenic response and degradation of therapeutic DNA by serum nucleases is a big obstacle for functional therapeutic delivery to the target cell. pDNA targeted to specific site can improve DDS towards gene therapy (29).

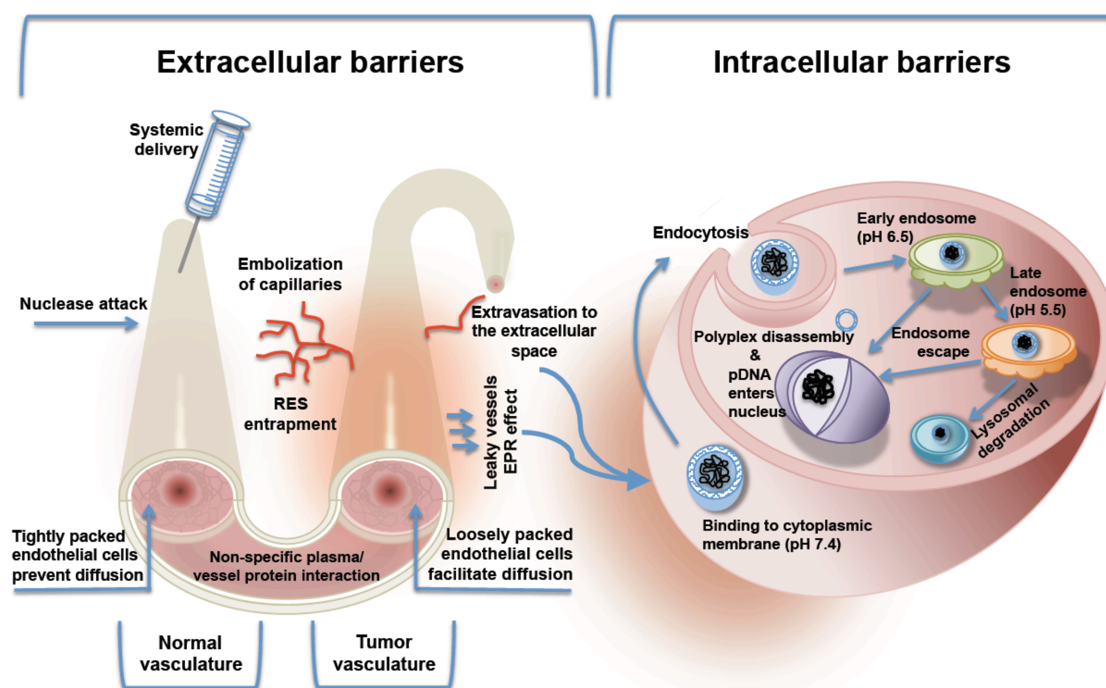

Figure 1-2. Extra and intracellular barriers faced by non-viral gene delivery system in systemic delivery (Ref. 69).

1.2 Strategies in gene therapy

Ex-vivo gene therapy is a strategy, especially well- suited for targeting a specific organ rather than a whole organism but ex-vivo gene therapy was not successful to treat internal disorders, because of that the concept of in vivo gene therapy came to existence. In vivo gene therapy is a method in which genetic material often in the form of pDNA, is applied to modify the genetic structure of target cells to achieve therapeutic goals. This technology is now being developed in clinical trials as a treatment for hereditary disorders, and is also being considered as a potential treatment of acquired diseases (30). There are two general approaches to deliver genes into a cell: viral and non-viral.

1.2.2 Viral vectors: For better understanding of how polyplexes and lipoplexes traverse cytoplasm, it is important to first understand how virus have evolved to access nucleus. Many viruses have expertise in delivering their genetic material to nucleus. Viral vectors with small diameter (~100 nm) have been used since long as stable transgene vectors. Viruses are able to use host cell machinery for protein transgene expression. Retrovirus, lentivirus, adenovirus, adeno-associated virus and herpes simplex virus have been investigated for the purpose of the gene therapy (31). Among them adenovirus are most widely used viral vectors for human gene therapy due to their unbeatable ability to deliver their genomes directly into host cell nucleus. Multiple cytoskeletal networks appear to be involved in the intracellular trafficking of retrovirus. Viruses such as retrovirus are RNA to double stranded DNA, they can enter cells in a variety of ways such as endocytosis and fusion with cell membrane, they un-coat in the cytoplasm to free RNA, the conversion of RNA to DNA occurs in the cytoplasm. The resulting pre-integration complex consists of the reverse transcribed DNA and other viral proteins. Access to nucleus depends on how virus entered the cell (32). Adenoviruses enter cells via clathrin-mediated endocytosis. Low pH in endosome causes conformational changes in adenovirus capsid that leads to endosomal escape and entry into cytoplasm and migrate to nucleus with intact DNA to release into the nucleus with essential help of microtubule network (31,32). Leopold et al. reported that once in the cytoplasm, GFP-labeled adenovirus particles use the microtubule network, they found that nocodazole treatment to depolymerize microtubule resulted in nuclear localization inhibition of virus, and that virus itself can not travel via directed movement towards the nucleus without microtubule

network (32). HSV viruses enter cell by membrane fusion when HSV capsid binds to dynein present in very close proximity to nucleus it results in deposition of DNA-coat capsid at periphery of the cytoplasm. Dynein helps aggregation of viruses to nuclear rim and subsequent translocation into the nucleus. Transport of individual capsids was monitored and it was found that most of the virus was located at the nuclear rim (34). Despite of having better transgene efficiency than nonviral vectors, viral vectors show strong immunogenic response, pathogenicity and size limitations of cargo genes (Table 1-2). (32)

1.2.2 Non viral vectors:

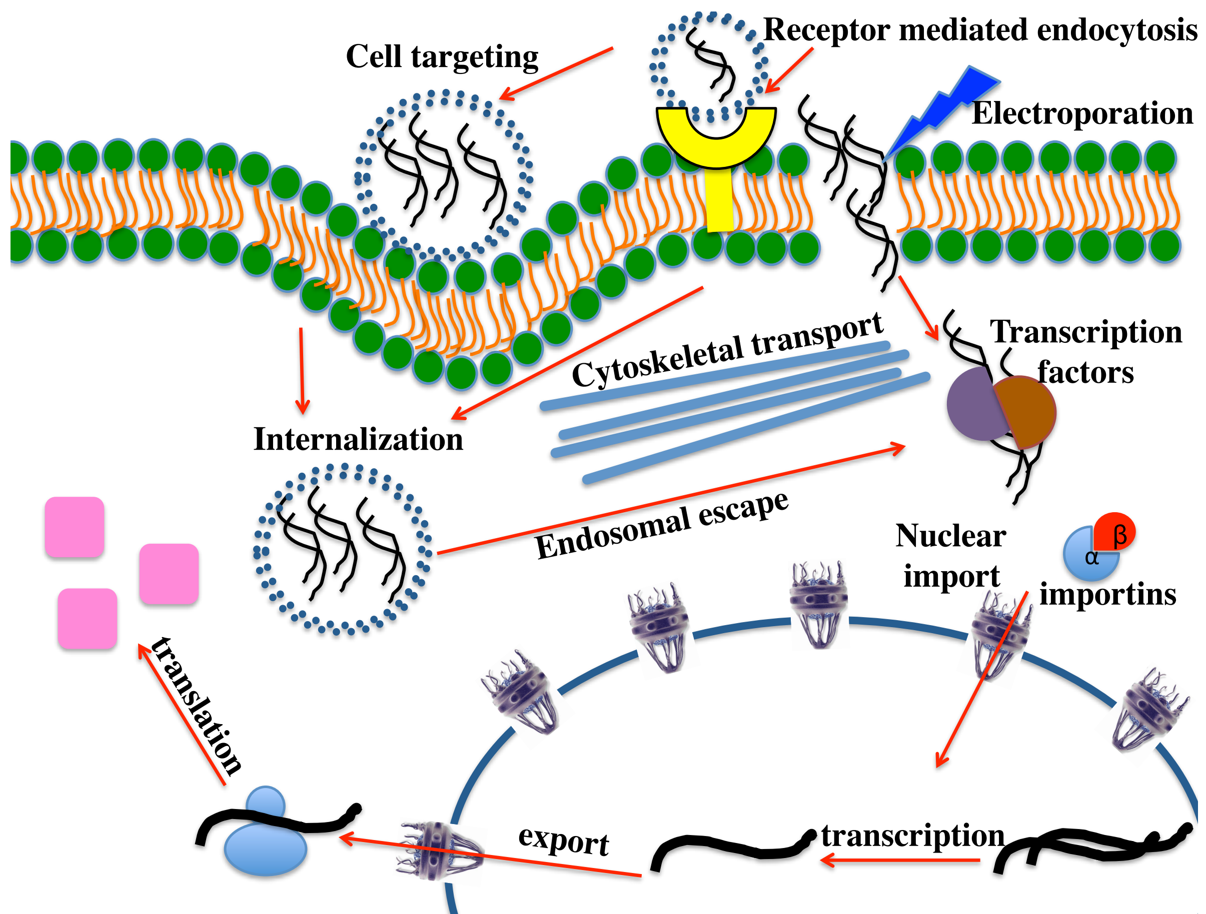

Figure 1-3. Different possible pathways of non viral vectors for gene delivery.

DNA can be internalized via physical methods such as electroporation, receptor mediated endocytosis, liposomal endocytosis or through direct diffusion. Safety

concerns over use of viral vectors make nonviral vectors preferred choice for gene delivery. Nanoparticles are attractive tools for nonviral gene transfer.
Using non-viral vectors is a better choice for gene therapy but this system has numbers of limitations. Eukaryotic cells have advanced subcellular structures. Since plasmid DNA is large and negatively charged molecule, it is impossible to allow the plasmid DNA to interact with cell membrane with negative charge; for that it is required to condense the DNA with cationic materials. These cationic lipids impose a positive charge on DNA and so it can enter the cell. It has been already reported that on condensation of DNA with poly-ion causes decrease of molecular size (35,36). DNA and vector with positively charged polymers such as PEI, interacts with negatively charged cell membrane electrostatically and finally initiates internalization and also buffering effect protects plasmid DNA from degradations inside endosome. Reports suggest that lipid-DNA complex directly injected into nucleus does not result in gene expression, and so for successful gene expression, the DNA must escape from endosome and dissociate from lipoplex or polyplex. Several studies with different transfection agents have shown that plasmid is efficiently internalized into cells but less than 1% of the DNA present in the cytoplasm reached the nucleus (37). Physical methods also work well as an alternative such as electroporation and gene gun method. In addition, chemical methods such as lipoplexes (DNA-liposome complexes) and polyplexes (DNA-polymers) increase the stability of DNA and also facilitate the entry into cell. But regardless of the method (Table 1-1) by which DNA accumulates in cytoplasm there should be a way by which DNA can migrate to nucleus (36).

Table 1-1

Comparative analysis of Gene Delivery System

DNA delivery system	Advantages	Disadvantages
Viral Vectors	a) High transfection efficiency b) Suitable for systemic delivery c) Targeting for selected cell types is possible	a) Difficult manufacturing b) High quality control essential c) High cost d) Interference with pre-existing immunity against

		the biological vectors. e) Safety issues. f) Low temperature storage
Chemical Methods	a) Most effective for cultured cells b) Simpler manufacturing c) Less limit on gene size d) Easy storage	a) Limited clinical applications b) Preparation of consistent chemical formulations
Physical Methods	a) High local transfection efficiency b) No cell type dependence c) Easy to fix the process d) Less limit on gene size e) Useful for ex vivo application	Specific instruments and machinery is required.

Table 1-2

Utilization of viral vectors for gene delivery (Ref. 36)

Vector	Genome	Properties	Structure
Adenoviruses	dsDNA	Transient expression, strong immunogenicity	Capsid
Alphaviruses	RNA	Transient, but extreme expression levels; low immunogenicity	Envelope
HSV	dsDNA	Latent infection, long-term expression, low toxicity (mutant)	Envelope
Lentiviruses	RNA	Genome integration, long term expression, safety concerns low, inefficient production	Envelope
Retroviruses	RNA	Genome integration, long-term expression.	Envelope
Adeno-	ssDNA	Slow expression onset, genome	Capsid

associated viruses (AAV)		integration, long term expression, inefficient large-scale virus production.	

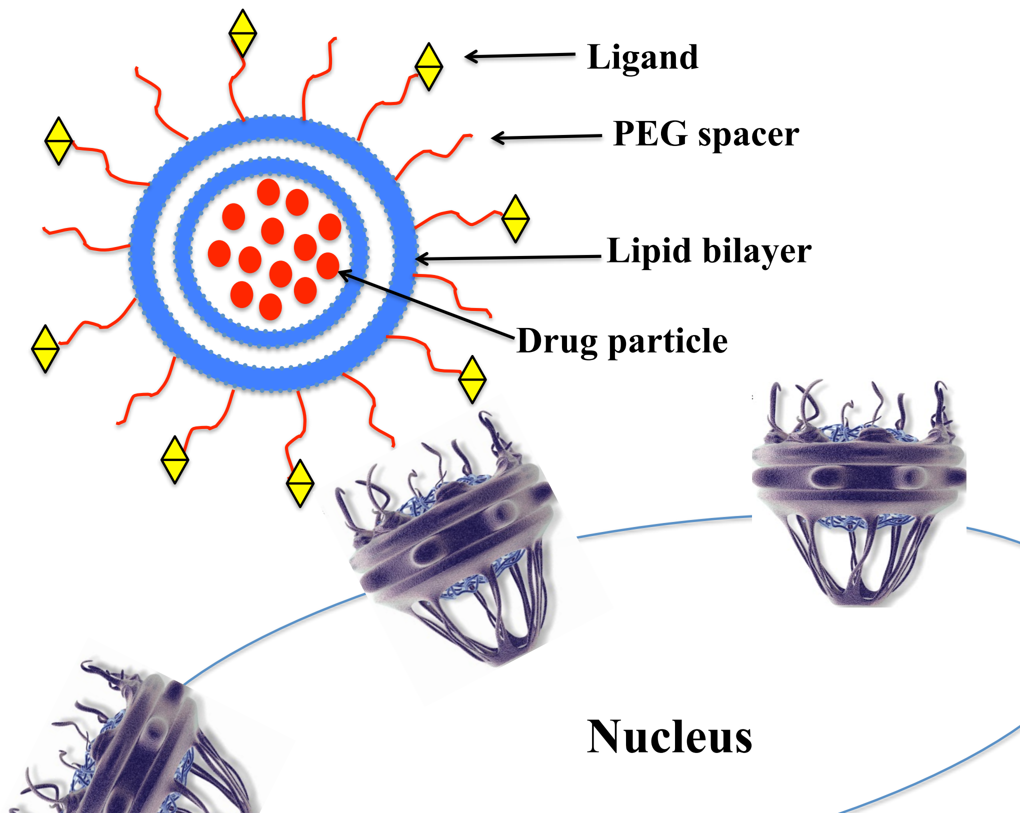

Figure 1-4. Aptamer (ligand) modified drug loaded PEG-LP binds to target receptor.

Drug delivery can be further enhanced if the LP-attached ligands bind selectively to receptors (Figure 1-4), which would help increase the concentration of drugs inside the site of action. This binding to cellular receptors induces receptor-mediated endocytosis of LPs into endosome compartments with low pH, where the LPs break down and release the encapsulated drugs into intracellular space. While to our hypothesis, dual targeting MEND can target cell and then specific subcellular organelles such as nucleus .

1.3 Targeted Drug Delivery:

Targeted drug delivery (Table 1-3) is a method of delivering medication to a patient

in a manner that increases the concentration of the medication in some parts of the body relative to others. The goal of a targeted drug delivery system is to prolong, localize, target and have a protected drug interaction with the diseased tissue. The conventional drug delivery system is the absorption of the drug across a biological membrane, whereas the targeted release system releases the drug in a dosage form. The advantages to the targeted release system is the reduction in the frequency of the dosages taken by the patient, having a more uniform effect of the drug, reduction of drug side-effects, and reduced fluctuation in circulating drug levels. This helps maintain the required plasma and tissue drug levels in the body, thereby preventing any damage to the healthy tissue via the drug. The drug delivery system is highly integrated and requires various disciplines, such as chemists, biologists, and engineers, to join forces to optimize this system. The disadvantage of the system is high cost, which makes productivity more difficult and the reduced ability to adjust the dosages (38). In traditional drug delivery systems such as oral ingestion or intravascular injection, the medication is distributed throughout the body through the systemic blood circulation. For most therapeutic agents, only a small portion of the medication reaches the organ to be affected. Targeted drug delivery seeks to concentrate the medication in the tissues of interest while reducing the relative concentration of the medication in the remaining tissues. For example, by avoiding the host's defense mechanisms and inhibiting non-specific distribution in the liver and spleen, (39) a system can reach the intended site of action in higher concentrations. Targeted delivery is believed to improve efficacy while reducing side effects. When implementing a targeted release system, the following design criteria for the system must be taken into account: the drug properties, side effects of the drugs, the route taken for the delivery of the drug, the targeted site, and the disease. Increasing developments to novel treatments requires a controlled microenvironment that is accomplished only through the implementation of therapeutic agents whose side effects can be avoided with targeted drug delivery. Advances in the field of targeted drug delivery to cardiac tissue will be an integral component to regenerate cardiac tissue (40). There are two kinds of targeted drug delivery: active targeted drug delivery, such as some antibody medications, and passive targeted drug delivery, such as the enhanced permeability and retention effect (EPR-effect).

1.3.1 Passive targeting

Passive drug targeting refers to the accumulation of therapeutic drug or drug carrier system at a particular site such as in case of anti cancer drugs size and surface properties of drug delivery nanoparticles must be controlled specifically to maximize circulation times and targeting ability. To attain such conditions optimal size must be of lesser diameter than 100 nm and surface should be hydrophilic. Passive targeting corresponds to transport in the organism according to the normal distribution pattern of the liposomes that are composed of phospholipids or phospholipids and sterols.

1.3.2 Active targeting

Active targeting is a strategy that describes special interaction of ligand modified drug loaded carrier to receptor (Figure 1-5). This method promotes binding of drug carrier to target site just as missile are launched. Targeted drug delivery can be broadly divided into 2 areas: 1) Systemic targeting: based on blood circulation and extravasation. 2) Intracellular targeting: targeting of drug carriers to the target cells. There are different types of drug delivery vehicles, such as polymeric micelles, liposomes, lipoprotein-based drug carriers, nanoparticle drug carriers, dendrimers, etc. An ideal drug delivery vehicle must be non-toxic, biocompatible, non-immunogenic, biodegradable, (41) and must avoid recognition by the host's defense mechanisms.

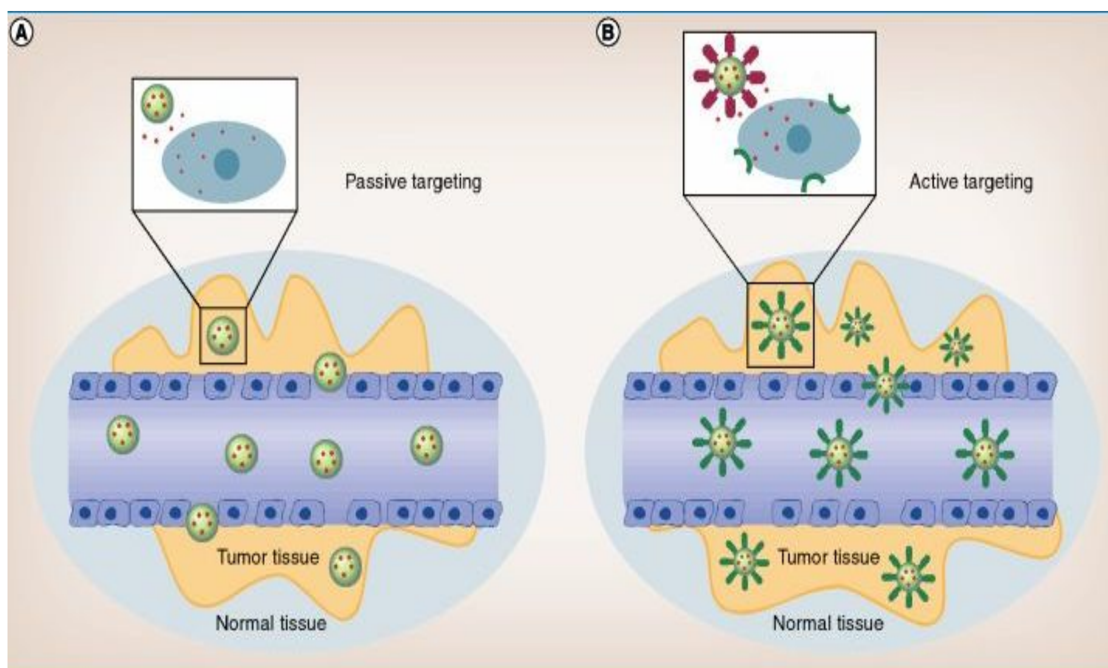

Figure 1-5. Active and Passive targeting (ref 70)

Table 1-3. Division of targeted drug delivery system.

Targeted drug delivery system
1. Systemic targeting based
a. Ligand-receptor interaction b. Locally-activated delivery i. Self-triggered release of the drug at the target cells ii. Externally-activated release of the drug at the target cells
2. Intracellular targeting
a. Low- pH activation technologies that use default pathway delivery to lysosomes b. Mechanism that avoid lysosomal delivery

1.4 Nuclear Drug Delivery

Nuclear drug targeting means targeting of drug loaded system to the site of action that is nucleus. But the nuclear delivery process is a crucial barrier to successful gene delivery, most importantly in non- dividing cells.

With evolution of life eukaryotic cells and microbes both developed themselves to survive better. Cells developed many complex intracellular organelles to protect cell interior while pathogens developed special machinery to invade the cell barriers. For the successful gene delivery, the nanocarrier has to pass through several cellular hindrances, 1) stability of DNA and carrier in extracellular environment, 2) endocytosis or attachment of nanocarrier to cellular membrane, 3) endosomal escape, 4) transport of pDNA through cytoplasm and finally 5) nuclear import. For successful targeting of drugs to individual cell, the pDNA must enter the cell, translocate in cytoplasm, and enter the nucleus for transcription and gene expression (Figure 1-6).

