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Nanocarriers for Cytosolic Drug and Gene Delivery in Cancer Therapy

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1. Introduction

In this burgeoning era of personalized medicine we have witnessed a humongous increase in novel therapeutics encompassing wide range of modalities including small molecule drugs which can elicit their action upon encountering certain cellular component, protein macromolecules interfering cellular signaling pathways and nucleotide and DNA based therapies which alter protein/gene expression (Gonzalez-Angulo et al., 2010). The major factor which underscores the success of these novel therapeutic modalities is their propensity to reach the target site of action. Undoubtedly, the ultimate target for all these therapeutic modalities according to traditional paradigm is the cell. But there is a need for change in this paradigm since many of these modalities are targeted towards very specific subcellular organelles. Even though the major subcellular target even today is nucleus, there is growing body of evidence that other organelles also have role in many diseases (Davis et al., 2007). Targeting therapeutics to subcellular organelles would positively improve treatment in a myriad of diseases of metabolic, genetic and oncologic nature. Oncology is perhaps the most demanding area for organelle specific targeting since the standard therapy for oncology involves random interaction with cellular components and is harbinger of potential problems like toxicity and immunogenicity (Fulda et al., 2010; Galluzzi et al., 2008). Subcellular organelles in eukaryotic cells comprise of a complex organization of distinct membrane-bound compartments and these form the cellular basis of human physiology. These subcellular organelles by virtue of highly specialized metabolic functions interact with each other to uphold various cellular functions. Organelle biogenesis regulated by transcriptional networks modulating expression of genes encoding organellar proteins results in inheritance and proliferation of subcellular organelles such as nucleus, mitochondria, endoplasmic reticulum, peroxisomes and lysosomes (Hill et al., 1995; Nunnari et al., 1996; Warren et al., 1996). The recent developments in molecular and cellular biology opened up new vistas in the development of metabolic disorders due to disruption of organelle biogenesis. The disorders pertaining to organelles are not limited to genetic and metabolic origin. They are also involved in metabolic disturbances occurred during diseases due to infections, intoxications and drug treatments (Dhaunsi, 2005). The subcellular organelles are involved in wide array of diseases known to human nature like myopathy, obesity, type 2 diabetes, Zellweger syndrome, cancer etc., and these diseases are explained in detail further in the review. Thus, appropriate targeting of subcellular organelles not only

provides direct amelioration of genetic and metabolic disorders but also aid in cure for diseases whose causes underlie subcellularly.

The approach of using nanocarriers for subcellular delivery of drugs, macromolecules and DNA therapeutics is proved to be more effective. This is because inherent physiochemical properties of the carriers such as size, shape and molecular weight are bestowed upon the molecule it is carrying. There is huge body of evidence reported in literature where nanocarriers were able to passively and actively target tumor vasculature and tumor cells (Magadala, 2008; Sawant, 2006; Soman, 2009; Torchilin, 2007: Yang, 2010). Now the major task ahead is to tailor these nanocarriers to cater the needs of subcellular targeting. This can be achieved by developing nanocarriers either by virtue of their inherent predilection toward a cellular compartment, or by attaching subcellular targeting ligands to direct nanocarriers to organelle of interest. For example, dequalinium (DQA)-based liposome like vesicles DQAsomes have inherent capability to target mitochondria for DNA and small molecule drugs (D'Souza et al., 2005; D'Souza et al., 2003; Weissig et al., 2001; Weissig et al., 2000). The examples of targeting using ligand involve use of folic acid, low density lipoprotein, mannose-6-phosphate, transferrin, riboflavin, ICAM-1 antibody etc.,(D'Souza et al., 2009). This ability to control the intracellular trafficking and fate of nanocarriers is by far the most important advantage of using nanocarriers for organelle targeting.

The major challenge posed for subcellular trafficking of nanocarriers is the constitution of the cell interior. This cell interior is very different from an aqueous buffer and it contains many large molecules mainly proteins, nucleic acids and complex sugars. The high concentration of these molecules (up to 400 grams per liter) causing the 'macromolecular crowding' is an important barrier for intracellular trafficking of nanocarriers (Ellis et al., 2003). The complex array of microtubules, actin, and intermediate filaments organized into a mesh resembling lattice also influence the diffusion of solutes inside cell. The other factors that might perpetuate hindrance of diffusion of nanocarriers are fluid phase viscosity, binding to cytosolic components and collisional interactions due to macromolecular crowding (Garner et al., 1994). Hence, it is important to consider these factors while designing nanocarriers for subcellular targeting.