1.4.1 Cytoplasmic Diffusion

Recently researches have proved that it is possible to deliver drugs into cytoplasm while earlier it was viewed as black box. Although, exact mechanism behind how DNA moves through the cytoplasm and how it reaches into the nucleus, is largely unknown. After pDNA enters the cell by diffusion or by active transport it can reach to the nucleus in size dependent manner. Previous reports

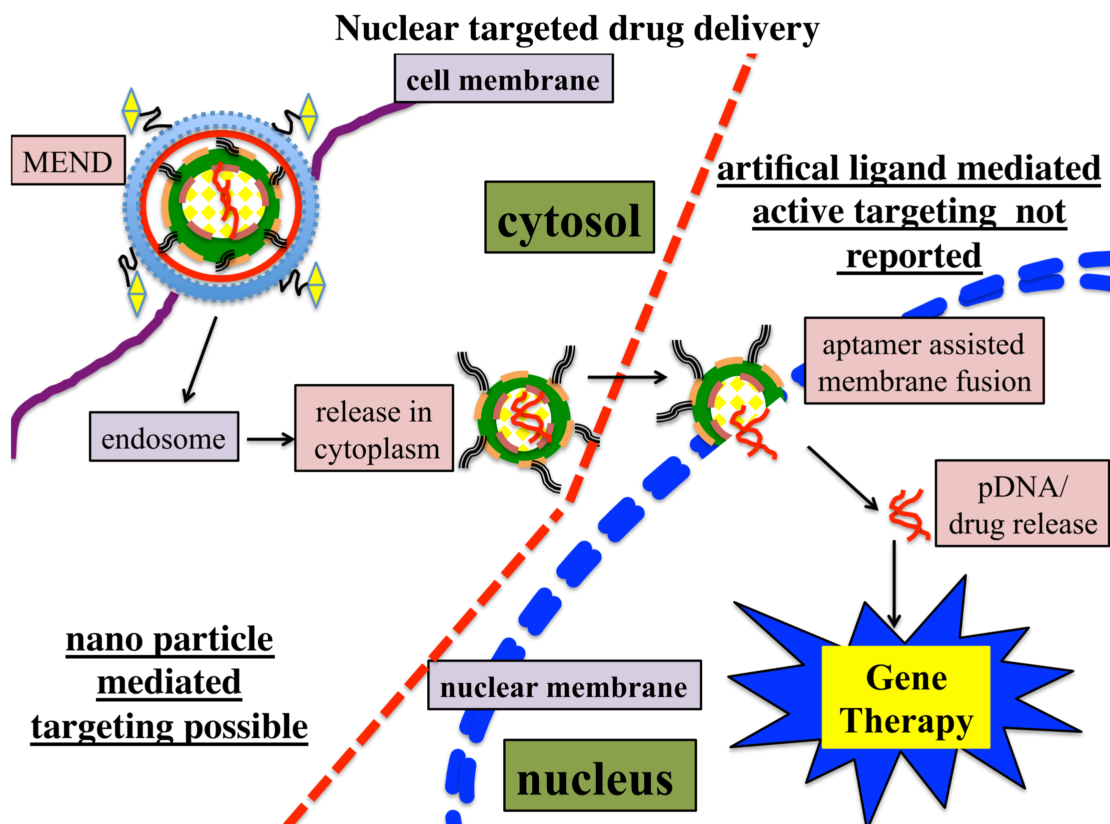

Figure 1-6. Nuclear targeting drug delivery system.

suggest that small solutes could diffuse freely and rapidly in the cytoplasm and nucleus (42,43) so it was thought that diffusion of macromolecules such as DNA may be possible in cytoplasm but Verkman et al in his report explained that it is highly improbable to freely diffuse large DNA into the cytoplasm (44). Reduced mobility of DNA in cytoplasm is considered to be because of high viscosity of cytoplasmic fluid (due to highly cross-linked actin cytoskeleton) as on disruption of viscosity of cytoplasm by cytochalasin D resulted in elimination of size dependent reduction in DNA diffusion (45). So, as diffusion cannot explain transfer of the DNA to the nucleus directed targeting is one of the best alternatives.

1.4.2 Endosomal escape

Plasma membrane is negatively charged and because of it naked DNA (negatively charged molecules) does not cross membrane barriers efficiently. So, DNA has to be attached or encapsulated on a carrier to gain access to individual cells. With the help of carriers attachment of positively charged vector to negatively charged plasma membrane becomes possible, but this binding could be more specific in presence of a specific targeting ligand attached on the vector (46). Once the drug-loaded nanocarrier attaches to plasma membrane it is taken up by endocytosis. Eukaryotic cells exploit various endocytic pathways like, clathrin mediated endocytosis via coated pits, and non-clathrin mediated pathways including caveolin mediated endocytosis, clathrin and caveolin dependent endocytosis and macropinocytosis. Clathrin dependent endocytosis pathway is more efficient in transfection as in various reports internalization of lipoplexes are found by clathrin mediated endocytosis. After the intracellular vesicle carrying the vector is taken up via endocytosis, they usually fuse with endocytic compartments. First the vector locates itself in a vesicle with slightly low pH known as early endosome. The late endosome then access the nanocarrier to the lysosome where DNA is destroyed by broken down finally. So to get therapeutic effect of a drug endosomal escape is a must.

1.4.3 Nuclear membrane

Nuclear membrane is considered as one of the most potent barriers for DNA delivery to the cells. The nucleus is separated from the cytoplasm by nuclear envelope (NE) composed of a lipid bilayer called as nuclear membrane. The nuclear membrane has several thousands of perforations referred as nuclear pore complex (NPCs) (125 kDa) that constitute large aqueous transport channels that mediated and regulate the bidirectional exchange of macromolecules between the nucleus and the cytoplasm.

The size of nuclear pore is ~9 nm to ~39 nm that restricts macromolecules to enter inside nucleus freely. Size limitation, network mesh of FG repeats and complex of different but important ~30 nucleoporins make nuclear pore complex a big barrier in nuclear drug delivery system. The first report came in 1980, when pBR322-based plasmids were injected into the cytoplasm but there was no gene expression found, while the plasmids injected directly into the nucleus showed gene expression in 50-100% of cells (47). Further studies confirmed that on microinjection of 1000-3000 copies of plasmids into the cytoplasm, the levels of gene expression were only 3% in

comparison to plasmids microinjected directly into the nucleus (48). Zabner et al also demonstrated that nuclear injected DNA injected resulted in high-level gene expression, but the same DNA injected into the cytoplasm produced no gene expression (49).

1.4.4 Nucleocytoplasmic trafficking of exogenous DNA

Cationic liposome/DNA complexes can enter the cells by endocytosis and traverse the cytoplasm through various compartments has been published in many papers (50). There can be 2 fates of these internalized complexes, after internalization either they are targeted to lysosomes for degradation, or released into the cytoplasm. Once DNA is released in cytoplasm it is available to enter into the nucleus for expression. To increase the DNA delivery to the cytoplasm incorporation of cationic peptide DNA condensing agents or endosomal lytic peptides (51-52) is used. The insufficiency of drug to nucleus is thought to be the main hurdle of a non-viral drug delivery system. After endocytosis, the drug or pDNA has to be released into the cytosol so that it can enter nucleus. After release of DNA, cytosolic diffusion and stability of pDNA seem to be important factors, which may inhibit the efficiency of gene transfer in the nucleus.

1.4.5 Stability of DNA

Lechardeur et al., 1999 reported about their experiments to check the stability of pDNA, delivered by microinjection into the cytosol. On observation using by fluorescence in situ hybridization (FISH) and quantitative single-cell fluorescence video-image analysis they found that both single- and double-stranded circular pDNA disappeared with an apparent half-life of 50– 90 min from the cytoplasm of HeLa and COS cells. DNA compaction with vectors used in gene transfer experiments may offer an increased resistance to nucleases (53). The carrier complexes have to breakdown to release the DNA. In such case the metabolic instability imposed by cytosolic nucleases may cause hindrance for DNA translocation into the nucleus (54).

1.4.6 Nuclear importation

Eukaryotic cells have very complex cellular machinery, in non-dividing cells there is an additional barrier of nonviral gene transfer that is the translocation of DNA

through the nuclear envelope composed of lipid bilayer. The macromolecular and ionic exchange between cytoplasm and nucleus are regulated by nuclear pore complex (NPC) (55). The pore size of NPC is approximately 55 Å in diameter, and is allows free diffusion to molecules with a molecular weight lower than 40 kDa. Despite of constant working and knowledge about Nucleocytoplasmic transport very little is known about the nuclear import of exogenous plasmid DNA. Short macromolecules such as nucleic acids can diffuse freely through the NPC, but pDNA is a macromolecule and the ability of a macromolecule to undergo nuclear import strongly depends on its molecular weight. pDNA entry in the nucleus by an active transport process involves the NPC and is energy dependent as per reported *in vitro* studies results (56). The formation of complexes of pDNA with proteins prior to transport to the NPC and translocation is observed (57).

1.5 Nuclear Localization Signal (NLSs)

After endocytosis, once the drug-loaded carrier can escape from endosome transportation of drug to nucleus is essential to attain gene expression and therapy.

After escaping from the endosome when drug is inside the cytosol, drug is actively transported to nucleus using motor proteins. The particles bind to cytoplasmic phase of the nuclear pore complex and transfer DNA into the nucleus. Viruses have developed their transfection machinery by generating NLS like layers over their surface. Certain peptide sequences or NLSs or nuclear localization signals is an amino acid sequence, which 'tags' a protein for import into the cell nucleus by nuclear transport. Typically, this signal consists of one or more short sequences of positively charged lysine or arginine exposed on the protein surface. Karyopherins identify these NLSs signals and transport them into nucleus. To learn from viral transfection method is a key to gain nuclear access. So to enhance intracellular transport, complex must have ligand/ligands that are recognized by microtubules. Naturally during cell division the cell disassembles and so at that time the nanoparticles may trigger release of nucleic acids using some proteins such as c-myc. In the non-dividing cells, the nuclear membrane is considered as the ultimate barrier as it is a lipid bilayer that separates intranuclear compartment with cytosolic phase. It leaves nuclear pore complex (NPC) as only option for transport of the macromolecules. Nuclear pore complex are up to 3000-4000 in numbers per nucleus. The opening width of NPC is

restricted to 9 nm-39 nm (58). This small size allows only viruses to move in and out freely through it to enter nucleus with help of NLSs. NLSs may be broadly divide into two main types (Figure 1-7). Many researchers have tried coupling of NLSs to pDNA to consider it as an attractive strategy in nuclear targeting. NLSs specifically interact with karyopherins, which can then target molecules to the nucleus. NLS are peptides mostly composed of basic, positively charged oligopeptides containing several lysine and arginine residue that can be monopartile or bipartile, with no general consensus sequence. First and most studied classical NLS sequence is SV-40 large T antigen NLS sequence P^{126}**KKKRK**V^{132} (59). Different nuclear localized proteins may share the same NLS. To achieve a nuclear targeting method many types of NLSs have been developed.

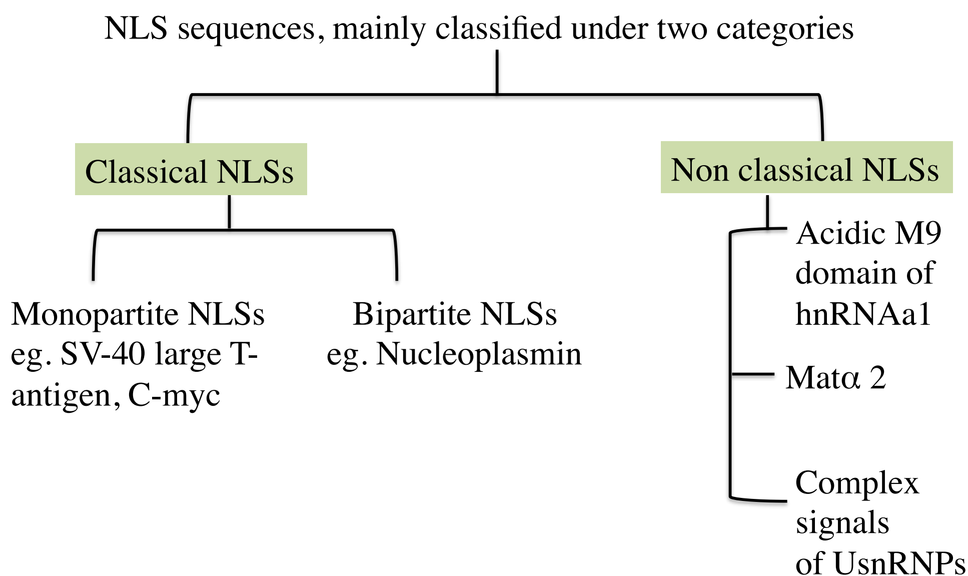

Figure 1-7. Division of Nuclear Localization Sequence (NLSs)

1.5.1 Mechanism of NLS mediated nuclear import

Covalent or non-covalent attachment of a nuclear localization signal to plasmids has been shown to improve transfection efficiency. Proteins containing an NLS interact with karyopherins, termed as importins. An import complex is formed in the cytoplasm between cargoes bearing NLSs, importin α and importin β. After passing through the nuclear pore complex (Figure 1-8), the binding of RanGTP to importin-β

dissociates importin-β from importin-α. The NLS-containing cargo is then displaced from importin-α and then importin-α is recycled to the cytoplasm by its nuclear export factors, CAS, complexed with RanGTP. In the cytoplasm, RanGAP stimulates GTP hydrolysis, releasing the importins for another import cycle (60).

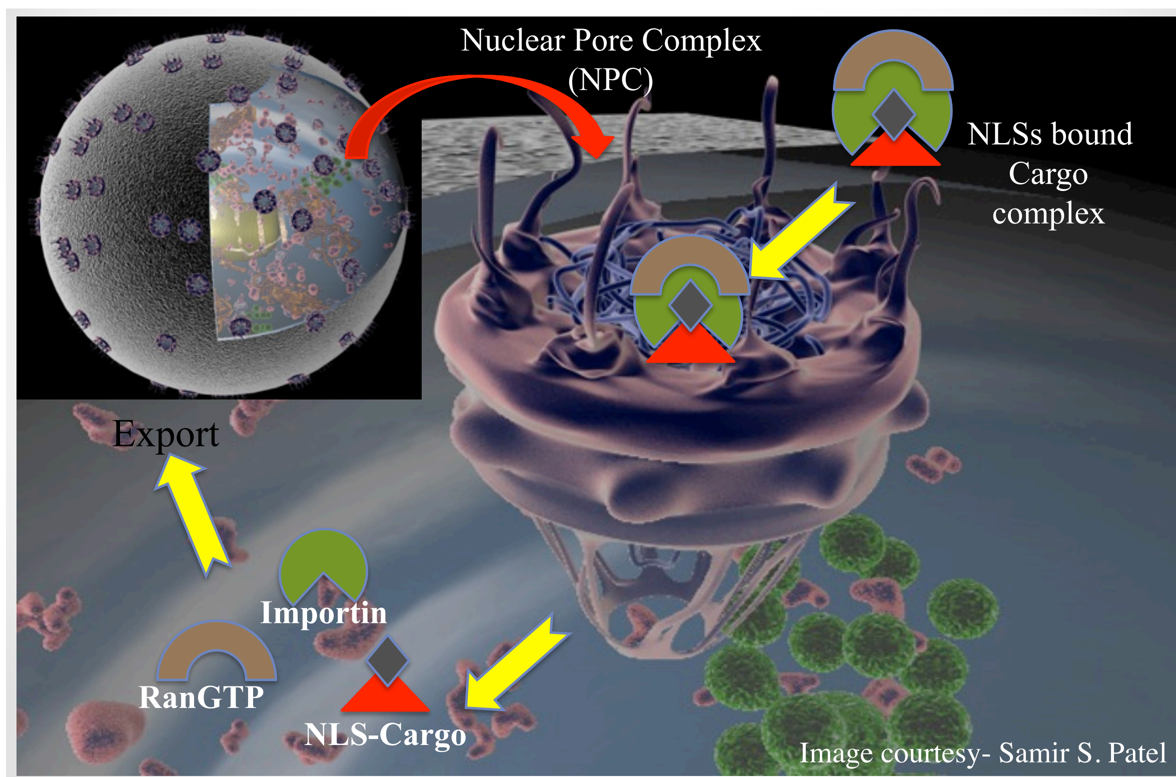

Figure 1-8. General scheme of classical NLSs mediated nuclear import.

The drug delivery can be divided into viral and nonviral. Viral drug delivery method is the most widely used method because viruses such as adenovirus and retrovirus have excellent ability to enter and express their gene in host cell. However, the major drawback of this system is immunogenicity. Non-viral gene delivery system includes use of receptor and cationic liposomes. Liposomes form complexes with DNA through charge interactions and mediate encapsulated DNA uptake through cell membrane to cytoplasm. Several cationic liposomes such as lipofectin, lipofectamine and DOTAP have been used for drug delivery but one disadvantage of nonviral delivery is its limited stability in serum. To achieve high transfection efficiencies it is important not only to cross the cell membrane but also once into the cytoplasm DNA

must get access to nucleus through nuclear pore complex for transcription and gene expression.

1.5.2 Use of NLSs pathway for nuclear import of gene delivery vectors

Nuclear localization signal pathway has been extensively studied (Table 1-4). The most widely used and studied pathway for macromolecules (protein, nucleic acids, small molecules) import appears to be mediated by NLSs. It has been hypothesized since many years that such peptide sequences if incorporated into synthetic gene delivery method could result in increased gene expression through an improvement in the delivery of gene into nucleus. With help of covalent linkers these NLSs sequences has been coupled with variety of proteins such as BSA and ferritin and with large molecules such as IgM (61,62). Although hypothetically it was assumed that NLSs coupling will increase gene expression for sure but practically the results are not very impressive as it is not very clear how best to incorporate the NLSs peptides into gene delivery system. There are mainly two methods of NLSs coupling to DNA, Non-covalent association of DNA with NLS sequence bearing synthetic polymers (Table 1.5) and Linkage of NLS sequence directly to DNA (Association of DNA with virus-derived proteins association of DNA with nuclear proteins, HMG box protein, Transcription factors, Histones).

Table 1-4.

Summery of reports of NLSs mediated gene delivery attempts

NLSs	**DNA**	**Attachment of NLSs to DNA**	**Transfection method/ reporter gene**	**NLS-specific increased transgene expression**	**Conclusion**	**Ref**
NLSs SV-40	Linear 900 bp	Streptavidin-biotinylated DNA	Microinjection/GFP	Yes	NLSs increases efficiency of 900 bp linear DNA	63

NLS-SV-40	Linear 3.4.kbp pDNA	oligonucleotide-NLSs-cap	EXGen500/luciferase	Yes	Covalently bound NLSs increases transfection efficiency of linear DNA	64
NLS^T	pDNA	Pso-ODN triple helix	Cationic lipid/β-galactosidase	ND	No effect of NLS on transfection	65
NLS^T/ mNLS^T	pDNA	Lissamine	Cationic lipid/β-galactosidase	No	No effect of NLS on transfection	66
NLS^T/ mNLS^T	pDNA	CPI	Lipofectin or microinjection/ luciferase	No	No effect of NLS on transfection	67
NLS^T/ revNLS^T	pDNA	PNA	25 kDa PEI/ β-galactosidase and luciferase	No	No effect of NLS on transfection	68
P101	pDNA	110 kDa PLL	HGM fusion protein/ β-galactosidase	~2 fold	Slight effect	69
M9	pDNA	Scrambled NLS^T	Lipofectamine/ β-galactosidase	~10 fold	Non covalent M9 enhances transfection efficiency	70
NLS^T/ revNLS^T	pDNA	Direct binding to DNA	Microinjection	Yes	Microinjected NLS/DNA	71

					complexes lead to transfection	
NLS[T]	pDNA	Direct binding to DNA	Transfection with DNA/peptide	ND	Sequence specific effect of NLS on transfection was not demonstrate d	72

Table 1-5

Other attempts to target nucleus for active drug targeting

Ligand	Attachment of ligands	Expression result	Ref
TAT	PEG-based vector with DNA binding peptide	Up to 15-fold increase in CHO cell transfection	75
NLS-1,2,3	Inclusion of NLS peptides in lipofectamine LPs for transfection into human and rat cells	Roughly two, four and six fold enhancement in luciferase gene expression	76
I-NLS	Iodinated NLS was complexed with pDNA and PEI luciferase transfection was assessed in MCF-7 breast cancer cells	130-fold improvement in transfection compared to absence of NLS. Iodination improved nuclear localization	77
Triamcinolone acetonide (TA)	TA was conjugated to PEI and nuclear localization determined	Low molecular weight PEI/TA efficiently targeted the nucleus	78
Dexamethasone	PEI with plasmid	10-100 fold increase in	79

	luciferase	transfection efficiency	
Trans-cyclohexane-1, 2-diol	Amphipathic alcohol collapses nuclear pore cores allowing macromolecules uptake	Improved lipofectamine 2000 mediated gene transfection to 293T cells *in vitro,* but not *in vivo*.	80
Antibodies ScFv short fragment of mAb	Conjugation with C-terminal p53 peptide	Transfection via electroporation, ScFv-p53 peptides penetrated cells and localized into the nucleus.	81

1.6 Aptamers

Nucleic acid aptamers are small single stranded, well structured RNAs or DNAs generated from large random pool of oligonucleotide library by several rounds of selection, that binds specifically and tightly with their target adapting three-dimensional (3-D) structure (82). Among several advantages over antibodies some major advantage of nucleic acid aptamers are that they are more stable in serum, can be chemically modified, production is cost effective, are non toxic and elicit no immunogenicity, 10-20 times small in size than antibodies that make them have exciting tissue penetration properties. RNA aptamers with 2'-F or 2'-amino pyrimidines are 1000-fold more stable and nuclease resistant (83). Two groups Ellington, A.D., Szostak, J.W. and Tuerk, C. and Gold, L. et al. (1990) published the very first reported independently using the term 'SELEX' for their process of selecting RNA ligands against T4 DNA polymerase; and the Szostak lab, coining the term *in vitro selection*, selecting RNA ligands against various organic dyes. The Szostak lab also coined the term aptamer (from the Latin, *apto*, meaning 'to fit') for these nucleic acid-based ligands (84,85). Two years later, the Szostak lab and Gilead Sciences, independent of one another, used *in vitro selection* schemes to evolve single stranded DNA ligands for organic dyes and human coagulant, thrombin, respectively. There does not appear to be any systematic differences between RNA and DNA aptamers, save the greater intrinsic chemical stability of DNA. Non-modified aptamers are cleared rapidly from the bloodstream, with a half-life of minutes to hours, mainly due to nuclease degradation and clearance from the body by the

kidneys, a result of the aptamer's inherently low molecular weight. Unmodified aptamer applications currently focus on treating transient conditions such as blood clotting, or treating organs such as the eye where local delivery is possible. This rapid clearance can be an advantage in applications such as *in vivo* diagnostic imaging. An example is a tenascin-binding aptamer under development by Schering AG for cancer imaging. Several modifications, such as 2'-fluorine-substituted pyrimidines, polyethylene glycol (PEG) linkage, etc. (both of which are used in Macugen, an FDA-approved aptamer) are available to scientists with which to increase the serum half-life of aptamers easily to the day or even week time scale.

Chemically stable aptamers also known as chemical antibodies possessing very high affinity for protein or non-protein target are generated from large complex combinatorial libraries (86). Pegaptanib sodium is an anti-VEGF RNA aptamer, marketed as Macugen (Pfizer and Eyetech) is used as medicine for treatment of age-related macular degradation. This aptamer got FDA approval in 2004 and is considered as biggest milestone in clinical therapy and nucleic acid aptamer research (87). In addition, the company NeoVentures Biotechnology Inc. (http://www.neoventures.ca) has successfully commercialized the first aptamer based diagnostic platform for analysis of mycotoxins in grain. Many contract companies develop aptamers and aptabodies to replace antibodies in research, diagnostic platforms, drug discovery, and therapeutics.

1.6.1 Advantages of aptamers over antibodies

There are several advantages of aptamers over antibodies (Table 1-6); during the last two decades since aptamers were first selected through SELEX and so in researchers prefer using aptamers in drug delivery system. Aptamers possess several advantages over other ligands typically used in drug delivery such as antibodies (Table 1-4). First, production of aptamers does not rely on biological systems hence is much easier to scale up with low batch-to-batch variability; Second, aptamers are quite thermally stable and can be denatured and renatured multiple times without significant loss of activity (86). Third, the smaller size of aptamers than intact antibodies (~150 kDa) can lead to better tissue penetration in solid tumors; Fourth, lack of immunogenicity is another favorable advantage of aptamers over antibodies; Lastly, conjugation chemistry for the attachment of various imaging labels or functional groups to

aptamers are orthogonal to nucleic acid chemistry, hence they can be readily introduced during aptamer synthesis. On the other hand, the disadvantages of aptamers include faster excretion than antibodies due to smaller size, potentially weaker binding to targets than antibodies, unpredictable toxicity and other systemic properties, susceptibility to serum degradation when unmodified aptamers are used, and intellectual property-related issues (81-85).