Traditionally, the interactions of nanocarriers with cells and intracellular organelles were considered to be strongly influenced by size. But recent advances in microscopy and particle fabrication techniques has led us to understand the interdependent role of size, shape and surface chemistry on cellular internalization and intracellular trafficking (Geng et al., 2007). Once internalized into the cell, the most important determinant of successful delivery of therapeutics is the intracellular fate of endosomal content. The intracellular fate of the nanocarriers can be controlled depending on endocytic pathway. For example clathrin dependent endocytosis results in lysosomal degradation whereas clathrin independent internalization results in endosomal accumulation and sorting to a nondegradative path. The major aim of subcellular targeted delivery system is to avoid lysosomal trafficking so as to protect the drug or biomolecule from enzymatic degradation (Bareford et al., 2007). As cellular uptake and fate can be controlled by endocytic mechanism, the subcellular distribution can be directed by presence of additional peptide sequences that direct the nanocarrier to a desired subcellular site.

Concept of targeting chemotherapeutic drugs to malignant tissue by identifying certain overexpressed receptors and proteins has been investigated in great detail. The concept of targeting to cancer can be studied by dividing the therapeutics into two classes. The first one being the category where drug itself is capable to act specifically on mechanisms unique to

malignant cells. For example, imatinib inhibits Bcr-Abl tyrosine kinase which is overexpressed in chronic myelogenous leukemia and trastuzumab binds and inhibits HER2/neu receptor which is overexpressed in breast cancers (Droogendijk et al., 2006; Hudis, 2007). The second category is utilization of structural moieties such as ligands and antibodies which will be attached to the drug to direct it toward certain features unique to cancer cells. For example, folate is a very good ligand to target cancer cells as folate receptors are over expressed in many cancers and anti-CD22 antibody epratuzumab was conjugated with ^{90}Yttrium for specific diagnosis of B cell lymphoma (Allen, 2002). However, the selectivity to the certain tissue or cell is not sufficient to produce the desired therapeutic effect if the drug is not accumulated at appropriate subcellular target organelle. There also exists other complications such as, efflux of drug after internalization by efflux pumps such as p-glycoprotein (P-gp) and multidrug resistance associated protein (MRP). Thus, subcellular targeting of cancer therapeutics is of prime importance since drugs are designed to act against specific subcellular targets. For example, certain DNA therapeutics are expressed only after they reach nucleus and certain drugs intended for tumor regression by reducing endoplasmic reticulum stress response have to act at endoplasmic reticulum (Nori et al., 2005).

The present review is an attempt to elucidate the importance of nanocarriers in subcellular targeting. The scope for subcellular targeting lies in understanding diseases affected due to malfunctioning of organelles. It is also very important to understand the challenges posed by intracellular environment for effective transport of nanocarriers. The recent targeting strategies employed to target each subcellular organelle is explained in detail. Thus, a comprehensive understanding of role of the nanocarriers in subcellular targeting and their application in amelioration of diseases like cancer is provided to the reader through this review.

2. Cellular organelles and related disorders

Subcellular organelles are responsible for cellular metabolic state which in turn is responsible for maintaining physiologic functions of tissue. Important subcellular organelles like mitochondria, peroxisomes, lysosomes, endoplasmic reticulum and cytoskeleton carry out important functions like production of energy, sorting of proteins, supporting and providing shape to the cell. A defect in any of the components of the network of organelles leads to a serious pathological state. A better understanding of diseases of organelles is of paramount importance in developing efficient targeting strategies. The list of cellular organelle related disorders is tabulated as Table-1 at the end of this section.

Mitochondria, the powerhouse of eukaryotic cells plays a key role in energy metabolism in many tissues. The defects in mitochondrial functions such as respiratory coupling, reactive oxygen species production (ROS), enzymatic activity (fatty acid oxidation), and mitochondrial content and size may result in metabolic disorders such as aging, insulin resistance and type 2 diabetes. Most important diseases of mitochondria arise due to mitochondrial DNA (mtDNA) deletions which cause the formation of mutant mtDNA. Examples of these diseases include Kearns-Syare syndrome and Pearson syndrome which can be fatal in infancy or early childhood (Johannsen et al., 2009). Mitochondria also regulates cellular life cycle through release of *cytochrome c* which is an important stimulator of apoptosis thus indicating its role in cancer. It was also proved that mitochondrial oxidative and phosphorylation capacity and mitochondrial content are decreased with age

thus showing importance of mitochondria in aging. Mitochondrial dysfunction was also implicated in insulin resistance and type 2 diabetes. Recent reports suggest that `metabolic overload' of muscle mitochondria is a key player in insulin resistance (Koves et al., 2008). Another important mitochondrial dysfunction is increased damage by ROS, which in turn results in cancer and neurodegenerative diseases (de Moura et al., 2010).