Table 1-6

Merits of aptamers over antibodies.

Antibodies	Aptamers
Expensive and tedious production	Aptamers production is less laborious and production cost is much less.
Limitation of target	Toxins as well as molecules that do not evoke immune response can be used to generate high affinity aptamers.
Antibody-Antigen interactions cannot be changed on demand.	Kinetic parameters such as on/off rates changed on demand.
Limited shelf life and are sensitive to temperature and may undergo denaturation.	Denatured aptamers can be regenerated, stable to long term storage
Antibodies often suffer from batch to batch.	Aptamers are produced by chemical synthesis resulting in little or no batch-to-batch variation.
Requires the use of animals	Aptamers are identified through an in vitro process not requiring animals.
Labeling of antibodies can cause loss in affinity.	Reporter molecules can be attached to aptamers at locations not involved in binding.

1.6.2 RNA aptamers as a new artificial ligand: Despite of few successful reports of nuclear-targeted drug delivery using NLSs and NLSs modified nanocarriers the numbers of failures are bigger and there was a need of development of new ligand

that can enhance nuclear import. With successful and increasing use of nucleic acid aptamers in various fields of scientific research, RNA aptamer attracted us to use them as new artificial ligand for drug delivery system.

Chapter 2

Identification of CA-1 specific RNA aptamer using Protein-SELEX

2.1 Introduction

Carbonic anhydrases (CA) are family of widespread metallo-enzymes containing zinc ion consisting active site (88-90). Main functions of these enzymes in all life kingdoms are to interconvert carbon dioxide and bicarbonate to maintain acid-base balance in animal body (88,89). In higher vertebrates, 14 different CA isozymes have been described. Carbonic anhydrases according to their subcellular location are divided into 4 categories. CA-I, CA-II, CA-III, CA-VII and CA-XIII are cytosolic, CA-VA, CA-VB are present in mitochondria throughout the body, CA-VI is secreted in saliva and CA-IV, CA-IX, CA XII and CA XIV are membrane associated carbonic anhydrases (90,91). Two major CAs (CA-I and CA-II) are present in cytosol of erythrocytes with very high concentration. CA-I is one of the most abundant protein which molecular weight is 29 kDa in mammalian red cells. The physiological functions of CAs have been investigated since long and still much to be learned about them. CA-I is mainly involved in process of respiration and regulation of acid and base homeostasis (92). Carbonic Anhydrase-I is expressed in different organs such as in alpha cells of endocrine Langerhans islets, corneal epithelium, lens, ciliary body epithelium, sweat glands, adipose tissue, myoepithelial cells and in GI (gastrointestinal canal) tract. CA-I is five to six times more abundant as CA-II in erythrocytes and strongly expressed in colon. Physiological role of CA-I is still a mystery but CA-I is essential for survival if CA-II is absent (88,89, 93). However, Chang group recently reported that CA-I may play an important role in bio-mineralization and new bone formation as their *in vitro* assays demonstrate that CA-I promotes calcium precipitation in Ankylosing Spondolitis (AS) disease which is characterized by abnormal bone formation in the spine and sacroiliac joints (94). In another report it is found that CA-I may have great advantages in diagnosis of prostate cancer as serum prostate-specific antigen (PSA) level is checked in patients of prostate cancer, where in investigation it was found that level of CA-1 was highly increased in diseased patients in comparison of healthy ones, CA-I acts as the

biomarker for prostate cancer, in absence of early diagnostic signals of this disease increased level of (95). CA-I has role of acid-base balance in colon epithelium, according to another report in case of Ulcerative Colitis (UC) alteration in the expression of CA-I is found that shows some possible role of CA-I in UC (96). Aliphatic sulfamates and derivatives have been found to act as inhibitors of CA and especially for CA-I and CA-II (97). Many acetazolamide (AZA), ethoxzolamide (EZA) and sulfonamide (SA) inhibitors showed high affinity for many major carbonic anhydrase isozymes such as CA-II, CA-IV, and CA-V. CA-II possesses higher affinity towards sulfonamide inhibitors (CAIs) than CA-I. Some salicylic acid derivatives such as ellagic acid, caffeic acid, quercetin, gallic acid, ferulic acid and natural phenolic compounds such as resveratrol, catechin, silymarin, dobutamin and curcumin have shown weak inhibitory effect in mM range on CA-I (98,99).

2.2 Materials and Methods

2.2.1 Library Preparation

99mer ssDNA template was purchased from Sigma-Genosys and 80 nucleotide ssRNA was *in vitro* transcribed with Durascribe T7 *in vitro* transcription kit (Epicentre Biotechnologies) using it as template. RNA pool consists 20 nucleotide forward and reverse primer fixed regions at 3' and 5' ends with 40 nucleotide central random sequence.

Primer containing 5' and 3' template regions, T7 transcription promoter region is underlined- 5'-AGCTCAATTC<u>TAATACGACTCATAGGG</u>AGGACAATCAGA-N40-CAGTCTCGTCGCTGAGGACAAGAGA-3'

2.2.2 RNA preparation via *in vitro* transcription

Durascribe T7 *in vitro* transcription kit (Epicentre Biotechnologies) was used to transcribe RNA pool from the double stranded DNA template. (2 μl of water, 4 μl of 100 bp DNA template (1.0 μg), 2 μl of 10X Reaction Buffer, 2 μl each of 10 mM ATP, GTP, 2'-F-dCTP, 2'-F-dUTP, 2 μl 100 mM DTT, 2 μl of Durascribe T7 enzyme mix was incubated for 6 hours at 37°C. 1 μl (1 unit) of DNase I was added and further incubated for 30 minutes. For RNA precipitation equal volume of phenol-$CHCl_3$-isoamyl (25: 24: 1) (100 μl), to *in vitro* transcription product was added and

mixed well by vortexing, spun at room temperature for 2 minutes, aqueous layer was collected and equal volume of chloroform was mixed well by vortexing and spun at room temperature for 2 minutes. Aqueous layer was collected and added 0.1 volume of 3 M NaOAc, 1.0 µl glycogen (5 mg/ml) and 2.5 volume of ethanol. This mixture was incubated at -20 °C overnight to precipitate RNA. Resulting solution was centrifuged at 15000 ×g for 30 minutes under 4 °C. RNA pellet was washed with 1 ml of 70% ethanol and spun again with same condition, pellet was allowed to dry at room temperature and was dissolved with DEPC treated water. RNA was purified using NAP-5 column (GE healthcare) and concentrated with amicon-filter (MWCO 3000).

2.2.3 CA I selection with syringe filter method

1.0 nmol library in first selection and 500 pmol of RNA in subsequent selections was denatured in filter sterilized 1X selection buffer (50 mM Tris-HCl (pH=7.5), 5 mM KCl, 100 mM NaCl, 1 mM $MgCl_2$ and 0.1% sodium azide). For positive selection, lyophilized carbonic anhydrase I protein from human erythrocytes (Sigma-Aldrich, MO) was dissolved in 1 ml of water to make 1 mg/ml protein stock. DISMIC mixed cellulose acetate ester syringe filter (0.20 μm, Advantech, ROC) was used for positive selection. Nitrocellulose disc filter units (0.45 μm, Millipore) were used for negative selection. Washing was done with 5 ml of wash buffer (2 mM HEPES-NaOH (pH = 7.5), 3 mM $MgCl_{2,}$ 100 mM NaCl) and elution using 3 ml of elution buffer (400 mM NaOAc, 5 mM EDTA, 7 M Urea) was performed. RNA was precipitated as mentioned above.

2.2.4 Amplification of recovered library

Reverse transcriptional PCR (RT-PCR) was performed with QIAGEN one step RT-PCR kit and followed their procedure. 34.8 µl of water, 10 µl of (50 µM) 5X RT-PCR buffer, 2 µl of 10 mM dNTPs, 0.6 µl (50 µM) of reverse primer, 0.6 µl (50 µM) of forward primer, 1 µl of recovered RNA library, 2.0 µl of RT-PCR enzyme mix were mixed and applied for PCR reaction. The sequence of forward primer was TCATAGGGAGGACAATCAGA and reverse primer was TGTACTCAGCGACGAGACTG. PCR conditions- denaturation: 94 °C, 30 sec, primer annealing: 60 °C, 30 sec, extension: 72 °C, 1 min. At first, suitable cycle

number was investigated to prevent the amplification of undesired bands. 33 nmol of 80 bp of DNA was recovered through phenol-chloroform extraction and ethanol precipitation and applied for next PCR. 80bp dsDNA was amplified with PCR to prepare 100 bp dsDNA. The sequence of forward primer for 2nd PCR was AGCTCAATTCTAATACGACTCATAGGGAGGACAATCAGA and reverse primer was same as RT-PCR. Second PCR- 40.5 µl of water, 5 µl of 10 x reaction buffer with $MgCl_2$, 1 µl of 10 mM dNTP, 1 µl of (50 µM) reverse primer, 1 µl of (50 µM) forward primer, 1 µl of template cDNA, 0.5 µl of *Taq* polymerase. PCR conditions- Denaturation: 94°C, 30 sec, Primer annealing: 60 °C, 30 sec, Extension: 72 °C, 1 min. Suitable cycle number was investigated to prevent the amplification of undesired bands. 3.8% agarose gel was prepared and 1/10 fraction of PCR product was mixed with 1 µl of 6x loading buffer and loaded along with 20 bp ladder. Resolved for 40 min at 100V, gel was observed under UV-Vis spectrophotometer after staining in ethidium bromide solution (1 µg/ml) for 15 minutes.

2.2.5 Gel Shift Assay

FAM labeled RNA aptamer was heated at 80 °C for 10 min and set on room temperature. Suitable amount of CA I protein and RNA aptamer were incubated at 37 °C for 30 minutes. These mixtures were resolved in a 1.5% agarose gel in 1X TBE buffer 100 V for 40 minutes. The gel was then exposed to ImageQuant LAS4000 and estimated band intensities with ImageJ software.

2.3 Results

2.3.1 Selection led to enrichment of CA I specific RNA library

We have developed protein-SELEX to select RNA aptamers specific for CA I protein (Figure 2.1). A random RNA library containing 2'-F pyrimidine modification was first incubated with nitrocellulose disc filters to remove nonspecific binding species as the negative selection in our SELEX procedure. The unbound RNA was recovered and subsequently incubated with target protein for 30 minutes. Ratio of protein (CA I) and RNA pool was mixed equimolar amount in each round of SELEX. RNA-protein complexes were loaded on nitrocellulose filter to immobilize protein. After washing to remove unbound or weakly bound RNA, the bound RNA-protein complex were denatured by urea solution and collected. To check the enrichment after every

selection rounds, amounts of bound RNA were quantified with UV absorbance and binding amounts were estimated (data not shown). After 4 rounds of selection, 48% library was found to bind CA I, while filter binding species were only 2.0%. It meant that the RNA, which could bind to CA I, was amplified. Therefore the sequences of this library were investigated with TA-cloning and there were no conserved sequences. We considered that non-specific electrostatically binding species were contained, so we introduced to include counter selection. 5th cycle *in vitro* transcribed RNA was incubated with GST protein (non target), unbound library was recovered and 5th positive selection was performed with CA I. After this selection, 19% of RNA library was recovered as CA I bound RNA. EMSA of 6th cycle RNA pool showed clear band shift with protein CA I (Figure 2.2), while no complex formed with RNA with other protein such as GST and no shift was observed (data not shown).

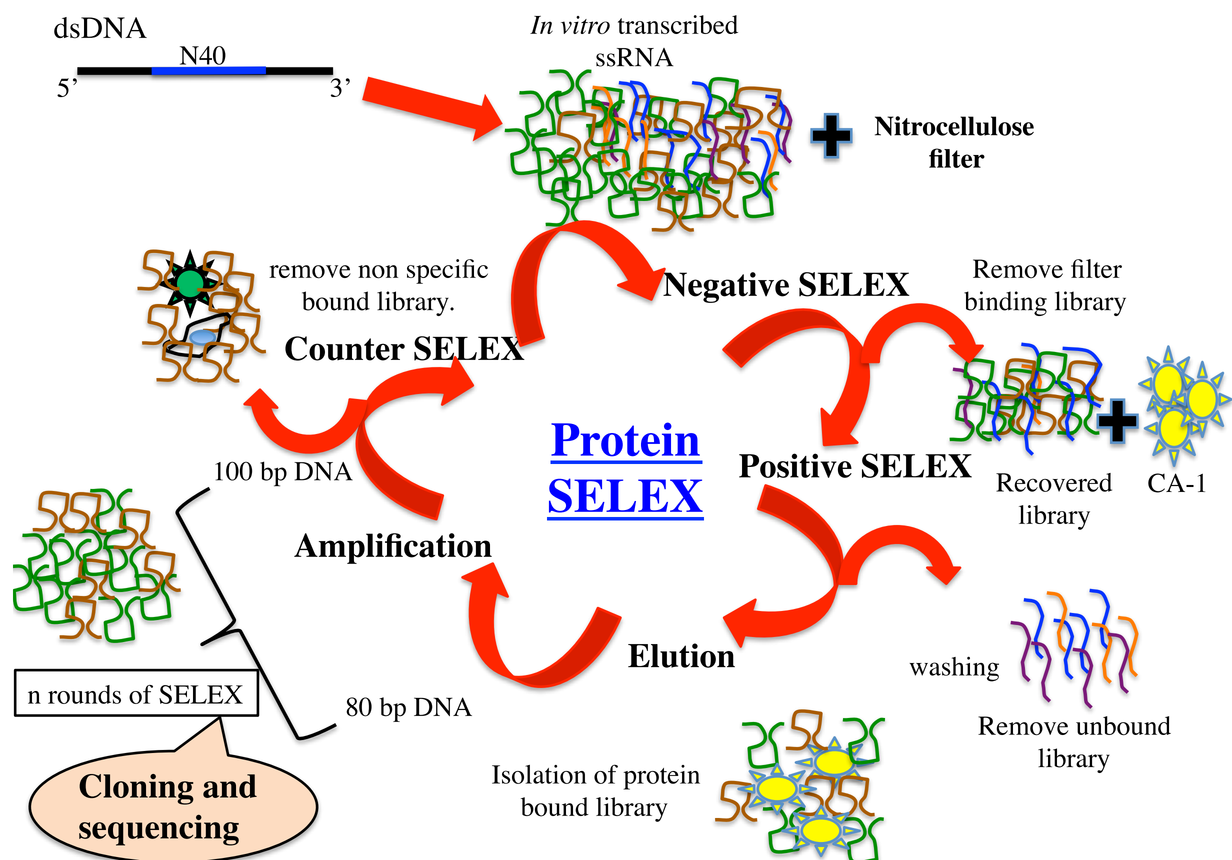

Figure 2-1. Diagrammatic representation of Protein SELEX

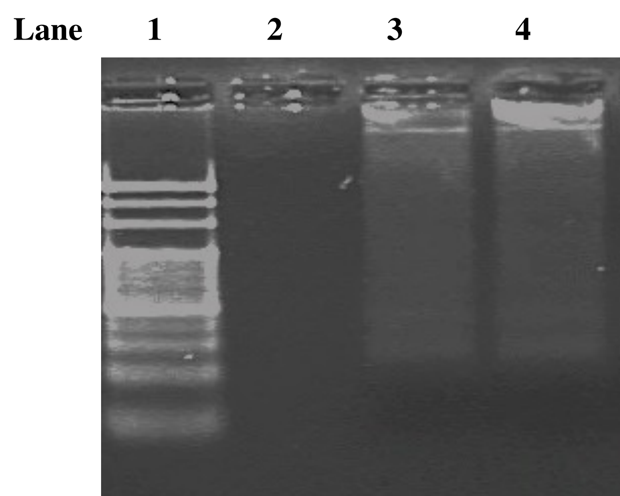

Figure 2-2. EMSA of 6th cycle RNA pool. 6th cycle RNA library pool, in 1X selection buffer with final concentration of 5 μM in 20 μl reaction was heated at 80 °C for 10 minutes and allowed to cool down slowly at room temperature, incubated with CA I protein (lane 3, 4) 10.0, 25.0 μM respectively at room temperature for 30 minutes. The reaction was resolved at 3.8% agarose gel, for 40 minutes, 100 V. For staining gel was dipped in ethidium bromide solution (250 μg/250 ml) for 15 minutes and shift was measured under UV- transilluminater. Lane 1 indicates 20 bp DNA ladder.

2.2.2 Identifying aptamer sequence posses affinity for CA I

After successful RNA library enrichment to CA I, RNA libraries were cloned (TOPO-TA cloning kit, Invitrogen) followed with their procedure (Figure 2.3). and sequenced (Figure 2.4). Homology among whole sequences was analyzed using ClustalW software. Out of 93 readable sequences, 3 distinct sequences were identified (Table 2-1). In order to confirm binding of all 3 obtained distinct sequences, dsDNA from respective pDNA was prepared and *in vitro* transcribed RNA libraries were applied for EMSA. Out of 3 sequences, one sequence referred as CAapt1 showed better binding affinity than others (Figure 2-5 A). On further investigations, we found that the RNA sequence posses no affinity for other proteins such as GST and BSA, also no affinity of 40 nucleotide FAM-labeled random library for CA I protein was observed (Figure 2-5 B, C). Specific complex formation of CA I protein and CAapt1 RNA aptamer confirmed that this single stranded RNA aptamer specific for protein CA I has been produced successfully. Dissociation constant (Kd) of CAapt1 was

calculated as 386 ± 105 nM using gel shift binding assay (100). ImageJ software was used to analyze gel bands. (Figure 2-6)

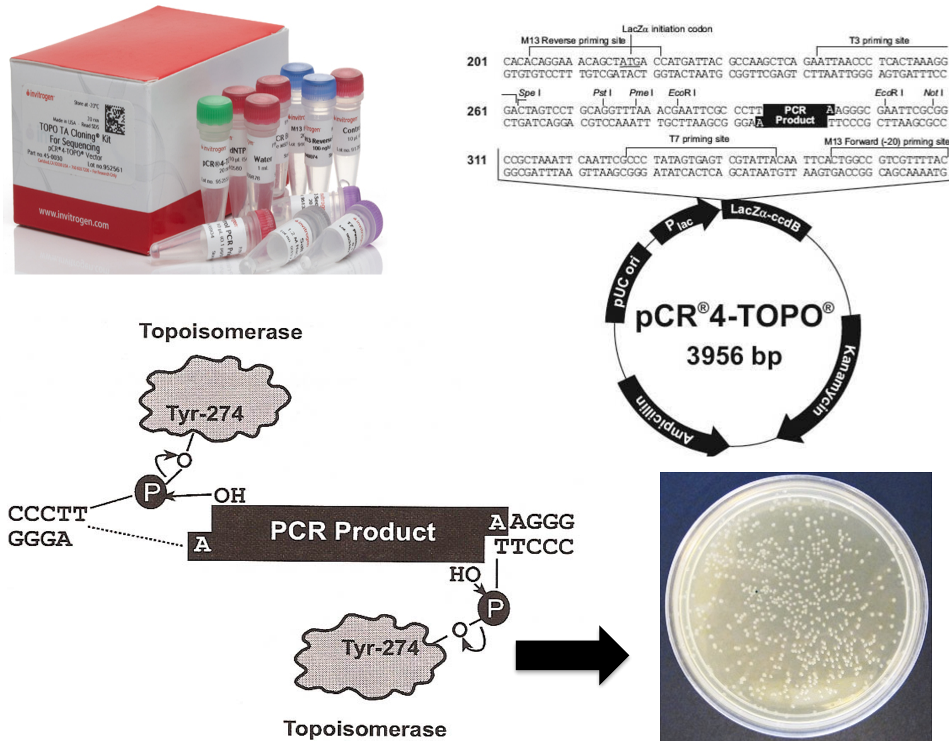

Figure 2. 3 Principle of TA cloning.

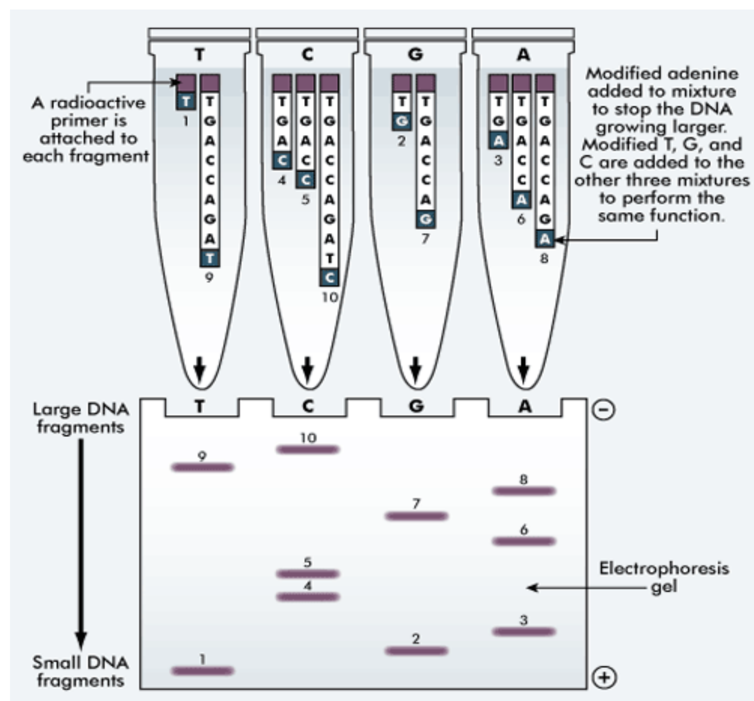

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Figure 2.4 Principle of sequencing. Aptamer sequences were sequenced using both forward and reverse primers. Not readable sequences were simply discarded.

Table 2.1.

Sequences obtained after cloning. Sequences were Multiple Aligned using ClustalW software. Nos. denoted copies of individual sequence in total no of sequences.