Impaired ribosome biogenesis and function due to genetic abnormalities result in a class of diseases called ribosomopathies. These ribosomopathies result in distinct clinical phenotypes most often involving bone marrow failure and craniofacial or other skeletal defects. The ribosomopathies are generally congenital syndromes due to mutations of genes encoding ribosomal proteins. The first discovered ribosomopathy was Diamond-Blackfan anemia (DBA) which is due to mutation in *RPS19* gene. DBA is a rare congenital bone marrow failure syndrome with a striking erythroid effect (Draptchinskaia et al., 1999). The other congenital syndromes linked to defective ribosome biogenesis are Schwachman-Diamond syndrome (SDS), X-linked dyskeratosis congenital (DKC), cartilage hair hypoplasia (CHH), and Treacher Collins syndrome (TCS). All of these ribosomopathies except TCS were reported to pose risk to cancers like osteosarcoma and acute myeloid leukemia (Narla et al., 2010).

Endosomes and lysosomes envisage important functions within cells including antigen presentation, innate immunity, autophagy, signal transduction, cell division, and neurotransmission. The cellular function will be compromised if undegraded substrates accumulate in endosomes and lysosomes due to lysosomal dysfunction. Lysosomal storage disorders constitute a group of genetic diseases involving dysfunction of lysosomal hydrolases resulting in impaired substrate degradation. Lysosomal diseases are manifested by enlarged lysosomes which contain partially degraded material due to 1) glycosaminoglycan, lipid or protein degradation defects, 2) transport across lysosomal membrane or 3) endosome-lysosome trafficking. The first discovered diseases of lysosomes are related to lipidoses and mucopolysaccharidoses. They include diseases like Tay-Sach disease, Gaucher disease, Fabry disease, Niemann-Pick disease, Hurler syndrome. However, much of the initial concept for the lysosomes and its dysfunction came from the studies of Pompe disease characterized by cardiomegaly, cardio respiratory failure, hepatomegaly and progressive muscle weakness (Parkinson-Lawrence et al., 2010).

Peroxisomes are single membrane bound organelles which contain more than 50 different proteins, mainly enzymes essential for various metabolic processes, which include hydrogen peroxide based respiration, β-oxidation of very long chain fatty acids, bile acid synthesis and plasmalogen biosynthesis. There exists several genetic disorders associated with peroxisomal system and are divided into two categories. The first category is related to peroxisome biogenesis and second is the single protein defects in which a single metabolic function is different. The examples of first category are heterogeneous group of autosomal recessive disorders including Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease and rhizomelic chondrodysplasia punctata. The examples of second category are X-linked adrenoleukodystrophy, hyperoxaluria type I and thiolase deficiency (Gartner, 2000).

The endoplasmic reticulum (ER) apart from playing an important role in many cellular functions is also involved in protein folding and trafficking. The important manifestation of failure of the ER's adaptive capacity is activation of unfolded protein response (UPR), which in turn affects various inflammatory and stress signaling pathways. UPR is closely integrated with inflammation, stress signaling and JNK activation. These pathways play a

critical role in chronic metabolic diseases such as obesity, insulin resistance, and type 2 diabetes. It was also reported that chronic ER stress and activation of the UPR may also result in oxidative stress, causing a toxic accumulation of ROS within the cell (Hotamisligil, 2010). Mice were subjected to obesity-induced stress and then treated chemical chaperones phenyl butyric acid and tauro-ursodeoxycholic acid. After treatment the stress was relieved and also there was observed an increase in insulin sensitivity and reduction in fatty liver disease in those obese mice, showing the link between ER induced stress and metabolic disorders. (Ozcan et al., 2004). A small molecule Salubrinal was reported to protect cells against ER stress induced cell death *in vitro* and *in vivo*. Salubrinal prevents the dephosphorylation of eIF2α (Boyce et al., 2005). ER stress associated disorders also include various neurodegenerative disorders. Recently, various neurological disorders including Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis have shown disruption of ER homoeostasis and up-regulation of UPR. Another recent ER related neurodegenerative disorder which was reported recently was `seipinopathy', which is a motor neuron disease related to protein seipin. This protein seipin activates the UPR and induces ER stress-mediated cell death (Ito et al., 2009). Thus, targeting ER would be an attractive approach to ameliorate inflammatory and chronic metabolic disorders.

The Golgi complex is an important organelle within the secretory system of the cell. Its organization is maintained by proteinaceous matrix, cytoskeletal components and inositol phospholipids. It carries out two important tasks, one being sorting of secretory cargo to various destinations in cell and other being modification of protein during its way to plasma membrane. Certain pathological conditions, pharmacological agents and over expression of golgi-associated proteins cause profound morphological changes of golgi apparatus. These morphological changes were shown by neuronal golgi apparatus in many neurodegenerative disorders like Alzheimer's disease, amyotrophic lateral sclerosis, Creutzfeldt-Jacob disease, multiple system atrophy, Parkinson's disease, spinocerebelar ataxia type 2 and Niemann-Pick type C (Fan et al., 2008). Protein glycosylation is another important function of Golgi apparatus. A defective glycosylation process by golgi network would result in disorders like congenital disorders of glycosylation and also cause acquired glycosylation defects associated with epidemic diseases such as cancer and diabetes (Ungar, 2009).

3. Role of nanocarriers in cytosolic delivery

During past decade numerous efforts were made to efficiently direct the polymeric and/or nanoparticulate carriers to the organelle of choice. Most of those efforts were successful in delivering small molecule drugs (S. R. Yang et al., 2006), proteins (Bale et al., 2010) and nucleic acids (Jensen et al., 2003) to specific organelle inside cell. The major advantage of use of nanocarriers in cytosolic delivery arises from their characteristic properties like nanosize (1-100 nm), ability to carry high drug/gene payload, feasibility to modify the surface functionality for active targeting, ability to utilize its surface charge for passive targeting. The arsenal of nanocarriers investigated for organelle specific targeting includes inorganic and organic materials. The major units of this arsenal are liposomes (Fattal et al., 2004), micelles (Bontha et al., 2006), quantum dots (QDs)(Hoshino et al., 2004), polymeric nanoparticles (Nori et al., 2003; Yessine et al., 2004), gold nanoparticles (Bergen et al., 2006), magnetic nanoparticles (Xu et al., 2008), dendrimers (Samuelson et al., 2009), carbon nanotubes (Z. Yang et al., 2010).

Cellular Organelle	Disease	Defective gene/Function	Clinical Features	Ref
Mitochondria	Leigh's syndrome	*mtDNA* deletion	Ataxia, seizures, hypotonia, lactic acidosis	(Corona et al., 2002)
	Type 2 diabetes	GLUT4 Translocation	Insulin resistance	(Johannsen et al., 2009)
	Progressive external ophthalmoplegia	*mtDNA* deletion	Developmental delay, lactic acidosis	(Holt et al., 1988)
Endoplasmic reticulum (ER)	Obesity	ER stress-induced autophagy	Increased body mass index	(Hotamisligil, 2010)
	Seipinopathies	*Seipin/BSCL2*	Spastic paraplegia, muscle weakness	(Ito et al., 2009)
Lysosomes	Pompe disease	Lysosomal glycogen hydrolysis	Cardiomegaly, hepatomegaly, Progressive muscle weakness	(Parkinson-Lawrence et al., 2010)
	Gaucher disease	Lipid degradation in macrophages	Hepatosplenomegaly, Osteonecrosis, neurodegeneration	
	Fabry disease	Glycosphingolipid hydrolysis	Growth restriction, Cardio-respiratory problems, Lipid accumulation	
Peroxisomes	Zellweger syndrome	*PEX* gene	Abnormal facial appearance	(Gartner, 2000)
	X-linked adrenoleukodystrophy	*ALD* gene	Progressive neurodegenration	
Ribosomes	Diamond-Blackfan anemia	*RPS19, RPS24, RPS17, RPL35A*	Macrocytic anemia, Short stature, craniofacial defects	(Narla et al., 2010)
	Shwachman-Diamond syndrome	*SBDS*	Neutropenia/infections, Pancreatic insufficiency, Short stature	
Golgi Apparatus	Alzheimer's disease	Dysregulation of Ca^{2+} signalling	Cerebral deposition of amyloid plaques	(Fan et al., 2008)
	Amyotrophic Lateral Sclerosis	Dysregulation of Ca^{2+} signalling	Progressive degeneration of cortical and spinal motoneurons	
Nucleus	Primary biliary cirrhosis	Antibodies against nucleoporins	Cirrhosis of liver with destroyed bile ducts	(Cronshaw et al., 2004)
	Triple A syndrome	*AAAS* gene	Hypoglycemia, achalasia, alacrima	

Table 1. Cellular organelle related disorders with specific genes involved and their clinical sysmptoms