Name	Sequence	Frequency
CAapt1	CACGGAACCCACUAAUGAUCACAAAGUGAUACUGCGCCUG	2/97
CAapt2	CCAGACAUGUCGUCGGGGGUUUAUGCUGUUCUCCACCCCG	3/97
CAapt3	GCUCUAAAAUUCGUCUAUCUGCGUCCUGCCUACGUACUUG	3/97

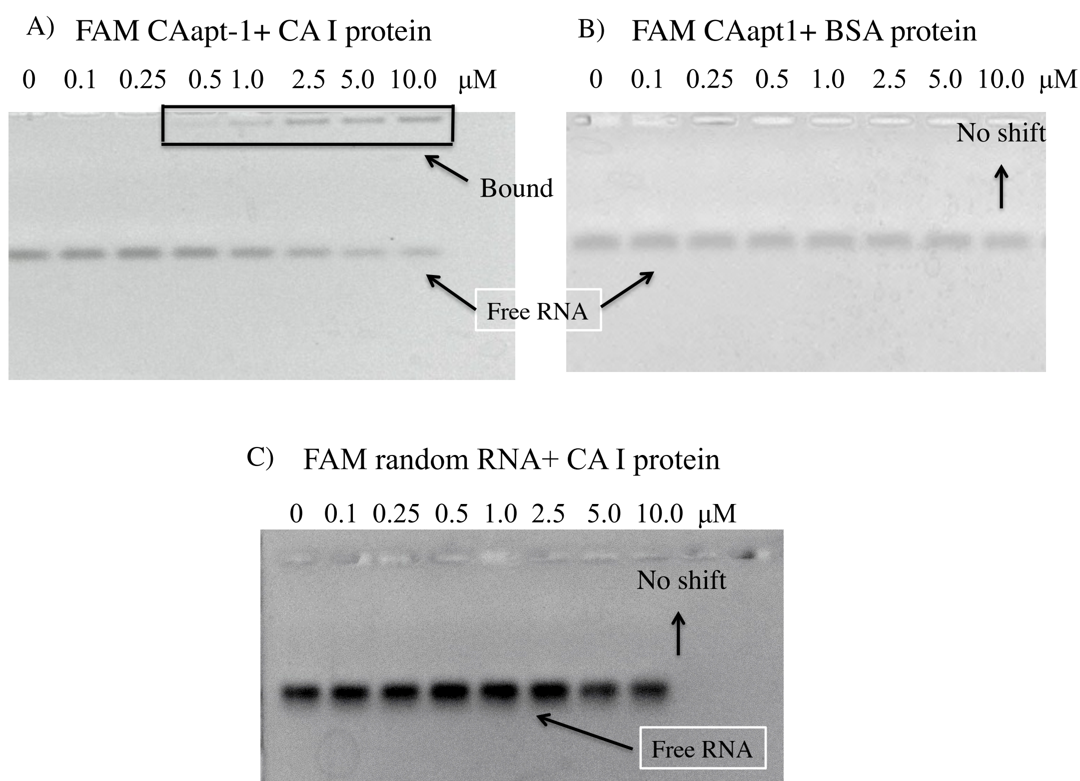

Figure 2-5. Selective binding of CAapt1 to CA I protein. EMSA was investigated with (a) FAM labeled CAapt1 vs CA I protein, (b) FAM tagged CAapt1 vs BSA

protein or (c) FAM tagged 40 nt random sequence (mixture of A, G, C and U) vs CA I protein. RNAs in 1X selection buffer were heated at 80°C for 10 minutes and cooled down slowly at room temperature. The 50 nM concentration of RNAs were incubated with increasing concentration of protein on lane A-H (0, 0.1, 0.25, 0.50, 1.0, 2.5, 5.0, 10.0 μM) in 10.0 μl volume for 30 minutes at room temperature. 2.5 μl of 6X loading dye was added and the binding was resolved at 1.5% agarose gel for 30 minutes at 100 V. The electrophoresis result was observed using ImageQuant LAS 4000. Protein position in gel was confirmed by CBB staining (data not shown).

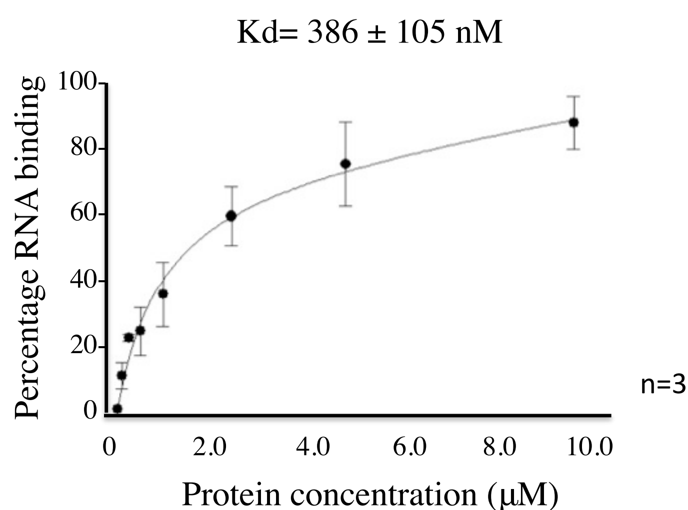

Figure 2-6. Binding Affinity (Kd value) determination of RNA aptamer CAapt1. Gel shift assay method was used to determine Kd value of CAapt1 to protein carbonic anhydrase I. FAM labeled aptamer was incubated with target protein on a scale of 20 μl. RNA aptamer CAapt1 was used at 50 nM with increasing concentrations of protein in 1X selection buffer. CAapt1 was first heated at 80 °C for 10 minutes and then was cooled down at room temperature. RNA and protein were mixed in 1X selection buffer for 30 minutes at room temperature, 4.0 μl of loading dye was added to resulting sample solution (total 20.0 μl). Samples were loaded on 1.5% agarose gel and run for 30 minutes at 100 V. The gel was checked under ImageQuantLAS 4000. The binding was measured by quantifying and calculating value of free RNA aptamer

using ImageJ software. The experiment was repeated three times and error bar representing standard deviation of means.

2.3 Discussion

Carbonic Anhydrase I is the most abundant protein in RBCs. According to reports, recently it has been found that expression of CA I enhanced in various diseases. In the diseased state of a cell, change in level of expression of CA I can be a sign for early diagnosis of various fatal diseases such as prostate cancer. Here, we have reported a RNA aptamer that bound to CA I protein *in vitro*. We have developed a simple method referred to as Protein- SELEX in which filter binding method (modified according to our requirements) was employed (101). Established Protein SELEX method could successfully produced the CA I protein specific RNA aptamers. RNA aptamer library pool after selection when applied on agarose gel it showed sharp shift of CA I bound RNA aptamers. For generating RNA aptamers, we had to make several modifications and most importantly we could improve cloning and sequencing results by introducing negative and counter SELEX during whole selection procedure. It was confirmed that it was very important to perform negative selection in beginning of each SELEX cycle in order to remove filter binding RNA species. The random RNA pool had maximum non-specific binding for nitrocellulose filters in initial selections and this binding might cause electrostatic interaction. Therefore removing nonspecific binding RNA species contributed to enrich the specific aptamer toward CA I protein. After 5th cycle of selection, we have tried sequencing and found no conserved sequences even the binding amount of RNA library to CA I protein was 48%. This meant that there were still large number of RNAs were remained which bind to CA I protein with electrostatic, hydrogen bonding or hydrophobic interaction. To get better aptamers, we had to remove non specific sequences and so we have introduced counter SELEX with GST protein. GST protein has almost same molecular weight with CA I protein (The molecular weight of GST is 26 kDa and CA I is 29 kDa) and also has almost same pI value (GST = 5.9 and CA I = 6.6) [102]. Therefore, these proteins can be said to have same kinds of physical properties therefore GST is the good candidate for the counter selection for CA I. After we have completed counter selection, the bound RNA amount was reduced to 19% compared

to starting RNAs. This result suggested that nonspecifically bound RNAs were removed. After we have applied this enriched library to sequencing, it was found that 12% of whole sequences were conserved after counter selection while 0% of sequences were conserved before counter selection. During the selection, it was difficult to add any other protein to remove non-specific binding because it could result in generation of RNA library that has binding affinity for other proteins (non target) added during selection. In case of addition of several proteins during selection, RNA library itself can not identify between target and non-target protein. So we did not add any other protein during selection and so it was must to perform counter selection after few rounds of selection. Counter selection as per our experience should be performed after at least 2 or 3 rounds of selection because complexity of library may decrease drastically if counter selection added in very initial cycles. Also we found that it was essential to monitor increasing or decreasing percentage of filter binding RNA library. It must decrease constantly after initial selections and completely remove after few selections finally. In initial cycles, if it could not be removed efficiently it may result in failure of selection. After six selection cycles we could successfully remove non-specific as well as filter binding species and could generate RNA aptamer CAapt1 specific for Carbonic Anhydrase I. EMSA was employed to check RNA aptamer library and after sequencing selected individual aptamer binding to protein CA I. It was found that on agarose gel protein-RNA complex could not move at all and so the band always appeared close to agarose gel well. It was confirmed by locating the position of protein in agarose gel by Commassie Brilliant Blue (CBB) staining. Free RNA in gel is also a significant parameter to confirm percentage binding of RNA library to protein. Same concentration of BSA protein was used to check non-specific binding of CAapt1 aptamer but no shift could be observed on gel, also, when random FAM-40 nucleotide RNA library was applied over agarose gel with CA I protein, there was no shift. It confirmed the specificity of CAapt1 RNA aptamer for CA I protein. We have estimated the dissociation constant from EMSA with FAM-tagged RNA and CA I protein and the Kd value was 386 nM. Inhibitor constant of selected sulphonamides/ sulphamates/ sulphamites inhibitors to CA I ranges between 21 nM to 54000 nM (102). Phenolic acids are also the CA I inhibitors and its inhibitor constant value range is between 99-1061 μM. Topiramate is one of the carbonic anhydrase inhibitor

approved by FDA as antiepileptic drugs for the treatment of epilepsy. But sulfonamides and their derivatives use in systemic therapy causes significant side effects (103,104). From these results, in presence of very limited strong inhibitors for CA I enzyme, CA I aptamer is the candidate for better CA I inhibitor because this aptamer can specifically bind to CA I protein, it might not show side effect because the aptamer doesn't bind to other proteins.

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Chapter 3

Identification of Nup358 specific RNA aptamers

3.1 Introduction

Intracellular targeted nano drug delivery has gained major attention in last few years to overcome toxicity and increase therapeutic effects. Many drug carriers composed of metal, oxides, semiconductors and polymers are in practice to achieve successful delivery but the major concern is access of the nanoparticles to the nucleus. The nucleus, a highly specialized main controlling device of a cell, contains genetic material that is essential for cell survival. The main role of the nucleus is to contain and preserve hereditary material in an undamaged and intact state and coordinate various cell activities with other cell organelles, such as cell division, growth and protein synthesis. The nuclear envelope contains perforations, which are referred to as nuclear pores, and participate in the import and export of macromolecules between the nucleus and cytoplasm. As the nucleus contains genetic material, in a diseased state it is essential for drugs to exert their action inside the nucleus in order to achieve a complete cure. Active targeting or receptor based targeting of nanoparticles to different organelles in a cell has been successfully achieved in an attempt to attain the cellular uptake of delivered therapeutic drugs and to reduce toxicity. Most of the present nano drug delivery systems target the cytoplasm of cancer cells and not nuclei, because of the many biobarriers associated with the nucleus (1). Therefore, a new active targeting system for the nucleus would be expected to provide site specific drug delivery and gene therapy. Nuclear Pore Complex (NPC) is a big complex of 125 MDa, acts as barrier for macromolecules such as nucleic acids and various proteins restricting their bidirectional traffic. NPC opening is considered to be ~39 nm in diameter and is composed of various copies of ~30 different proteins called as nucleoporins. (1-4). Nucleoporin-358 also known as RanBP2 is main and unique constituent of cytoplasmic filaments ~50-100 nm long of higher eukaryotic nucleus. Largest known nucleoporins, Nup358 of 3324 residues (in humans) interacts with karyopherins, transport receptors including Ran, SUMOylated RanGAP1, the NxF1-p15 mRNA export heterodimer and exportin CRM-1 and hence mediate in nuclear

import and export. First report on exact location of RanBP2/Nup358 was published by Jian Wu *et al* (1995). Nucleoporin358 (Nup358) consists of several distinct regions- ~830 residue α-helical N Terminal Domain (NTD) followed by four Ran binding sites, 8 Cys2-Cys2 type zinc finger motifs, an SUMO E3 ligase domain, phynyl-glycine (FG) repeats characteristic of nucleoporins and a C-terminal (CTD) cyclophilin A homology domain (6) (Figure 3-1). Apart from essential role in nuclear transport Nup358 has others important functions too. Up regulated expression of Nup358 was found in multiple myeloma tumerogenesis that is an incurable malignant neoplasm and mutation in Nup358 gene is related to inflammatory myofibroblastic tumor and acute necrotizing encephalopathy. (7-9). HIV-1 capsid (CA) binds directly to the cyclophilin domain of Nup358/RanBP2, C-terminus of Nup358/RanBP2 comprising a cyclophilin-homology domain mediates docking of HIV-1 cores on NPC cytoplasmic filaments (10).

Polypeptide chain of Nup358 contains 3324 residues-

An N-terminal domain (NTD) (red)

~830-residue alpha helical, leucine rich region (yellow)
Functions in protein protein interaction.

4 RanGTP binding domains (green)

8 consecutive zinc finger domains (blue)
Functions in protein: protein, protein RNA or protein DNA interaction in zinc dependent fashion.

A C-terminal cyclophilin A homology domain.(navy blue) associated with viral entry inside nucleus

Figure 3-1. Sequence detail of N-terminal domain of Nucleoporin358

Aptamers are nucleic acids or peptide ligands that bind to various targets by adapting

a 3-D structure with a very high affinity and specificity. Unlike antibodies, they are non-immunogenic, are smaller in size, and can be produced easily and cheaply (81). The SELEX method for selecting an aptamer from a random library pool by an iterative *in vitro* selection procedure was first reported by two labs independently in 1990. (83,84)**.** In case of nuclear drug delivery system apart from NLS there is no ligand available, NLS being a natural ligand has been extensively used for drug delivery but the success ratio is very limited. So we hypothesized to develop a new artificial ligand. Increasing success of aptamer as ligand attracted us to attempt to prepare nucleic acid aptamer as artificial ligand to target the nucleus. We hypothesized that an RNA aptamer that binds to the nucleus could act as a ligand for MEND-mediated active drug targeting (liposomal drug delivery).

3.2 Materials and Methods

3.2.1 Library Preparation

99mer ssDNA template was purchased from Sigma-Genosys and 80 nucleotide ssRNA was *in vitro* transcribed with Durascribe T7 *in vitro* transcription kit (Epicentre Biotechnologies) using it as template. RNA pool consists 20 nucleotide forward and reverse primer fixed regions at 3’ and 5’ ends with 40 nucleotide central random sequence.

Primer containing 5’ and 3’ template regions, T7 transcription promoter region is underlined- 5’-AGCTCAATTC<u>TAATACGACTCATAGGG</u>AGGACAATCAGA-N40-CAGTCTCGTCGCTGAGGACAAGAGA-3’. Durascribe T7 *in vitro* transcription kit (Epicentre Biotechnologies) was used to transcribe RNA pool from the double stranded DNA template. (2 µl of water, 4 µl of 100 bp DNA template (1.0 µg), 2 µl of 10X reaction buffer, 2 µl each of 10 mM ATP, GTP, 2’-F-dCTP, 2’-F-dUTP, 2 µl 100 mM DTT, 2 µl of Durascribe T7 enzyme mix was incubated for 6 hours at 37°C. 1 µl (1 unit) of DNase I was added and further incubated for 30 minutes. For RNA precipitation equal volume of phenol-$CHCl_3$-isoamyl alcohol (25: 24: 1) (100 µl), to *in vitro* transcription product was added and mixed well by vortexing, spun at room temperature for 2 minutes at table top centrifuge. Aqueous layer was collected and equal volume of chloroform was mixed well by vortexing and spun at room temperature for 2 minutes. Aqueous layer was collected and added 0.1 volume of 3 M NaOAc, 1.0 µl glycogen (5 mg/ml) and 2.5 volume of ethanol. This

mixture was incubated at -20 °C overnight to precipitate RNA. Next day, resulting solution was centrifuged at 15000 ×g for 30 minutes under 4 °C. RNA pellet was washed with 1 ml of 70% ethanol and spun again with same condition, pellet was allowed to dry at room temperature and was dissolved with DEPC treated water. RNA was purified using NAP-5 column (GE healthcare) and concentrated with amicon-filter (MWCO 3000).

3.2.2 Protein SELEX method

This method of selection is specially designed to work well with all kinds of recombinant proteins. 1.0 nmol library in first selection and 500 pmol of RNA in subsequent selections was denatured. The 2'F-modified RNA library (1nmol) was first heated in a thermo-block at 80°C for 10 minutes, and then cooled slowly to form secondary structures. Secondary structures are essential to fit well with target protein, so RNA library with secondary structures was then incubated with nitrocellulose filter (0.45 μm, Millipore, moisten in filter sterilized 1X selection buffer (50 mM Tris-HCl (pH=7.5), 5 mM KCl, 100 mM NaCl, 1 mM $MgCl_2$ and 0.1% sodium azide) to remove filter binding RNA species (negative selection). Nup358 is 3324 amino acid proteins, for selection we used 724 amino acids of GST tagged recombinant fusion Nup358 protein (specific sequence 2502-3324 amino acid which is C-terminal of Nup358 or GST-$Nup358_{2501\text{-}3324}$). This protein was provided as a kind gift from Dr. Shigehiro Yoshimura (Kyoto University). Unbound RNA library after negative selection was precipitated and incubated with target protein recombinant Nup358 (62.5 pmol) and 1X SELEX buffer at 4° C for 30 minutes in eppendorf tube (500 µl). DISMIC mixed cellulose acetate ester syringe filter (0.20 μm, Advantech, ROC) was used for positive selection. After incubation protein RNA mixture was loaded over filter unit. Washing was done with 5 ml of wash buffer (2 mM HEPES-NaOH (pH = 7.5), 3 mM $MgCl_{2,}$ 100 mM NaCl) and elution using 3 ml of elution buffer (400 mM NaOAc, 5 mM EDTA, 7 M Urea) was performed. Protein bound RNA was precipitated as mentioned above. Upto 7 rounds of selections, we performed two rounds of counter SELEX to remove non specific binding followed by positive selection. For the first counter selection at cycle no 5th we used same amount of proteins other than target protein such as BSA, CA-1 and GST and in 7th cycle we used cultured HeLa cells. Recovered library from the previous selection cycle was

first subjected to negative selection and then was incubated with non target protein (counter SELEX 1) and with cultured HeLa Cells (counter SELEX 2) at 4°C for 30 minutes. The unbound library was incubated with target protein to complete subsequent positive selection.

3.2.3 Amplification of recovered library

Reverse transcriptional PCR (RT-PCR) was performed with QIAGEN one step RT-PCR kit and followed their procedure. 34.8 µl of water, 10 µl of (50 µM) 5X RT-PCR buffer, 2 µl of 10 mM dNTPs, 0.6 µl (50 µM) of reverse primer, 0.6 µl (50 µM) of forward primer, 1 µl of recovered RNA library, 2.0 µl of RT-PCR enzyme mix were mixed and applied for PCR reaction. The sequence of forward primer was TCATAGGGAGGACAATCAGA and reverse primer was TGTACTCAGCGACGAGACTG. PCR conditions- denaturation: 94 °C, 30 sec,; primer annealing: 60 °C, 30 sec; extension: 72 °C, 1 min. At first, suitable cycle number was investigated to prevent the amplification of undesired bands. 33 nmol of 80 bp of DNA was recovered through phenol-chloroform extraction and ethanol precipitation and applied for next PCR. 80bp dsDNA was amplified with PCR to prepare 100 bp dsDNA. The sequence of forward primer for 2nd PCR was AGCTCAATTCTAATACGACTCATAGGGAGGACAATCAGA and reverse primer was same as RT-PCR. Second PCR- 40.5 µl of water, 5 µl of 10 x reaction buffer with $MgCl_2$, 1 µl of 10 mM dNTP, 1 µl of (50 µM) reverse primer, 1 µl of (50 µM) forward primer, 1 µl of template cDNA, 0.5 µl of *Taq* polymerase. PCR conditions- Denaturation: 94°C, 30 sec, Primer annealing: 60 °C, 30 sec, Extension: 72 °C, 1 min. Suitable cycle number was investigated to prevent the amplification of undesired bands. 3.8% agarose gel was prepared and 1/10 fraction of PCR product was mixed with 1 µl of 6x loading buffer and loaded along with 20 bp ladder. Resolved for 40 min at 100V in 1X TBE, gel was observed under UV-Vis spectrophotometer after staining in ethidium bromide solution (1 µg/ml) for 15 minutes. Conditions for colony PCR to identify transformed colonies were also fixed with finding suitable cycle number and amplified DNA was subjected to agarose gel electrophoresis.

3.2.4 Aptamer binding estimation

To estimate the value of enrichment of binding of the RNA libraries as well as to track constant increment in binding of RNA aptamer and decrease in filter binding species, 1 µl of the selected libraries (filter binding and protein binding separately), dissolved in 1X selection buffer was subjected to nanodrop machine (ND, Thermo Scientific, MA) to get accurate amount of RNA present in solution.

3.2.5 Gel Shift Assay

FAM labeled RNA aptamer was heated at 80 °C for 10 min and set on room temperature to cool down slowly in order to form secondary structures. Suitable amount of recombinant Nup358 protein and RNA aptamer were incubated at 37 °C for 30 minutes. These mixtures were resolved in a 1.5% agarose gel in 1X TBE buffer 100 V for 40 minutes. The gel was then exposed to ImageQuant LAS4000 and band intensities were estimated with ImageJ software. The equilibrium dissociation constant Kd was measured by fitting the dependence of the fluorescence intensity of unbound aptamer on increasing the concentration of the protein to the equation $Y = B\text{max}\, X/(K\text{d}+X)$ using the SigmaPlot 12 (Systat Software Inc., USA).

3.2.6 Cloning and Sequencing

The final and the important step of selection is cloning and sequencing of the transformed colonies in order to identify the possible RNA candidates for binding. RNA libraries of final selection were cloned (TOPO-TA cloning kit, Invitrogen) followed by manufacturer's protocol. To determine the individual sequence sequencing was performed. Homology among whole sequences was analyzed using ClustalW software. Secondary strucuture of RNA sequences were determined using Mfold software.

3.3 Results

3.3.1 Enrichment of RNA aptamer library

We have established a new method referred as protein-SELEX method, steps of which were designed specially to target recombinant proteins in order to produce nucleic acid aptamers. We used this SELEX method to generate RNA aptamers

specific for recombinant Nup358 protein (Figure 3-2). A random RNA library containing 2'-F pyrimidine modification was first heated to form secondary structures and cooled down and then was incubated with nitrocellulose disc filters in 1X selection buffer to remove nonspecific binding RNA species, this process is referred as the negative selection in our SELEX procedure. After about an hour of incubation, the unbound RNA library was recovered and precipitated overnight at -20°C in 100% ethanol containing 3 M NaOaC and glycogen. Next day, to get the RNA pellet the mixture was centrifuged at 15000 xg for 30 minutes at 4°C. Pellet was washed with 1 ml of 100% ethanol and spun again at same condition. Obtained pellet was allowed to dry at room temperature until the ethanol was all evaporated completely. For positive selection the pellet was first heated, cooled down at room temperature and subsequently incubated with target protein for 30 minutes at 4°C. With subsequent selections (after 1st selection) the amount of RNA library was decreased to increase strictness of selection. After 30 minutes incubation on ice RNA-protein complexes were loaded on nitrocellulose syringe filter to immobilize protein over its surface. Strict washing was done 3 times to remove unbound or weakly bound RNA. Now the filter containing only protein bound RNA was treated with elution buffer. The bound RNA-protein complex were denatured by urea containing elution buffer and collected to precipitate RNA. RNA was purified and extracted with phenol chloroform and precipitated with 100% ethanol and 3M sodium acetate. The amounts of protein bound RNA and filter bound RNA were quantified with UV absorbance to check the enrichment after every selection rounds, and binding amounts were estimated (Table 3-1).

Table 3-1.

Library binding (%age library binding) measurement after each selection

Round	Input RNA	Input Protein	% bound to protein	% bound to filter
1	1 nmol	62.5 pmol	1.3%	0.9%
3	100 pmol	62.5 pmol	3.0%	2.0%
4	100 pmol	62.5 pmol	4.9%	2.5%
5	100 poml	62.5 pmol	9.7%	Under detection limit

For successful protein SELEX it was mandatory to remove all filter binding species and so it was important to monitor its constant loss after each selection. After 5 rounds of selection, 9% library was found to bind recombinant Nup358, while filter-binding species could be removed successfully as the amount of filter binding species after 5th selection was under detection limit. It meant that the RNA, which could bind to target protein, was amplified and enriched constantly. After the filter binding RNA was removed successfully, the first counter SELEX was introduced in order to remove any non-specific binding sequences in library. In the first counter selection the library from 5th cycle (100 pmol) was first forced to negative selection and then the unbound RNA library was recovered and incubated 30 minutes on ice with CA I, GST and BSA protein (100 pmol each). After 30 minutes the protein RNA mixture was immobilized over nitrocellulose membrane filter and this unbound RNA was collected by washing. The unbound recovered RNA was used for subsequent regular selection. In 7th (final round) selection, second counter selection was introduced using cultured HeLa cells, after incubation unbound library was recovered and precipitated.

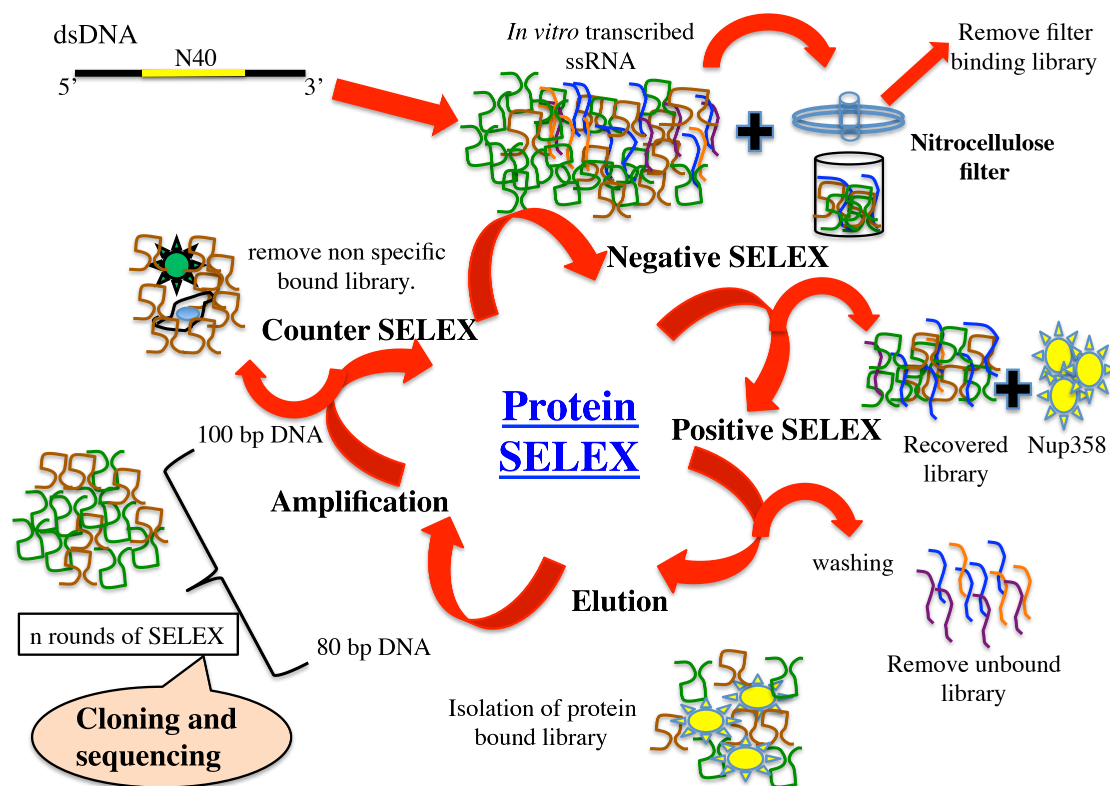
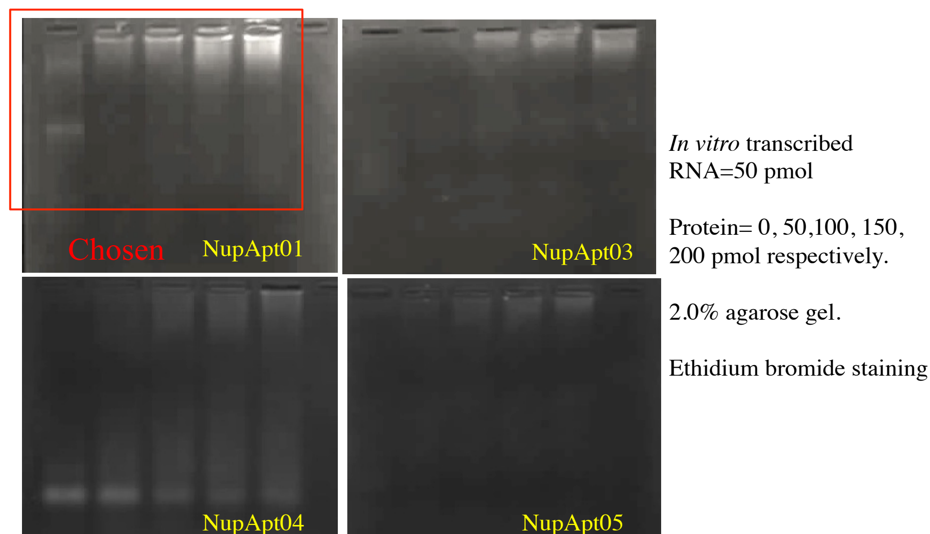


Figure 3-2. Protein SELEX for Nup358

After 7th selection, ~11% of RNA library was recovered as recombinant Nup358 bound RNA. EMSA of 7th cycle RNA pool showed clear band shift with protein Nup358 (data not shown).

3.3.2 Identification of aptamer sequences binding to recombinant-Nup358 (Cloning and sequencing)

RNA library after 7th selection and successful enrichment to Nup358 were cloned using TOPO-TA vector (TOPO-TA cloning kit, Invitrogen) as per user manual, their procedure and sequenced. Transformed colonies were selected using colony PCR and applied for sequencing. Homology among whole sequences was analyzed using ClustalW software and guide tree was prepared (Figure 3-4) Out of 50 readable sequences, 7 distinct sequences were identified in multiple copies (Table 3-2). Secondary structure was predicted using Mfold software (Figure 3-5). In order to confirm binding of all 7 obtained distinct sequences, dsDNA from respective pDNA was prepared and *in vitro* transcribed RNA libraries were applied for EMSA. 6 out of 7 sequences showed binding to Nup358 over agarose gel (Figure 3-3).



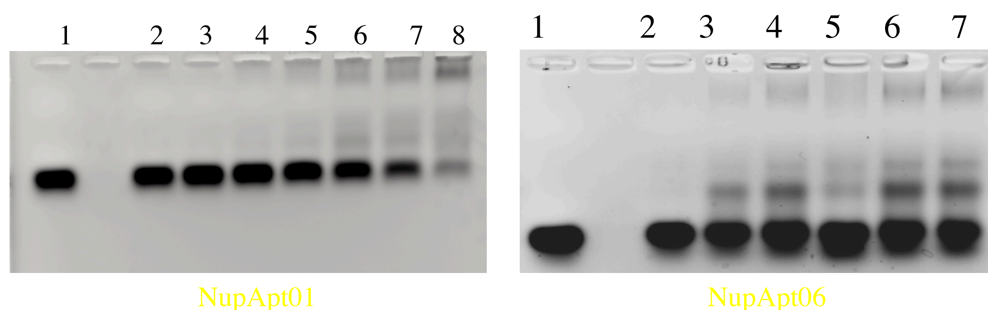


Figure 3-3. Agarose gel electrophoresis of all 6 sequences showed binding. 50 nM aptamer were incubated with increasing amount of protein (0-200 pmol) for 30 minutes on ice. The mixture was then electrophoresed on 2.0% agarose gel in 1 X TBE for 30 minutes at 100 V. The gel was stained with ethidium bromide (except aptamer NupApt01 and NupApt06, they were FAM labeled aptamer and after electrophoresis bands were sighted under ImageQuant machine) for 20 minutes and bands were observed under transilluminator.

Two out 6 were stronger binding aptamers than other sequences referred as NupApt01 and NupApt02 (Figure 3-6 A). On further investigations, we found that the RNA sequence posses no affinity for other proteins such as GST and BSA, also no affinity of 40 nucleotide FAM-labeled random library for recombinant Nup358 protein was observed (Figure 3-6 B, C). Specific complex formation of recombinant Nup358 protein and NupApt01 or NupApt02 RNA aptamer confirmed that these single stranded RNA aptamers, specific for protein Nup358 has been produced successfully. Dissociation constant (Kd) of NupApt02 was calculated as 1.6 μM using gel shift binding assay (ImageJ software was used to analyze gel bands (Figure 3-7).

Table 3-2.

Sequences obtained after cloning. Sequences were Multiple Aligned using ClustalW software. Nos. denoted copies of individual sequence in total no of sequences.

Aptamer	Sequence	Frequency
NupApt01	UGACAGUGGCGGCAGUCACUGAGAAAAACGAAACCGGAGC	6/50
NupApt02	CCACGCAGAUAGACGCUACUCUACUACAUCGCAGCCAAC	3/50
NupApt03	CCGCAGGAAAGUGGGAGUAGUAGCCGUAGUCUUUUAUGGU	3/50
NupApt04	CCGGUAUCUACGCACUAGGUACGCUCAGGUACUACGGAGG	3/50
NupApt05	CACGACCGCUACCCCACAAACUGUAUGCCGCCUGCUCGUA	3/50
NupApt06	CCACAUCACCCCAGGCACCCUACAUCGAGGAAGGAUCCU	3/50
NupApt07	GGGACGGGAGCUGACCAUAGACUAGCAACCCUCGCCACUC	2/50

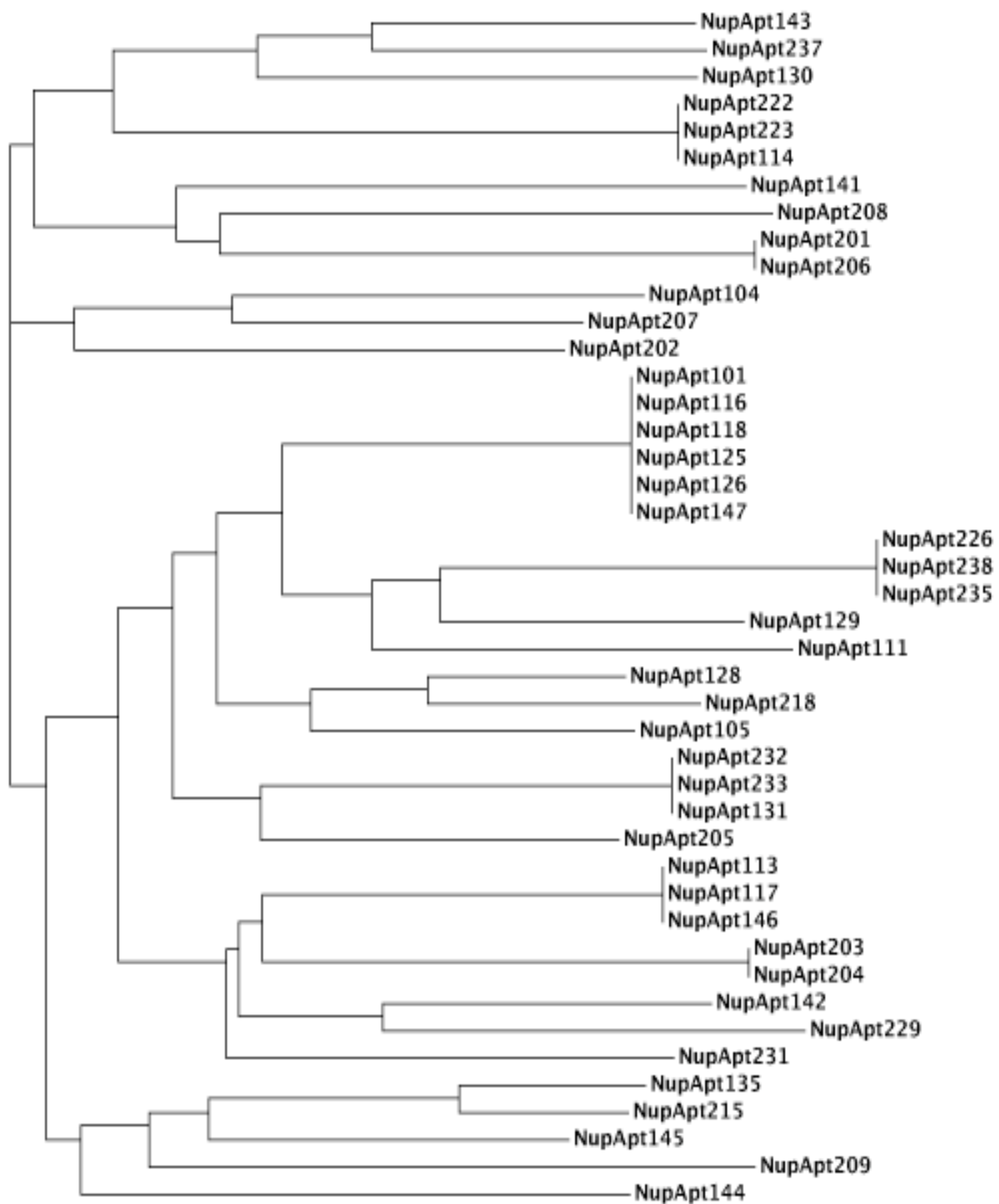
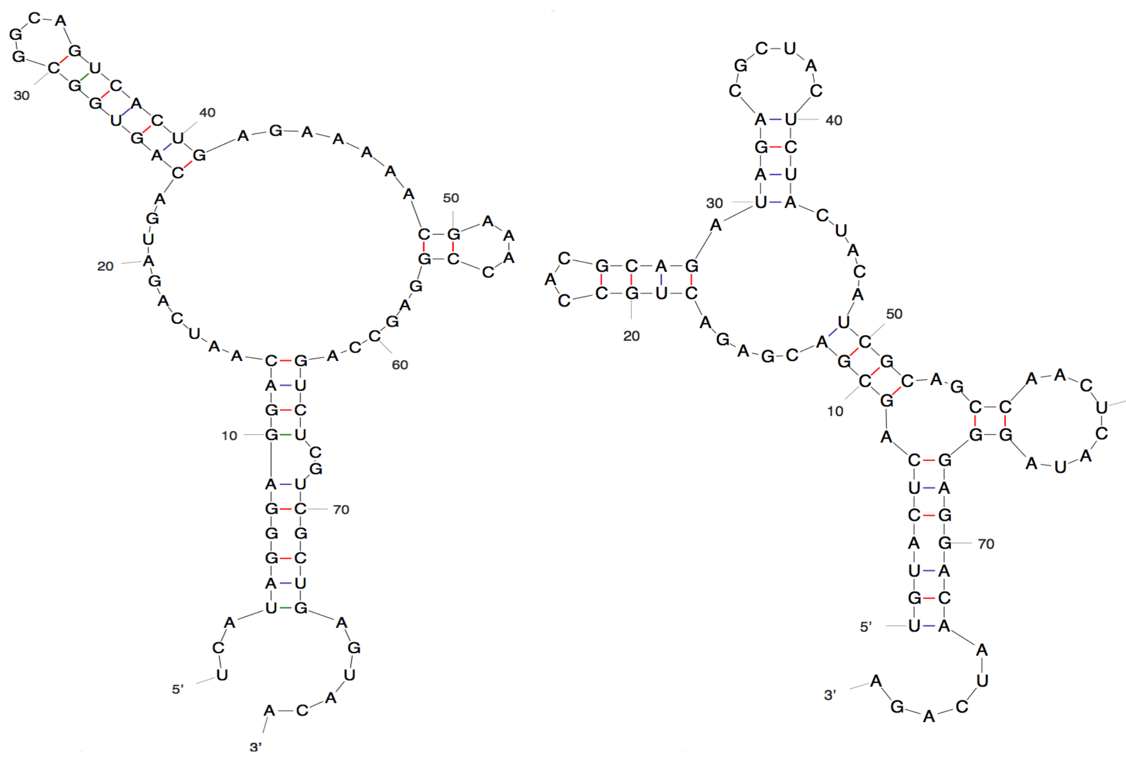


Figure 3-4. Multiple sequence alignment guide tree for aptamers after 7th SELEX

7 different conserved sequences were obtained from 50 sequences (highlighted)


5'
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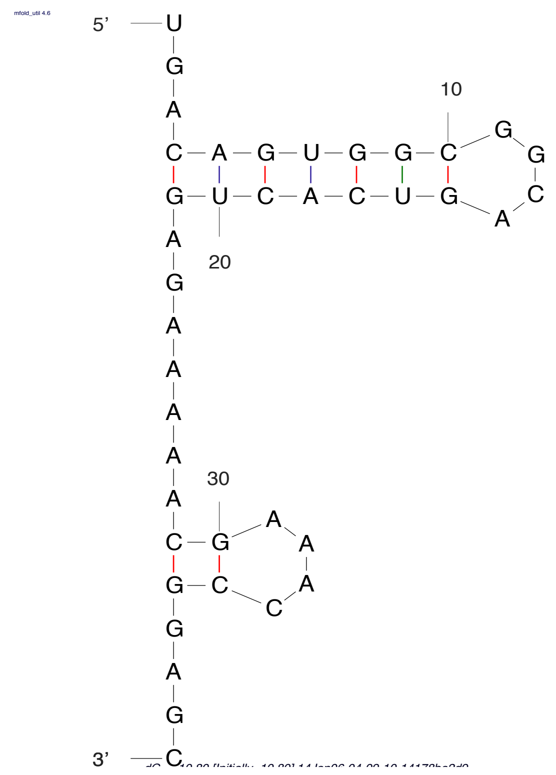

mfold_util 4.6
5'
3'
10
20
30

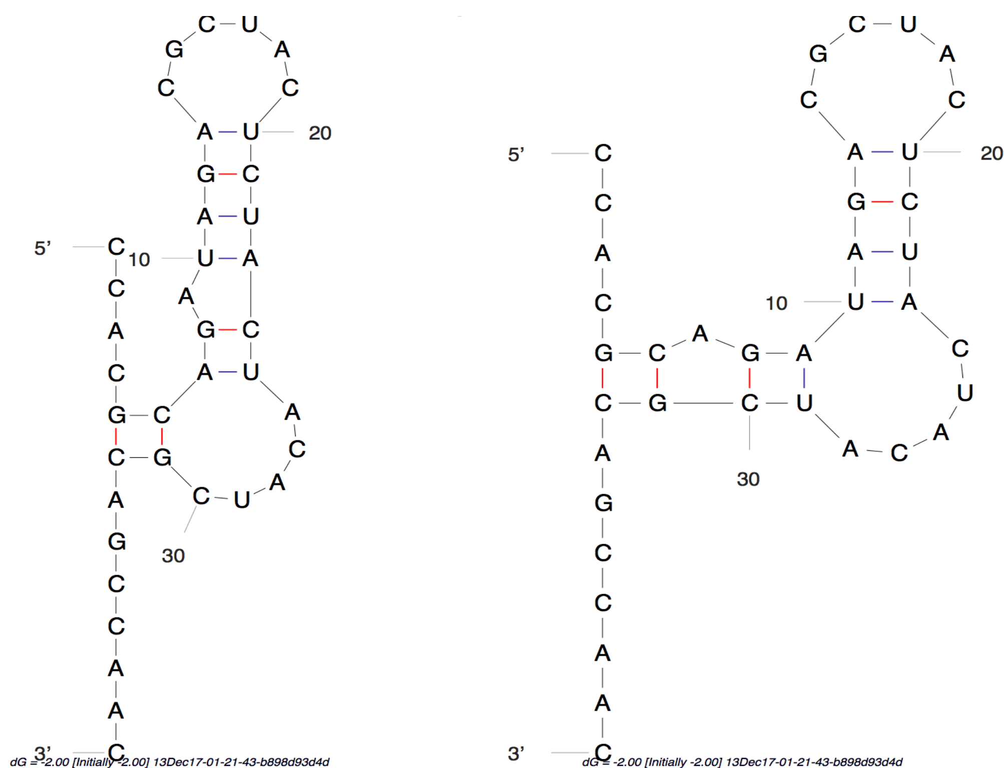


Figure 3-5 Secondary structure of 80 nucleotide NupApt01 (1), NupApt02 (2), 40 nucleotide NupApt01 (3) NupApt02 (4a), (4b).

3.3.3 Identification of strongest aptamer candidate

Identification of the strongest aptamer candidate was done by gel shift assay (Figure 3-6). After identification of 7 individual aptamer sequences I tried gel shift assay and 6 sequences were found binding but out of 6 sequences 2 of them NupApt01 and NupApt02 showed better binding ability than others, as at smaller amount of protein produced better shift and lesser free aptamer on gel shift. On further investigations, we found that the RNA sequence posses no affinity for other proteins such as BSA, also no affinity of 40-nucleotide FAM-labeled random library for recombinant Nup358 protein was observed (Figure. 3-6 B, C).

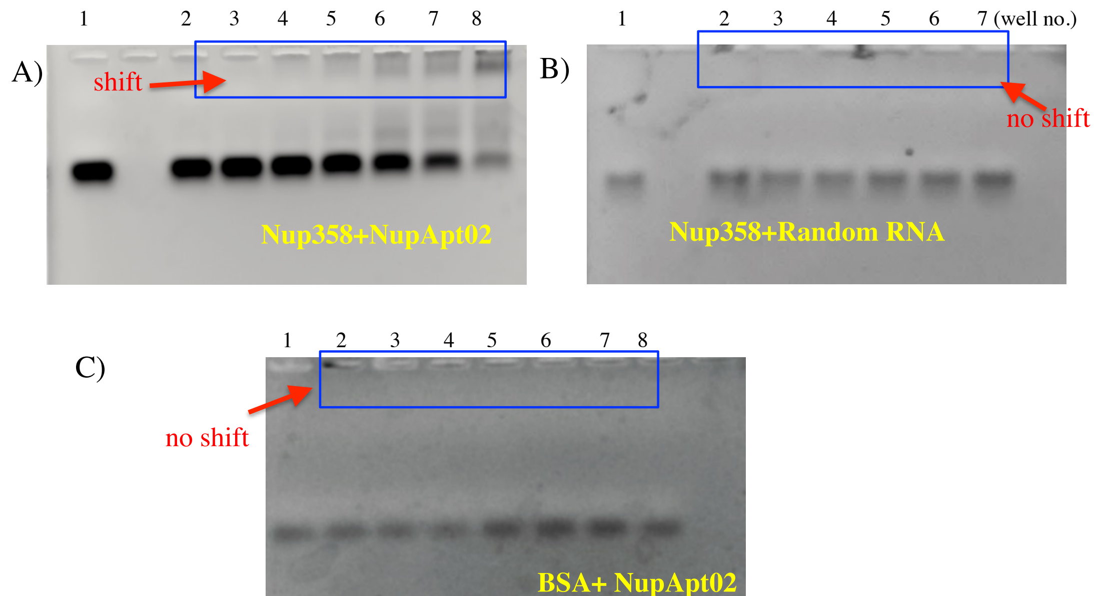


Figure 3-6. Selective binding of NupApt02 to Nup358 protein. EMSA was investigated with (a) FAM labeled NupApt02 vs recombinant Nup358 protein, (b) FAM tagged NupApt02 vs BSA protein or (c) FAM tagged 40 nt random sequence (mixture of A, G, C and U) vs recombinant Nup358 protein. RNAs in 1X selection buffer were heated at 80 °C for 10 minutes and cooled down slowly at room temperature. The 50 nM concentration of RNAs were incubated with increasing concentration of protein on lane 1-8 (0, 0.14, 0.28, 0.42, 0.57, 0.85, 4.28, 8.57 µM) in 10.0 µl volume for 30 minutes at room temperature. 2.5 µl of 6X loading dye was added and the binding was resolved at 1.5% agarose gel for 30 minutes at 100 V. The electrophoresis result was observed using ImageQuant LAS 4000. Protein position in gel was confirmed by CBB staining (data not shown).

3.3.4 Kd value determination of NupApt02

Specific complex formation of recombinant Nup358 protein and NupApt02 RNA aptamer confirmed that a single stranded RNA aptamer specific for protein recombinant Nup358 has been produced successfully. Dissociation constant (Kd) of NupApt02 was calculated as 1.6 µM using gel shift binding assay. ImageJ software was used to analyze gel bands (Figure 3-7).

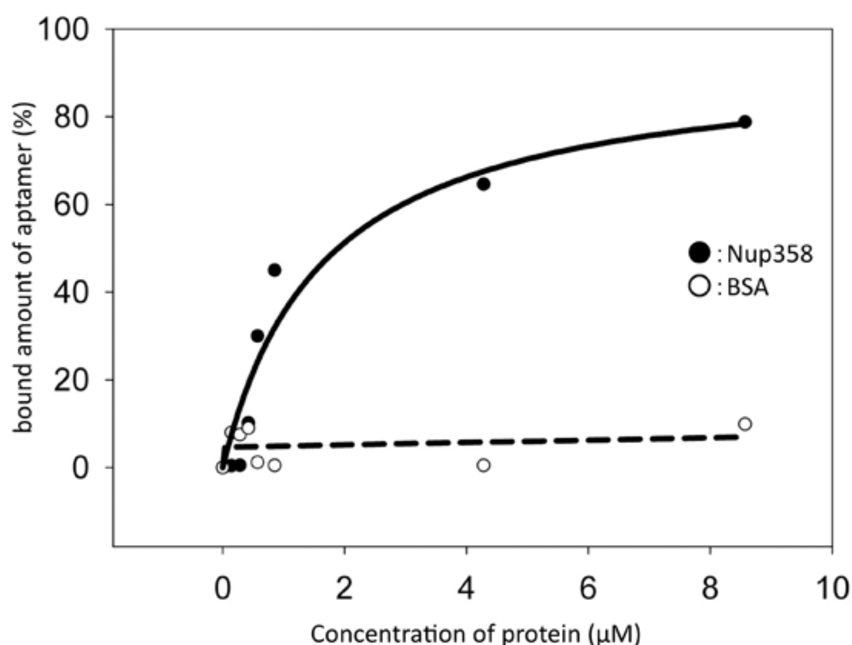


Figure 3-7 Binding Affinity (Kd value) determination of RNA aptamer NupApt02.

Gel shift assay method was used to determine Kd value of NupApt02 to recombinant protein Nup358. FAM labeled aptamer was incubated with target protein on a scale of 35 µl. RNA aptamer NupApt02 was used at 50 nM with increasing concentrations of protein in 1X selection buffer. NupApt02 was first heated at 80 °C for 10 minutes and then was cooled down at room temperature. RNA and protein were mixed in 1X selection buffer for 30 minutes at room temperature, 4.0 µl of loading dye was added to resulting sample solution (total 35.0 µl). Samples were loaded on 1.5% agarose gel and run for 30 minutes at 100 V. The gel was checked under ImageQuantLAS 4000. The binding was measured by quantifying and calculating value of free RNA aptamer using ImageJ software.

3.4 Discussion

The nucleus is the controlling device of the cell. The nucleus of an eukaryotic cell controls gene expression mechanism and selectivity of translocation of macromolecules between the internuclear and intranuclear space. Proliferating cells such as tumors become resistant to a particular drug after a while in a course of time. Because of genetic changes that ultimately lead to production of new daughter cells that are drug-resistant too. 3.2 billion base pairs (two copies each) makes the nuclear

genome of humans, only 2% of that are coding DNA while rest of the genetic material is considered "junk" DNA (13-15). Mutations in these 2% genes may lead to form of diseases such as cancer, neurodegenerative diseases, heart dysfunction, brain disorders, etc. So, a new way of drug delivery 'nanoparticle drug delivery systems' is highly required that may directly deliver drugs or plasmids into the nucleus that too effectively.

Macromolecules larger than 50 kDa require assistance from NLSs and NPC. NLSs-mediated transport involves energy-dependent recognition of NLSs by the NLS receptor, which subsequently docks the cargo at the NPC. This is followed by an energy-dependent translocation via NPC and release of cargo into the nucleus (11). Cationic lipids with DNA (lipoplex), and cationic polymers with DNA (polyplex), are being used for nucleic acid delivery into the nucleus (12). Lipoplexes were used in clinical trials for the treatment of melanoma (13). Liposomes (small percentage of cationic lipid) complexed with DNA and modified with Tat peptide, have shown translocation into the cell cytoplasm and subsequently into the nucleus (14) but the results are variable and most of the time non reproducible. So, a new ligand that can target nucleus effectively was required.

Here, we have reported a RNA aptamer that bound to Nup358 protein *in vitro*. We have successfully established a new selection method referred as Protein SELEX, aimed to work for selection of all kinds of recombinant proteins *in vitro*. The selection was first tried with other protein CA I (we reported earlier) and then with Nup358 recombinant protein, and we found that this method can be used for any specific protein to generate aptamers. We started selection with complex 2'F base modified random RNA library containing 20 nucleotide fixed regions at 3' and 5' position and 40 nucleotide random region in between. Removal of filter binding species was found must for successful selection and so it was very important to perform negative selection before each selection. To remove non specific binding it was important to introduce counter selection after few selections, most importantly we could improve cloning and sequencing results by introducing negative and counter SELEX. After 5th cycle of selection, we tried sequencing and found 2 conserved sequences while the binding amount of RNA library to Nup358 protein was 9.8%. This meant that there was scope of removing RNAs, which bind to Nup358 protein

with electrostatic, hydrogen bonding or hydrophobic interaction. To get better aptamers, we had to remove non-specific sequences and so we tried two more selections and introduced second counter SELEX with cultured HeLa cells. After we completed second counter selection and 7th selection, the bound RNA amount was reduced to 11% compared to starting RNAs. To confirm library enrichment we applied this enriched library to sequencing and in 50 sequences 7 different kinds of conserved sequences were obtained in multiple copies (38% of total sequences were conserved). NupApt02 was a sequence that repeated after 5th selection and after 7th selection cloning result. In strict conditions of selection NupApt02 sequence survived till last means it is a strong aptamer candidate. NupApt01 was one of the conserved sequences, which was identified in 6 copies making it an even stronger candidate. During the selection, it was difficult to add any other protein to remove non-specific binding because it could result in generation of RNA library that has binding affinity for other proteins (non target) added during selection. In case of addition of several proteins during selection, RNA library itself can't identify between target and non-target protein. So we did not add any other protein during selection and so it was must to perform counter selection after few rounds of selection. Counter selection as per our experience should be performed after at least 2 or 3 rounds of selection because complexity of library may decrease drastically if counter selection was added in very initial cycles. Also we found that it was essential to monitor increasing or decreasing percentage of filter binding RNA library. It must decrease constantly after initial selections and completely removed after few selections finally. In initial cycles, if it could not be removed efficiently it may result in failure of selection. After seven selection cycles we could successfully remove non-specific as well as filter binding species and could generate 2 strongest RNA aptamer NupApt01 and NupApt02 specific for recombinant Nup358. EMSA was employed to check RNA aptamer library binding and after sequencing, selected individual aptamer binding to protein Nup358. Six out of seven sequences found binding to protein. It was found that on agarose gel electrophoresis protein-RNA complex could not move at all and so the band always appeared close to agarose gel well. It was confirmed by locating the position of protein in agarose gel by Commassie Brilliant Blue (CBB) staining. Free RNA in gel is also a significant parameter to confirm percentage binding of RNA library to protein. Same concentration of BSA protein was used to check non-specific

binding of NupApt02 aptamer but no shift could be observed on gel, also, when random FAM-40 nucleotide RNA library was applied over agarose gel with CA I protein, there was no shift. It confirmed the specificity of NupApt02 RNA aptamer for CA I protein. We have estimated the dissociation constant from EMSA with FAM-tagged aptamer and Nup358 protein and the Kd value was 1.6 μM. Our protein SELEX method is a cheap, convenient selection procedure that could produce high affinity aptamers for 2 different proteins (in our case Nup358 as well as CA I). To our knowledge this is SELEX method for recombinant protein and nucleic acid aptamer that binds specifically to a nucleus protein that can help in nuclear import is first attempt in nuclear drug delivery regard.

Chapter 4

Binding of RNA aptamer-modified PEG liposomes in isolated nuclei

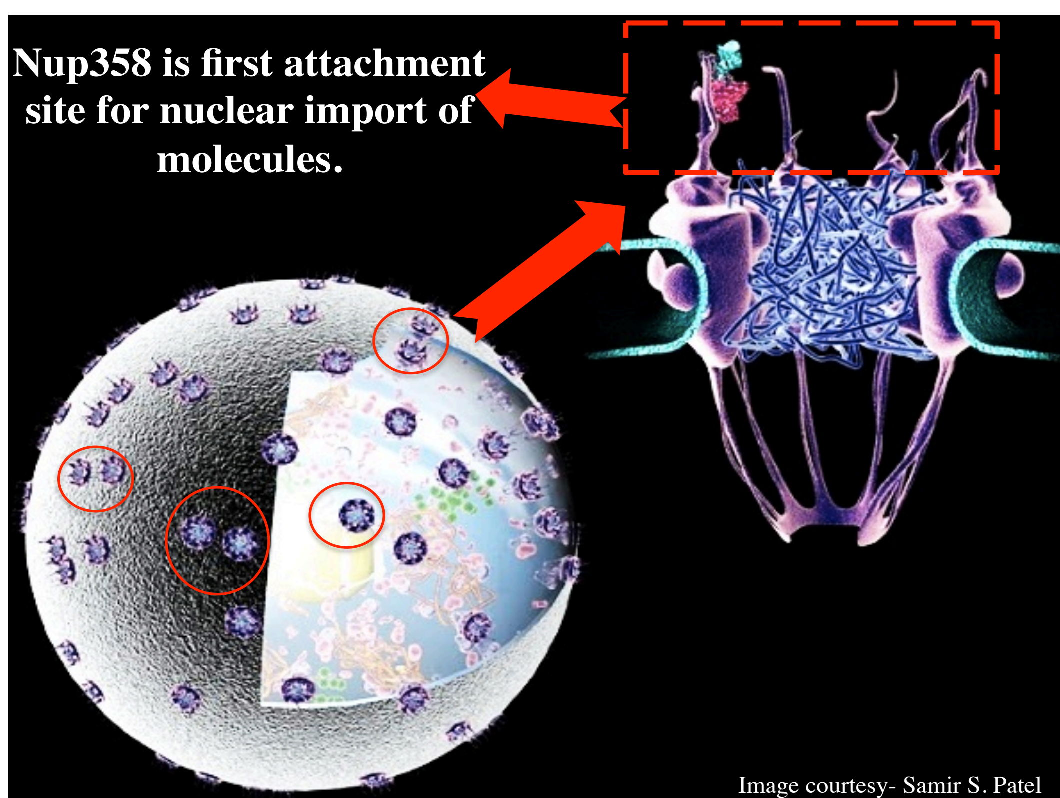
4.1 Introduction

Nanoparticle based drug delivery have been found to be very effective nuclear drug delivery method in DDS. Nanoparticle carriers must also overcome biological barriers similar to other drug delivery systems. However, attaching a ligand to nanoparticle carrier makes nanoparticles able to target selectively. After escape from endosome and release into cytoplasm, the pharmaceutical agent must deliver into the nucleus for its therapeutic effect (15). Apart from various peptide systems for nuclear drug delivery (detailed in chapter1) there are various nanoparticle transporter systems for nuclear delivery have been prepared such as chitosan oligosaccharide first conjugated with folic acid by a PEG linker to selectively target folic acid receptors of KB cells, Ch-PEG-FA-DNA nanoparticles were synthesized and gene expression was observed (16). Recombinant modular nanotransporters (MNT) composed of multifunctional polypeptides were designed to optimize *in vivo* nuclear delivery. Nuclei localization was observed by DTox-HMP-NLS-EGF in A431 cells and DTox-HMP-NLS-αMHS by B16-F1 and Cloudman S91 melanoma cells. As conclusion they found that by adjusting maoieties, MNTs are novel cell-specific nuclear delivery of variety of pharmaceutical molecules (17). There are many groups have showed increasing interest in biodegradable polypeptide delivery systems. DOX was encapsulated on 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine [poly(AA-*co*-LA)/DPPE], and this nanoparticle was degraded in acidic compartment releasing DOX into cytoplasm, followed by nuclear transportation of DOX (18). In another report, labeled-single walled chitosan-coated carbon nanotubes (CS-SWNTs) with FITC and labeled them with fluorescent DOX. The internalization of the FITC-CS-DOX-SWNTs into lysosome of EPC cells was tracked by green FITC fluorescence. The acidic lysosome broke the stacking between DOX and SWNTs, that resulted in release of DOX into the nucleus confirmed by red fluorescence into the nucleus (19).

In addition, inorganic systems for nuclear drug delivery are also practiced. The method has been found very fruitful as many drugs can be loaded on the central metal

(20), but the drawback is toxicity that results in adverse side effects as the metals naturally are not found it human body. To this series, gold nanoparticles (AuNPs) have gained major popularity and success. In a report, PEG linkers when conjugated to AuNPs and oxaliplatin, the [Pt(R,R-dach)]-PEG-AuNPs were observed to enter nuclei while PEG-AuNPs were not able to enter nuclei, while the cytotoxicity was very low (21). Chemical modifications to improve AuNPs delivery method had been tried such as $(GC)_2$ peptide and RGD-$(GC)_2$ peptide to target integrin receptors on cancerous cells, the RGD-$(GC)_2$-AuNPs were observed in the nucleus by trafficking through NPCs and nucleolus in *in vitro* experiments (22,23).

In a report from Odom et al., they reported visualization of interaction between gold nanostars (AuNSs) and the nuclei of cancer cells. The aptamer AS1411 (Apt, this aptamer is known to bind to nucleolin, phosphoprotein present in nuclei,



Yoshimura SH. ***et al*****,** ***J. Cell Sci.,*** **2013,126:3141-3150**

Figure 4-1. Importance of Nucleoporin358

overexpressed in cytoplasm of cancer cells) was conjugated to AuNSs. The Cy5-labeled-Apt-AuNSs were observed to enter HeLa nuclei by CLSM studies while the

control conjugate could not enter nuclei (24).

After study of several recent progresses in field of nuclear drug delivery, it was clear that, a new nuclear targeting active targeting ligand will provide site specific nuclear drug delivery and gene therapy. With this in mind, we attempted to target the nucleus using a RNA aptamer ligand, as with results of previous reports it seemed that there was a scope of new ligand that can target nucleus directly. The nuclear envelope contains perforations, which are referred to as nuclear pores, and participate in the import and export of macromolecules between the nucleus and cytoplasm. Each NPC contains 8 filamentous nucleoporins called as Nup358. To our hypothesis if Nup358 specific aptamer can provide site-specific binding to nanocarrier (Figure 4-1), it would be possible to deliver drug into nucleus through nanoparticle via nucleus membrane fusion.

4.2 Materials and Methods

4.2.1 Materials

Egg phosphotidylcholine (EPC), cholesterol (Chol), N-(lissamine rhodamine B sulfonyl)-1,2- dioleoyl-sn-glycero-3-phosphoethanolamine (rhodamine-DOPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine- N-[methoxy (polyethylene glycol)-2000] (PEG-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). N-[(3-maleimide-1-oxopropyl) aminopropyl polyethyleneglycol- carbamy] distearoylphosphatidyl-ethanolamine (maleimide-PEG-DSPE) was purchased from Nippon Oil and Fat Co. (Tokyo, Japan).

4.2.2 Preparation of aptamer lipid conjugate

For the preparation of the aptamer-modified liposomes, first we needed to prepare the aptamer lipid conjugate. To prepare the conjugate at first the individual aptamer NupApt01 and NupApt02 were mixed with Mal-PEG-DSPE in separate tubes. Briefly, first 20 nM of aptamer (NupApt01 and NupApt02 separately) was incubated with 100 mM Tris-HCl and 200 mM DTT forming 200 μl reaction in water for 2 hours at 37 °C with continuous shaking on a Bio-shaker. Next, after 2 hours this solution was purified by NAP-5 column (GE Healthcare, UK). The purified product was incubated overnight with 100 nM of 1 mM DSPE-PEG and 5% SDS at 37 °C

with continuous shaking on a shaking incubator. The conjugate, next day was subjected to dialysis to remove excess lipid in 3000 MW cutoff dialysis membrane, submerged in 0.1% PBS, 0.5% SDS buffer overnight (the buffer was changed at every 4 hours). Next day, 50 mM sodium bicarbonate buffer was prepared and membrane was transferred to this buffer for next 24 hours dialysis (the buffer was changed at every 4 hours). The purified product was concentrated upto 100 μl with centrifugal concentrator (TOMY, Japan). The conjugation of aptamer to PEG-DSPE was quantified and confirmed by 3.8% agarose gel electrophoresis and quality was confirmed by HPLC (Elite Lachrom, Hitachi, Japan)

4.2.3 Preparation of liposomes

Liposomes (LPs) composed of egg yolk phosphatidylcholine (EPC) and / cholesterol (Chol) (molar ratio: 7/3) were prepared by lipid hydration method. A lipid film was formed by evaporation of the solvents (Chloroform and ethanol) from a lipid solution in a glass tube. Ice cold HEPES buffer (10 mM, pH 7.4) was added and the solution was incubated for 10 minutes to hydrate the lipid film. The glass tube was then sonicated for approximately 30 sec in a bath-type sonicator (AU-25C, Aiwa, Tokyo, Japan). During the formation of the film, 1 mol% Rhodamine-DOPE (Avanti Polar lipids) was incorporated, to serve as a label for the lipid component. To modify the prepared liposomes with aptamer-lipid conjugate, the required amount of pre-prepared lipid conjugate was added and incubated at 37 °C for 1 hour. Modification of the aptamer conjugate over liposomes is successful or not was confirmed by characterizing liposomes after modification.

4.2.4 Characterization of liposomes

For, experiments several kinds of liposomes were prepared based on their ligand modification. To check physical properties of liposomes their size, PdI and zeta potential values were measured. The mean size was measured using a quasi-elastic light scattering method, and zeta potential of the prepared liposoms were determined electrophoretically using laser Doppler velocimetry (Zetasizer Nano ZS; Malvern Instruments Ltd., Worchestershire, UK). Liposomes size and zeta potential of all types of liposomes were measured before and after conjugation.

4.2.5 Cell Culture

For experiments, Human cervical carcinoma cells (HeLa) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (100 μg/ml). The cells were washed with luke warm 1XPBS and detached by cell scraper. The cells were cultured under an atmosphere of 5% CO_2 and 95% humidity at 37 °C.

4.2.6 Nuclei Isolation

$1x10^6$ HeLa human cervix carcinoma cells (RIKEN Cell Bank, Tsukuba, Japan) were cultured in DMEM media, one day before experiment. After removing the medium, the cells were washed twice with 1XPBS (0.1% BSA, 0.1% NaN3). 1 ml of 1XPBS (0.1% BSA, 0.1% NaN3) was added after washing and cells were scrapped with the help of a cell scrapper and collected in 1.5 ml appendorf tubes. All the steps were performed on ice. Isolation of washed cells was done by centrifugation (800 xg, 4 °C, 2 min). After centrifugation supernatant was removed and the pellet was suspended in ice cold HBSS 0.1% BSA. After that lysis buffer containing detergent NP-40 to lyse cellular structures (HBSS 0.1% BSA, 0.2% NP-40) was added and cells were set on ice for 5 minutes. To neutralize the effect of lysis buffer again HBSS 0.1% BSA buffer was added in solution and mixed gently by inverting tube upside down 2-4 times. To remove cellular digest tube were centrifuged (800 xg, 4 °C, 2 min). Supernatant was removed slowly without disturbing the pellet. The steps with lysis buffer were repeated until clean nuclei (white, delicate, less thicker pellet) were obtained. As a final step, nuclei were resuspended in 1 ml of HBSS 0.1% BSA buffer. Quality of nuclei was checked under inverted microscope (Olympus U-CMAD3, Tokyo, Japan) and counting was done using heamocytometer with trypan blue (0.3% v/v) staining method.

4.2.7 *in vitro* binding studies of aptamer modified liposome to isolated nuclei

After successful preparation of NupApt01 and NupApt02 modified liposomes and only PEG liposomes, nuclei were freshly isolated and used to check liposomes binding. 20 μM of aptamer modified liposomes (NupApt01 and NupApt02 modified liposomes) were incubated in individual tubes containing ~3000 nuclei in 200 μl reaction at 4 °C for 30 minutes. After 30 minutes $1/10^{th}$ of this mixture was separated

in 96 wells plate. Nuclei were centrifuged at 1000 xg, 4 °C, 2 minutes, supernatant was removed to remove unbound liposomes and nuclei were washed with 1X selection buffer and spun again at the same condition. Now the nuclei were resuspended in 1X selection buffer and 100 μl of mixture was put on 96 well plate to check fluorescence absorbance using Varioskan Flash 2.4 (Thermo Fisher Scientific, Japan).

4.2.8 CLSM studies to study binding pattern of aptamer modified liposome to isolated nuclei

A binding study was performed by confocal microscopy (Nikon NIS Elements).At first nuclei were isolated and counted. Separate tubes were prepared containing ~3000 nuclei in each. 20 μM of NupApt01 modified PEG liposomes, NupApt02 modified PEG liposomes and only PEG liposomes were added to respective tubes. The nuclei, liposomes mixture was incubated at 4 °C for 30 minutes on ice and then spun on 1000 xg, 4 °C for 2 minutes. Supernatant was removed and nuclei were washed again using 1X selection buffer and spun. The nuclei were then stained by incubation with Hoechst33342 (1 mg/mL). Next, different glass slides were prepared and ~300 nuclei were put over it and fixed with coverslip to observe binding pattern under CLSM. The excitation/emission range of Rhodamine and Hoechst33342 were 530/586 nm and 350/461 nm respectively. To check specificity of liposomes binding to nuclei, nucleolin (-) aptamer modified liposomes were also prepared and their binding toward isolated nucleus was studied under CLSM using same conditions in individual experiment.

4.2.9 Inhibition assay

Inhibition assay was performed to check if binding of liposomes to nuclei is ligand mediated or not. Two types of liposomes, aptamer modified PEG labeled liposomes and aptamer modified PEG unlabeled liposomes were prepared. In this experiment freshly isolated nuclei were first incubated with 10 times excess of aptamer modified PEG unlabeled liposomes for 30 minutes on ice, and then aptamer modified PEG labeled liposomes were added and incubated for more 30 minutes on ice. The ratio of unlabeled liposomes to labeled liposomes was (10:1). After incubation the nuclei were washed, resuspended in HBSS 0.1% BSA buffer. For staining nuclei were

incubated with Hoechst33342 (1 mg/ml) for 15 minutes and centrifuged at 1000 xg, 25 °C for 5 minutes to remove excess dye. Finally, nuclei were fixed on a glass slide covered with coverslip and studied under confocal microscope (Nikon NIS Elements) at 100X magnification.

4.2.10 Kd value measurement of aptamer modified liposome to isolated nuclei

Freshly isolated nuclei (~3000 per tube) were incubated with increasing concentration of ligand 0, 0.015, 0.031, 0.125, 0.5, 2.0, 12.5 μM at 4 °C for 30 minutes, after incubation nuclei were centrifuged to remove unbound liposomes in supernatant. Nuclei were washed with HBSS 0.1% BSA buffer and centrifuged again. Supernatant was removed and pellet was resuspended in 0.1% HBSS 0.1% BSA buffer and put in 96 well plate serially as per ligand concentration. The Binding affinity of NupApt01 modified PEG liposomes and NupApt02 modified PEG liposomes to nuclei were determined by measuring fluorescence intensity using Varioskan machine. The average mean fluorescence intensity of varying concentration of aptamer modified PEG liposomes was plotted to determine dissociation constant Kd.

4.3 Results

4.3.1 Preparation of aptamer modified liposomes with post insertion method

As the goal of my research was to achieve success in preparing an RNA ligand facilitating nuclear-targeted drug delivery, it was very important to produce results that confirm that the ligand is capable of binding to nuclei along with a liposome. To verify this, we used isolated nuclei to bind NupApt01 and NupApt02 modified liposomal nano-carriers respectively. For the establishment of this system, first we conjugated NupApt with Maleimide DSPE-PEG followed by modification of a PEGylated liposome (EPC:Chol= 7:3) 1 mol% rhodamine-DOPE with the pre-prepared aptamer-lipid conjugate (2.5% ligand) by means of a post modification method. At first the the conjugation of aptamer and PEG was prepared successfully (Figure 4.2), it was confirmed over 3.8% agarose gel by electrophoresis (Figure 4.3)

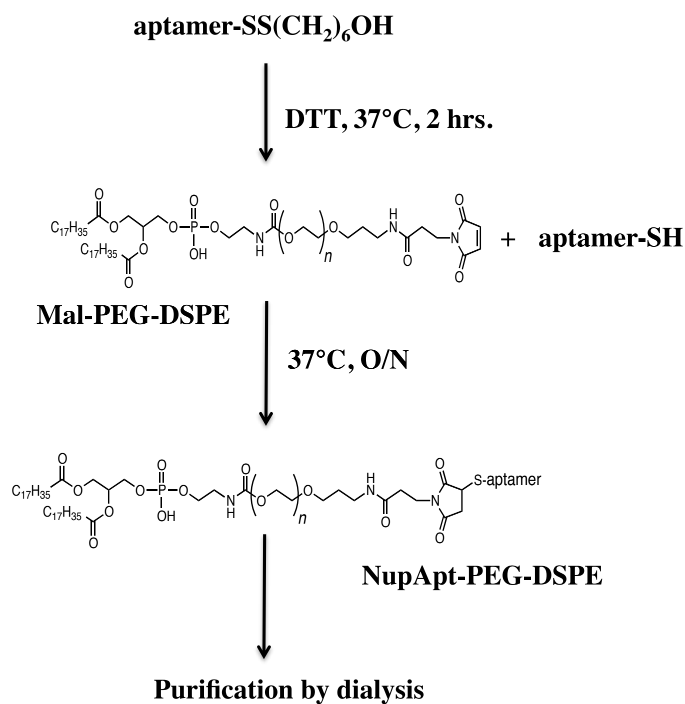


Figure 4-2. Scheme of Nup358 aptamer lipid conjugate preparation.

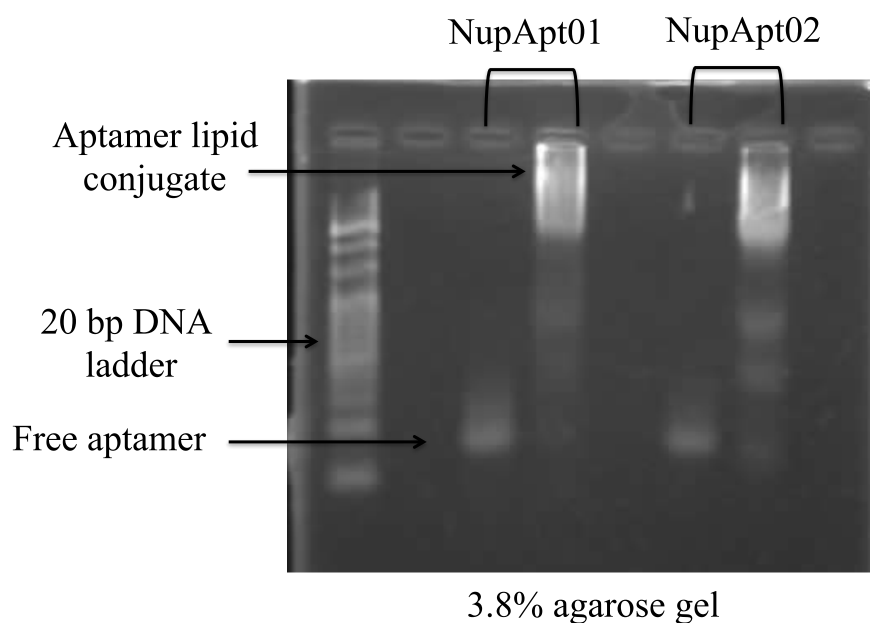


Figure 4.3 Confirmation of aptamer lipid conjugation preparation Lane 1 is 20bp DNA marker, 3 is NupApt01-lipid before conjugation, 4 is Apt-PEG-lipid conjugate,

6 is NupApt02-lipid before conjugation, 7 is Apt-PEG-lipid conjugate 2, 5, 8 are empty lane.

4.3.2 Physical properties of liposomes

Quantitative binding studies using aptamer modified PEG liposomes and PEG-liposomes were performed against isolated nuclei to determine the binding percentage of ligand-PEG versus only PEG. At first, aptamer lipid conjugates were prepared and, in the next step, EPC: Chol (7:3) liposomes were modified, using a method referred as the post-modification method. The average size in diameter and zeta potential was measured (Table 4-1). As zeta potential and size of liposomes were measured both before and after modification it was convenient to confirm the modification as zeta potential of liposomes successfully modified with aptamer lipid conjugate would become more negative due to aptamer negative charge. In our case also, after successful modification with the aptamer lipid conjugate the zeta potential turned more negative, thus confirming that the modification was successful and aptamer modified liposomes were successfully prepared.

Table 4-1
Physical properties of liposomes

Liposome	Size (dnm)	Zeta potential
DSPE-PEG	149.5	-38.8
DSPE-PEG-NupApt01	141.5	-41.2
DSPE-PEG-NupApt02	143.1	-46.7
DSPE-PEG-Nucleolin (-) Apt	146.2	-40.9

4.3.3 Nuclei Isolation

Setting up a reliable and good protocol for nuclei isolation was a prime requirement to start our experiments. For all experiments, nuclei were freshly isolated from HeLa cell line. Dr. Hidetaka Akita had provided us a protocol for nuclei isolation, after checking several conditions and different methods such as nuclei isolation using kits (Sigma Aldrich), we could finally found clean nuclei with some modifications in Akita sensei's protocol. Nuclei were counted using heamocytometer and quality were checked under microscope (Olympus U-CMAD3, Tokyo, Japan) (Figure 4-4) with trypan blue staining.

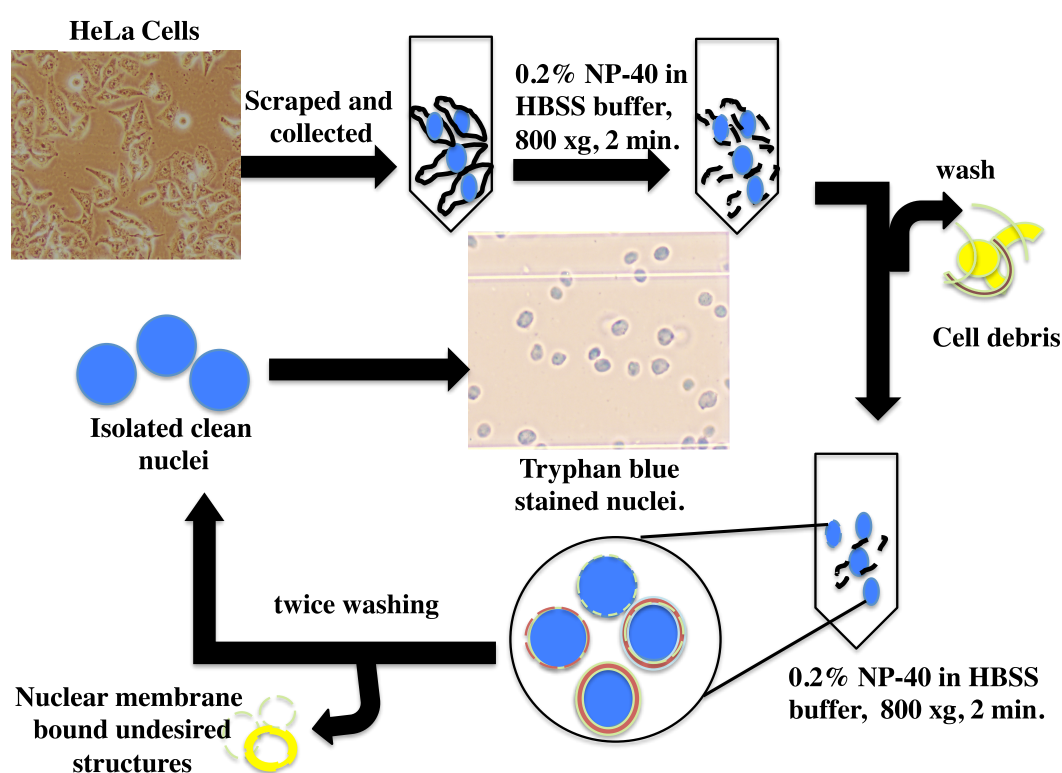


Figure 4-4 Nuclei isolation method. The nucleus isolation method is adapted from Dr. Hidetaka Akita's protocol. HeLa cell line was used and nucleus quality was obsereved under microscope with tryphan blue staining as shown in Figure 4-3.

4.3.4 Confirmation of binding of aptamer modified liposomes to nuclei (FI)

To check that the aptamer modified liposomes will bind to nuclei *in vitro* or not, first respective liposomes were prepared and nuclei were isolated. Nuclei with fixed numbers were incubated with aptamer modified and non-modified liposomes and washed. Binding was calculated by observing fluorescence intensity, both aptamer

modified PEGylated liposomes (NupApt01 modified PEG liposomes, NupApt02 modified PEG liposomes) showed significantly higher nuclear binding in terms of fluorescence intensity, while the non-modified or PEG-LPs did not show strong binding (Figure 4-5). NupApt01-PEG-LPs and NupApt02-PEG-LPs in comparison to only PEG-LPs showed 7 times and 6 times more binding to the nucleus respectively.

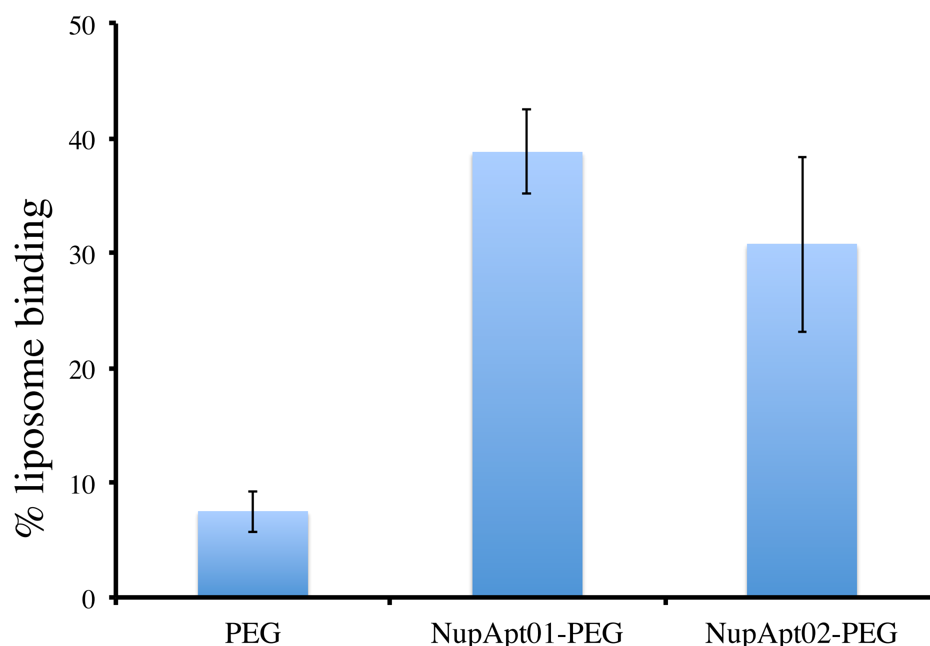


Figure 4-5. Aptamer modified liposome-binding studies into isolated nuclei. Fluorescence intensity (FI) results showed 7 to 6 times better binding of NupApt01 and NupApt02 modified liposomes to isolated nuclei, in comparison to PEG.

4.3.4 Confirmation of binding of aptamer modified liposomes to nuclei (CLSM)

Liposomes are carrier systems that take drugs to a specific site intracellularly. However, despite of several gene/ drug delivery attempts, there is no report of direct, aptamer mediated liposomal targeting to a nucleus. After confirming that the aptamer-modified liposomes show better binding *in vitro*, as evidenced by fluorescence intensity results in comparison to PEG liposomes, next we planned experiments to produce visible quantitative results that confirmed the localization of NupApt01/02-modified liposomes in nuclei, isolated from HeLa Cells. CLSM studies (Figure 4-6) clearly showed the specific binding of the NupApt01/02-modified liposomes to the

nucleus, compared to only PEG liposomes. NupApt01 and NupApt02 modified liposomes were found accumulated around the nucleus while only PEG liposomes failed to bind to the nucleus. The target protein Nup358 is cytoplasmic, an exposed fibre like extention (a few nano meters in length, 8 in numbers/ nuclear pore complex) attached to the nuclear membrane via the N-terminal domain and so, based on the CLSM results, we concluded that RNA aptamer mediated liposomes are able to bind to the nucleus while only PEG liposomes could not bind specifically and strongly. The issue whether binding is specific or not, was confirmed by using the negative sequence of the nucleolin aptamer (CCTCCTCCTCCTTCTCCTCCTCCTCC), a negative DNA aptamer that has already been reported for not binding to the nucleus (25). When in individual experiment nucleolin (-) aptamer modified liposomes were prepared and incubated with nuclei it showed no binding in comparison to NupApt (ligand) mediated liposomes. It confirmed that aptamer-mediated liposomal binding is specific to the nucleus.

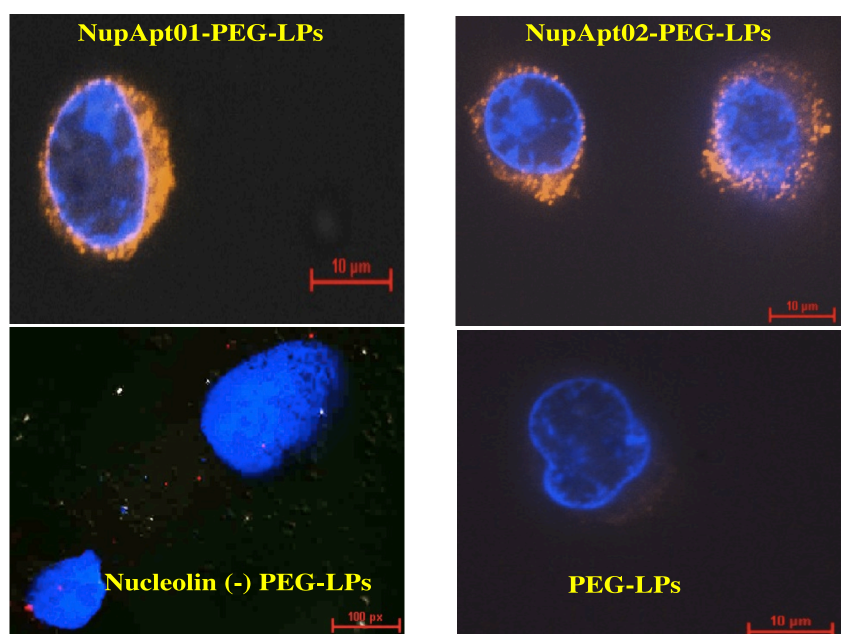


Figure 4-6. CLSM images of NupApt-PEG-LPs bound to the nucleus. NupApt01-PEG-LPs and NupApt02-PEG-LPs showed binding around nuclear membrane. Nucleolin (-) PEG-LPs and PEG-LPs did not show any strong signals.

4.3.5 Investigation of *in vitro* binding of aptamer modified labeled liposomes in presence of non-labeled liposomes or inhibition assay

To our hypothesis if the liposomes binding to nucleus are ligand mediated there must be a saturation point after which liposomes will stop binding to target protein. So, to confirm whether it is true in our case or not, first aptamer modified unlabeled and aptamer modified labeled liposomes were prepared. Isolated nuclei were first incubated with 10 times excess of unlabeled liposomes and then with labeled liposomes. Pre incubation with aptamer modified unlabeled liposomes saturated binding site for aptamer on nucleus and so aptamer modified labeled liposomes could not bind further. It confirmed that the binding of liposomes on nuclei was ligand mediated (Figure 4-7).

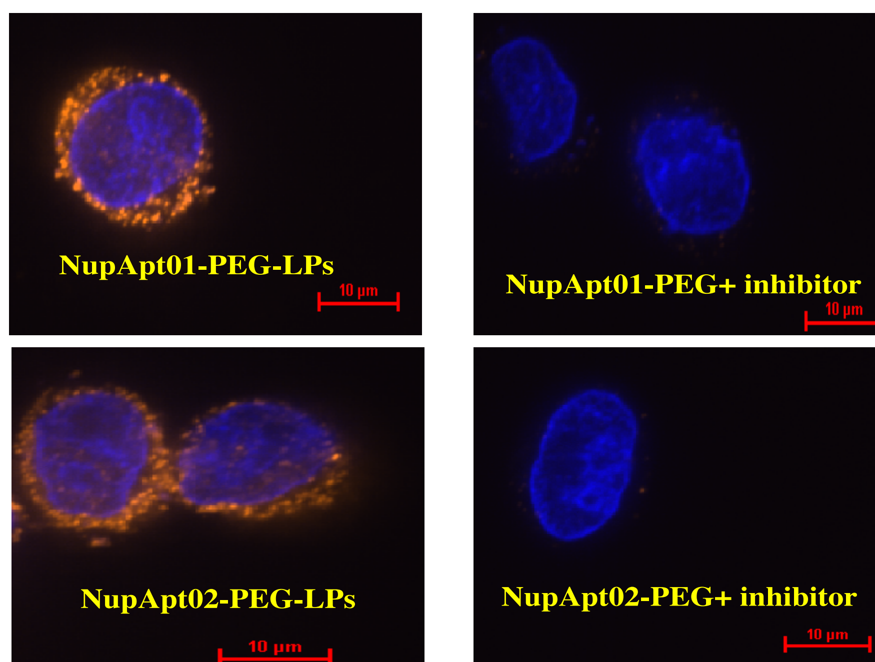


Figure 4-7. Inhibition assay. NupApt01-PEG-LPs and NupApt02-PEG-LPs (labeled) in absence of excess of inhibitory (unlabeled) liposomes showed strong signals while pre treatment of isolated nuclei with labeled liposomes reduced labeled liposomes binding drastically.

4.3.6 Kd value measurement of aptamer-modified liposomes

Dissociation constant of ligand indicates how strongly it can bind to its target protein. To checkdissociation constant of aptamer modified liposomes to nuclei was

calculated by observing fluorescence intensity. Different concentrations of aptamer modified liposomes were incubated with fix number of isolated nuclei, the liposomes were allowed to bound to nuclei and then centrifuged to remove unbound liposomes. Pellet of nuclei was washed, resuspended. Fluorescence intensity was measured using VarioSKan machine. Dissociation curve was plotted by measuring average mean fluorescence intensity of varying concentration of aptamer modified PEG liposomes. Dissociation constant of NupApt01 modified liposomes was calculated as 36 nM, and NupApt02 modified liposomes was 70 nM (Figure 4-8) respectively.

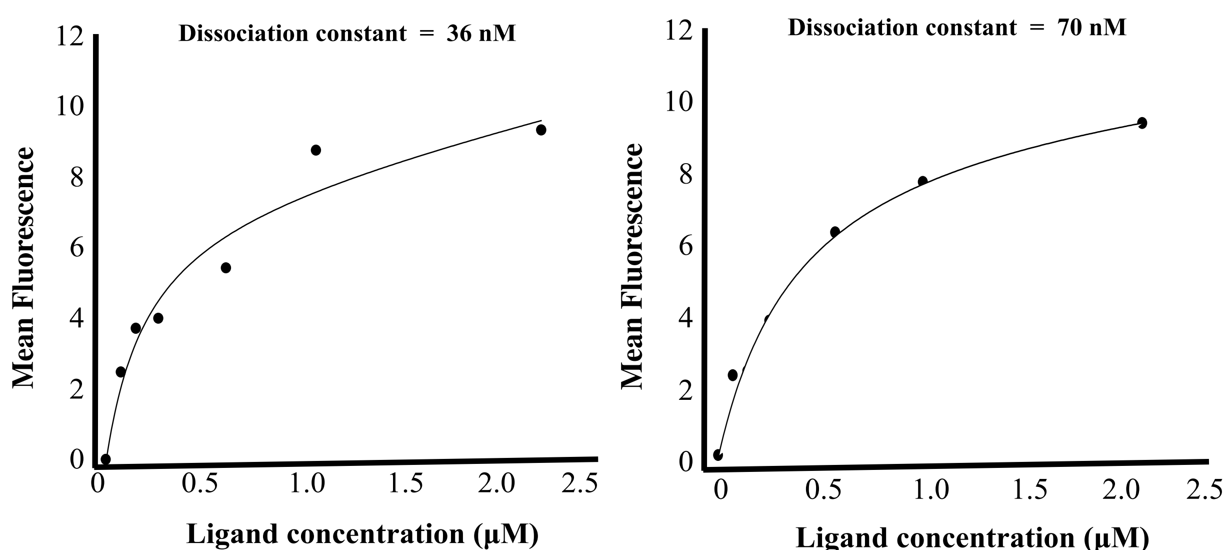


Figure 4-8. Dissociation constant of NupApt modified PEG liposomes. Isolated nuclei (~3000) with increasing concentration of ligands (0-12.5 μM) were incubated at 4 °C for 30 minutes. Fluorescence intensity was measured to check dissociation constant of NupApt01 and NupApt02 modified liposomes to isolated nuclei. Kd value of aptamer modified liposomes was 36 nM (NupApt01) 70 nM (NupApt02) respectively. (N=1)

4.4 Discussions

In this study, at first, we hypothesized to prepare recombinant Nup358 protein specific RNA aptamers that can identify specific protein over nuclear membrane. Newly established method Protein SELEX was used and a series of RNA aptamers were generated, identified which were good enough to use as a ligand for active

nuclear targeted drug delivery. At first the aptamer lipid conjugate was prepared and liposomes were prepared separately. The liposomes were then modified with aptamer lipid conjugate, and like this aptamer modified PEG liposomes were prepared by post insertion method. The two nucleus specific RNA aptamers NupApt01 and NupApt02 modified PEG liposomes showed very strong binding (6-7 times) towards the nucleus while PEG-LPs was not bound strongly to nucleus. Confocal studies confirmed the binding of aptamer modified liposomes to nuclear periphery. Binding of aptamer modified liposomes is specific or not was confirmed by binding comparison of Nucleolin (-) apt-PEG-liposomes and aptamer modified PEG liposomes, nucleolin (-) apt-modified liposomes did not bind to nucleus. Inhibition assay confirmed that the binding of liposomes to nuclei is ligand mediated, as on addition and pre incubation of nuclei in excess of aptamer modified unlabeled liposomes the binding of later added aptamer modified labeled liposomes reduced drastically. Kd value of single aptamer was not very strong but aptamer modified liposomes had very good binding affinity. The reason could be insufficient EMSA data. To get reliable Kd value with EMSA it very important to try gel shift at least more than three times, while the Kd value mentioned in the thesis is representation of just one EMSA result. By performing several EMSA reliable Kd value can be estimated. Aptamer modified liposomes however, showed strong binding towards isolated nuclei and so strong Kd value was estimated on the basis of aptamer liposome binding to nucleus, a decrease in dissociation constant of single aptamer and aptamer modified liposomes from 1600 nM to 36 nM was seen. Wilner et al. (2012) published a report, according to that the binding of transferrin specific RNA aptamer was improved 300 times (104 nM to 310 pM) when the aptamer was modified over liposome, as the aptamer present on liposome surface provides multivalent binding towards its target (131), similarly in an individual report Kibria et al. (2013) reported that large size liposomes modified with specific ligand provides multivalent binding (132). Because we prepared EPC:Chol liposomes and later aptamer-PEG conjugate was modified via post insertion, it is believed that several specific ligand (aptamer) present over nanoparticle provided attachment of nanoparticle to target protein more specifically and strongly with better binding probability due to multivalent binding.

As the target of all drug delivery systems is to ultimately reach the nucleus, either directly or indirectly, this system constitutes a new development in the field of

nuclear-targeted drug delivery system (DDS). Our report as per our knowledge represents the first successful attempt in the artificial ligand mediated nuclear-targeted drug delivery directly to the nucleus. However, further studies will be required to acquire sound detailed knowledge of aptamer modified liposomes that functions efficiently in system, such as via *in vivo* studies. As a result it can be concluded that a new SELEX method was successfully developed, along with first RNA aptamers that binds to specific nuclear protein Nup358. These aptamers are the pioneer artificial ligand for nuclear-targeted delivery that shows liposomal binding in intracellular experiments in cases of isolated nuclei.

Nanoparticle-aptamer conjugation is a new approach to facilitate delivery of nanoparticles to specific site of action; our finding is a stretch of this work that facilitates binding of nanoparticle-aptamer bioconjugate directly to the nucleus. However, aptamer based nanoparticles have to be engineered to work perfectly after systemic administration to avoid any toxicity. Modification includes, controlling optimal size of nanoparticles, surface modifications. We have showed results that prove that our RNA aptamer modified vehicles binds efficiently to nuclear surface protein Nup358 and so nanocarrier direct by this aptamer can also be used for direct nuclear targeting and drug delivery.

Chapter 5

Perspective

5.1 Perspective

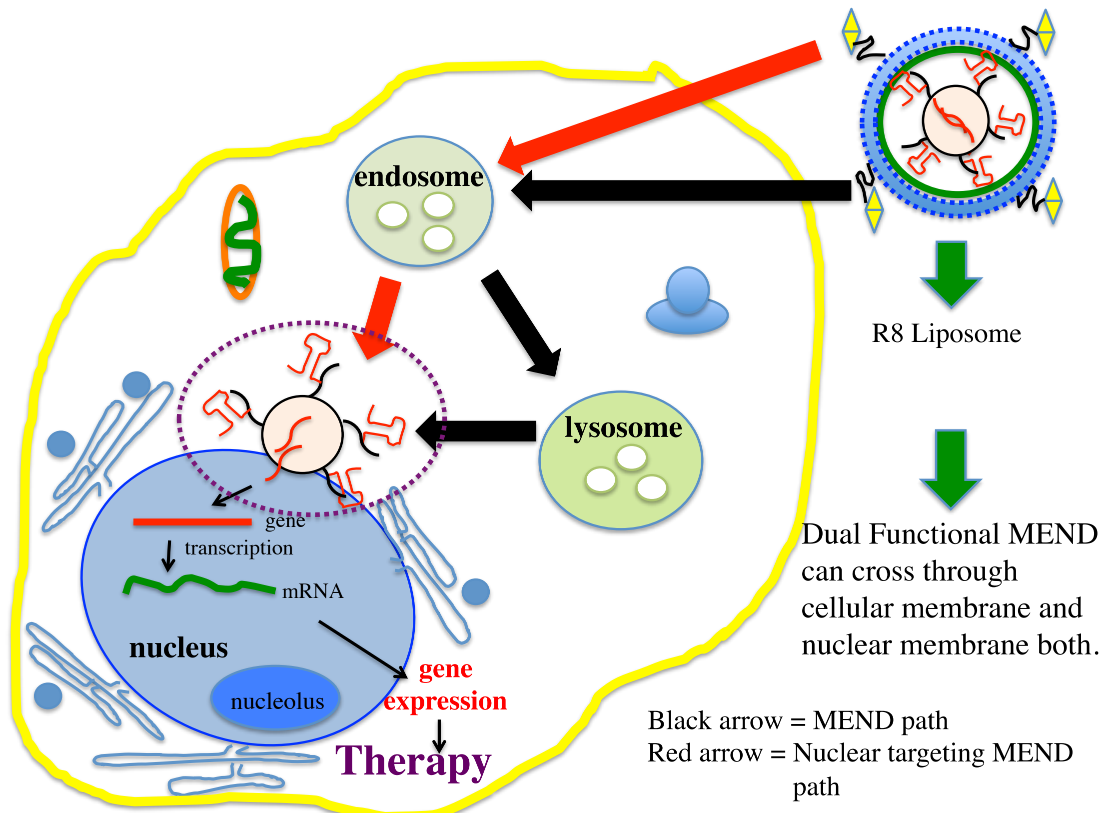


Figure 5.1 Perspective of current research

Akita *et al* (2009) (28), (our laboratory group) have already developed a nano-device referred as T-MEND (tetra-lamellar-multifunctional envelope type nano device). The T-MEND has coating of two nuclear membrane-fusogenic, two endosome fusogenic, inner envelopes and outer envelopes to overcome the endosomal and nuclear membrane barrier in a step vise manner. This nanoparticle acts like a spaceship because it interacts with individual cellular barrier through its individual component and finally free the drug in cytoplasm. Using this nanoparticle, a successful DNA delivery into the nucleus was observed in JAWS II cell line (133).

To make this process more accurate here we have prepared the very first artificial ligand that can target nuclear membrane. We have already mentioned intracellular fate of NupApt01, NupApt02 modified liposomes, to continue this work, the T-MEND nuclear fusogenic membrane can be pre modified with NupApt01 and/or NupApt02 ligand or with both to give direction to this nanoparticle towards nucleus and site specific binding. The RNA aptamer produced have specificity to nuclear protein Nup358. PEG chain provides flexibility to this nanoparticle and once the nanoparticle comes in contact with nuclear membrane specifically, it is possible that the nanoparticle lipid membrane and nuclear lipid membrane will fuse and intact pDNA can be delivered into the nucleus, and ultimately the transcription and translation of this gene will produce desired protein to attain gene therapy. This approach may solve all the disease problems, as all drug delivery system have just one goal to deliver drugs to nucleus in functional state.

5.2 Other important experimental results

5.2.1 Organelle based nucleus SELEX to prepare RNA aptamers for nucleus

Intracellular delivery can increase efficiency of various treatment protocols. Receptor mediated endocytosis results in delivery of drug carriers, drugs or pDNA to lysosomes followed by its degradation. The solution of this problem is an approach towards direct intracytoplasmic delivery of drugs or drug carrier that can overcome endocytic pathway. Organelle based selection is a hypothesis where we can target specific organelle of cell outside it to prepare aptamers and then check its binding efficacy within cell and *in vivo*. Pharmaceutical agents to act better need to deliver to its specific site of action such as specific organelles

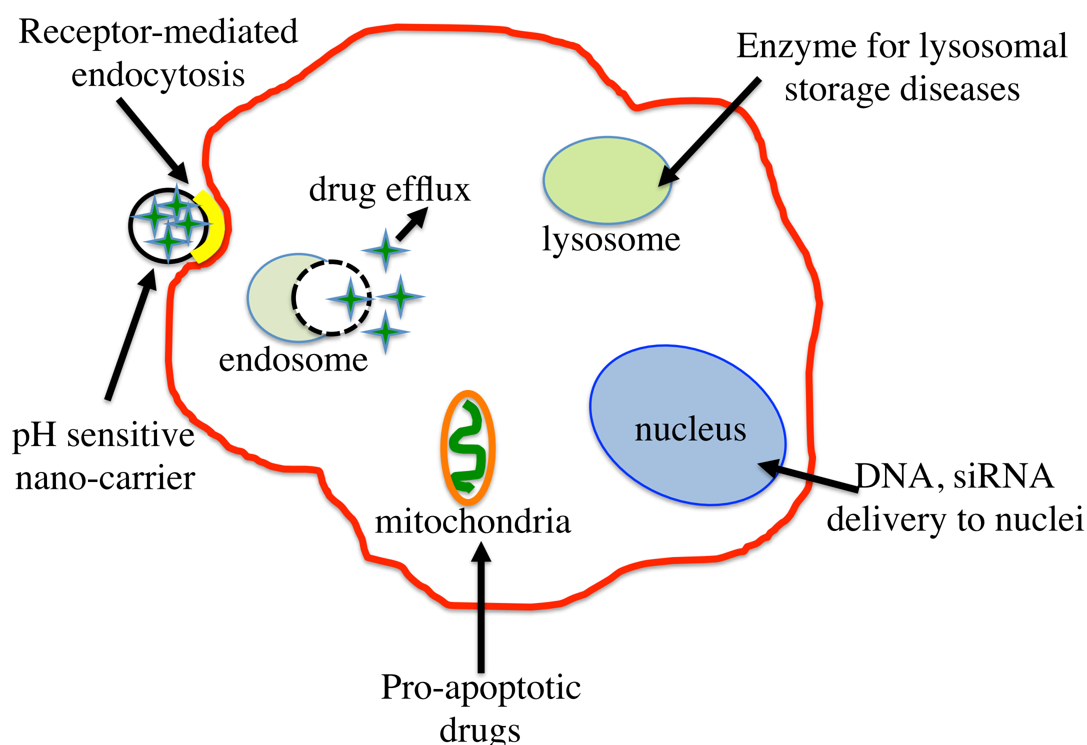


Figure 5.2 Benefits of Organelle based SELEX in disease treatment of specific organelle.

like, golgi apparatus, endosomes, mitochondria etc. to achieve maximum benefit and lowest toxicity (Figure 1). As mentioned earlier pharmaceutical agents need to overcome several biological barriers to reach cytoplasm in active state and then its

targeting to organelles however, this opting this way causes severe drug loss and toxicity. By the time drug reaches its targeted organ either the state is inactive or amount is insufficient. Organelle based selection can overcome this problem. This method is especially important in case of gene therapy or nuclear targeting where the biggest problem is to transfer drug inside nucleus. This was unique hypothesis to prepare aptamers for specific organs first and then check their binding intracellularly It was distinguish and first attempt to prepare aptamers in this way and there is no previous work has been reported earlier using organelle based SELEX for nucleus drug delivery, so, we tried to attempt this method. In this method, to prepare aptamers first we isolated nucleus and then used this isolated nucleus to prepare RNA aptamers

5.3 Results

I started Organelle based SELEX for preparation of RNA aptamers binding to

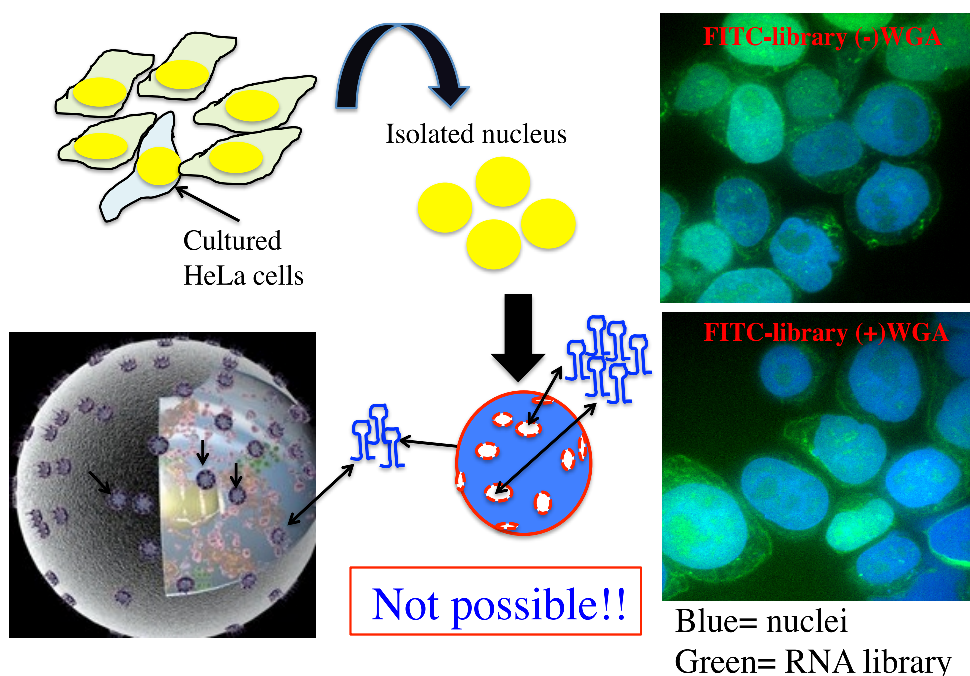


Figure 5.3 Organelle based nucleus SELEX

nucleus. HeLa cells were cultured and nuclei were isolated freshly for all selections. After completion of few rounds of selections CLSM studies were performed to check the binding location of RNA aptamers. FITC tagged RNA aptamers were incubated

with nuclei and washed but under confocal microscope we could not identify any specific binding site on or inside nucleus (Figure 2). To check whether we can prevent entry of RNA aptamers inside nucleus, nuclei were tried to block with WGA before incubation with RNA aptamer library. But as a result we could not identify the difference in blocked and unblocked nuclei and difference in binding pattern of RNA aptamer library.

5.4 Discussion

However, Organelle based selection is a promising method for preparation of aptamers for different organelle but it does not work for nucleus because of the physiology of nucleus. Nucleus has several nuclear pores (upt0 3000~4000) that mediates nucleocytoplasmic transport on the basis of size of macromolecules. The nuclear access to macromolecules is very difficult because of size limitation of nuclear pore and FG repeats but small molecules, nucleic acids and ion are allowed to move freely inside and outside of nucleus. The size of RNA aptamer I was using was so small (80 nucleotide) and it can gain access through nuclear pore complex freely without binding or with some non specific binding, it was close to impossible to use organelle based SELEX for specific RNA aptamers binding to nucleus.

5.4 Nup358 specific single aptamer binding to isolated nuclei (FI and CLSM studies)

NupApt01, NupApt02 binding assay to isolated nucleus

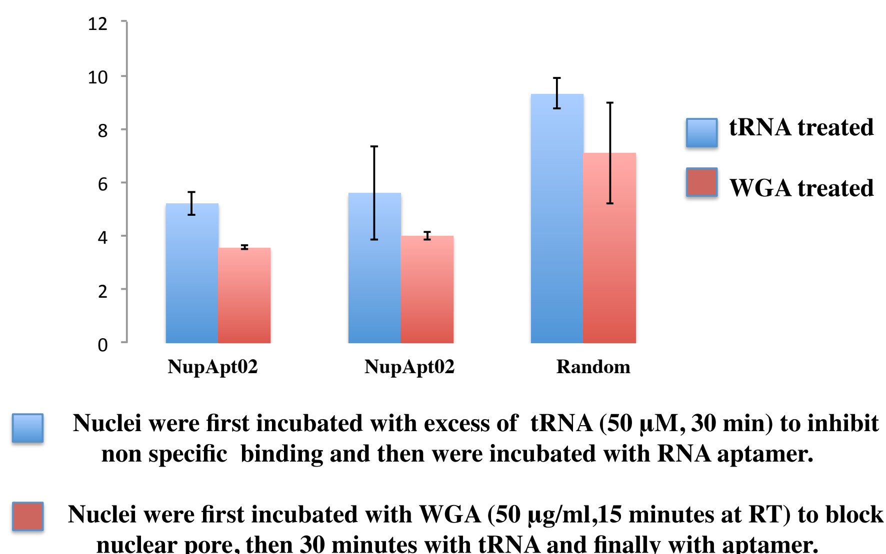
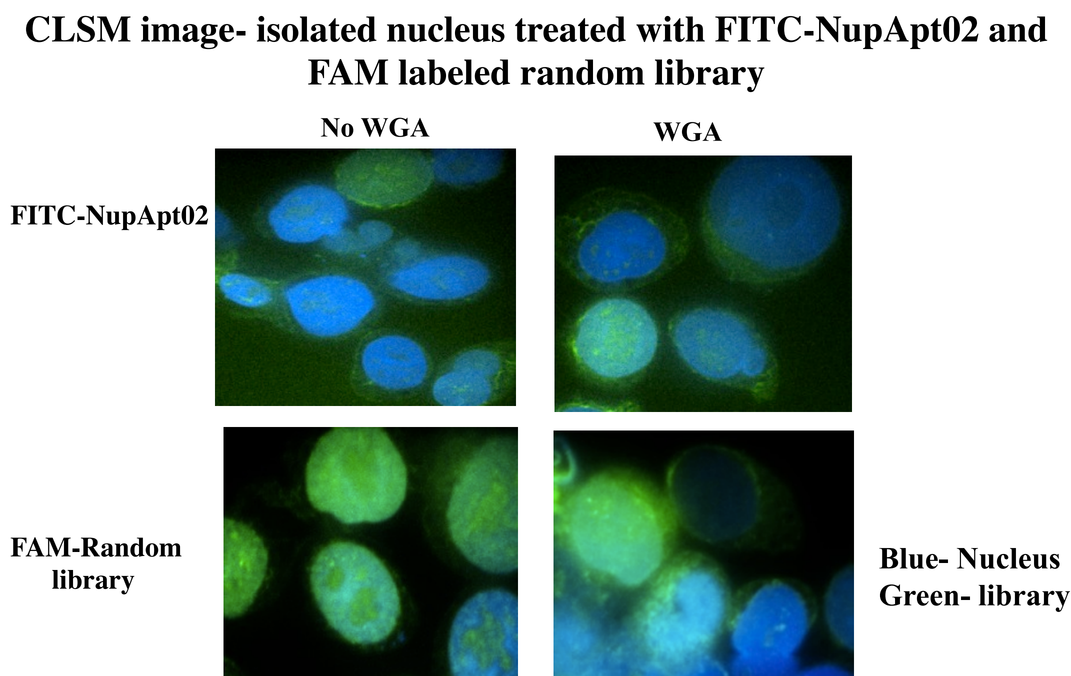


Figure 5.4 Fluorescence Intensity results, comparison of Nup358 aptamer and random RNA library binding pattern in WGA treated nuclei.



FITC-NupApt02 could not bind specifically to isolated nucleus, WGA treatment too had no major effect. Result showed consistency with our previous findings.

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Figure 5.5 CLSM studies to check Nup358 aptamer binding pattern in isolated nuclei

To explain that why recombinant protein was used for selection, here I mentioned all experimental results that were not talked within the thesis main results but have important information about aptamer based nucleus targeting. At first nucleus was targeted by using organelle based SELEX but RNA aptamer could not be prepared so recombinant protein Nup358 protein was used by establishing a new selection system referred as Protein SELEX. After identification of aptamers responsible for binding, the binding of single aptamer to isolated nuclei was checked. At first, isolated nuclei were treated with tRNA and then with WGA to block nuclear pore. The 40 nucleotide FAM labeled random library and 40 nucleotide FAM labeled RNA aptamer were heated at 80 °C for 10 minutes at thermo cool unit and then allowed to cool down

slowly at room temperature. WGA treated nuclei were incubated with random RNA and RNA aptamer for 30 minutes on ice and then spun to remove unbound RNA. Fluorescence intensity results showed excessive binding of random library while aptamer binding was lower (Figure 3). CLSM studies revealed that there was no big difference in binding pattern in random library and RNA aptamer (Figure 4). The result was same as we found earlier during organelle based SELEX where all library spread all over in nucleus due to small size of aptamer sequence. After this result, it was necessary to make aptamer-liposome conjugate and check binding. So it would be true to say that small length nucleic acid aptamer preparation, using organelle based SELEX for nucleus is not possible. As a different strategy, even after preparation of nuclear protein specific RNA aptamer, their binding to nucleus could not be determined. These ligands must have large size so that nuclear pore can prevent their import and export but in case of small ligands their free movement through nuclear pore limits their use as individual. So small ligands must be attached with carrier to attain specific binding.

Final Conclusion

This is the first report to demonstrate that liposomes modified with a aptamers (ligand) via short PEG stretch can be specifically binding to nuclear surface of HeLa cells. The intracellular experimental results suggest that a high affinity ligand could be prepared that has affinity for a specific nuclear protein Nup358 present at cytoplasmic phase of nucleus, in a flagella like structure. First of all a new selection method was established referred as Protein SELEX for producing high affinity RNA aptamers for recombinant proteins *in vitro*. After 7 rounds of selections, cloning and sequencing was performed and sequences were aligned and 7 sequences were identified. EMSA results suggested that 2 of 7 sequences showed very strong binding to recombinant protein and there was no non-specific binding could be observed. After identification of aptamers next these aptamers were used as ligand to modify liposomes by post insertion method. Fluorescence intensity results confirmed that aptamer modified liposomes have 6-7 times better binding capability towards isolated nuclei in comparison to PEG liposomes. CLSM studies confirmed that the aptamer-modified liposomes were binding specifically around nuclear membrane while PEG liposomes did not show binding. Nucleolin (-) aptamer modified liposomes did not show binding when incubated in it concentration indicated that the binding was specific. Inhibition assay was performed to confirm that the binding of liposomes to nuclei is ligand assisted, and we found that on adding excess of aptamer modified non labeled liposomes, binding of aptamer modified labeled liposomes decreased. The dissociation constant of aptamer modified liposomes were 36 nM and 70 nM (Apt01 and Apt02 respectively), both of them bound well on nucleus but based on all experimental results and finally the Kd value results we can say NupApt01 is more strong aptamer.

Taken together, the findings provide a proof of concept for nuclear drug delivery. RNA aptamer was used first time as a ligand, to target Nup358 (surface specific protein of nucleus) for assisting binding of nanoparticle to nucleus. Therefore, this new work may offer a new jump in discovery and development of nuclear drug delivery system As gene therapy is the ultimate goal of therapeutics, this work may fill the gap between assumptions and facts.

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