Nanocarriers are internalized into the cell by a process called endocytosis. After the carriers have been successfully endocytosed, depending on the intended target, these carriers are directed to respective organelles of cell by means of specialized mechanisms. There exists a wide plethora of enodocytic mechanisms depending on the physicochemical property of the internalizing nanocarriers. Heterogeneity in mechanisms of endocytosis can be utilized to efficiently translocate the cargo to specific cellular organelles and are subjected to required interactions during their journey towards the target (Maxfield et al., 2004). Process of endocytosis can be broadly classified into two categories, one is phagocytosis that involves uptake of large particles, and the other is pinocytosis which involves uptake of fluid and solutes. Phagocytosis is observed mainly in specialized mammalian cells such as macrophages whereas pinocytosis is observed in all cells. Pinocytosis is underscored by four major mechanisms macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis. The endocytic route which is of most interest in targeting of nanocarriers is clathrin-mediated endocytosis because it engages mainly receptor-ligand complexes (Conner et al., 2003). Upon ligand-receptor binding, certain adaptor proteins like adaptor protein 2 are engaged which interact with clathrin triskelion to trigger the formation of clathrin-coated pits. A small GTPase dynamin cuts the invaginated pits and release them into the cytoplasm as vesicles. Thus, the endocytosed cargo after being delivered into endosomes is then recycled, sorted for degradation or delivered to the golgi complex (Schmid, 1997). Some of the examples of receptors which are exploited for targeting drugs intracellularly are folate receptors, transferrin receptors and LDL receptors for tumor targeting, gene delivery and brain targeting respectively. There also exist other routes that are non-clathrin-mediated which involve internalization of many proteins, lipids, viruses and toxins. These are referred to as caveolae/raft-mediated endocytosis and cholesterol plays a crucial role in these mechanisms and role of dynamin is seen in some cargo (Rajendran et al., 2010). The other clathrin independent route of cellular internalization which involves internalization of glycophosphatidyl-inositol (GPI)-anchored proteins is GEEC (glycophosphatidyl-inositol (GPI)-anchored protein-enriched early endosomal compartment) pathway. The salient features of this pathway are that it is dynamin independent and bypasses the step of early endosome sorting by using long invaginations from the surface. Nanocarriers are thus directed towards their intracellular compartment using one of the above mentioned mechanisms and in some cases there exists interplay between the mechanisms also.

The strategies employed for targeting nanocarriers to organelles include making the nanocarriers pH responsive and thus rendering them endosmolytic (e.g Poly(methacrylic acid), PEG-Dendrimer), use of fusogenic peptides (e.g, GALA and KALA), use of cell penetrating peptides (e.g Tat, Antennapedia, Tp10), use of small molecule targeting sequences like triphenylphosphonium, attachment of nuclear localization sequence and making nanocarriers which have intrinsic endosmolytic escape capacity. However, each of these targeting strategies will be discussed in detail when dealing with individual organelle targeting.

4. Challenges posed by intracellular environment

The intracellular environment is very different from routine biochemical assay environment. Two complex fluids occupy a large portion of cellular interior, the cytoplasm and nucleoplasm. The cytoplasm is comprised of organelles which are dispersed,

macromolecules and the cytoskeletal network and chromosomal DNA constitutes nucleoplasm. This constitution of cytoplasm and nucleoplasm confer to their respective properties. Both cytoplasm and nucleoplasm thus, show a considerable degree of macromolecular crowding (Minton, 2006). It is very important to understand the spatial aspects of intracellular environment to obtain an appropriate description of cell's behavior. This would help in better design of nanocarriers for organelle targeting.

The very high total concentration of proteins, nucleic acids and complex sugars inside cell give rise to 'macromolecular crowding', which has energetic consequences which affect cellular functions like diffusion of proteins within cytosol. The important effects of macromolecular crowding can be formation of protein aggregates such as amyloid deposits and reduction in the diffusion rate of the diffusing particle. A new technique called cryoelectron tomography provided a direct evidence of crowded state of cell interior. The pictures showed a high density of actin filaments and ribosomes confirming that cytoplasm consists of huge compacts of macromolecules rather than freely diffusing and colloiding macromolecules (Medalia et al., 2002). Nanocarriers depict macromolecules because of their size, shape and surface functionality thus it is quite imperative that nanocarriers should be able to overcome this entire gamut of macromolecular crowding inside cell for efficient targeting.

The complex environment of intracellular milieu is mainly attributed by factors like immobile barriers, molecular crowding and binding interactions. These factors hinder the intracellular diffusion. The effect of immobile barriers and crowding agents on translational mobility was assessed using multi-photon fluorescence correlation spectroscopy. The immobile barriers were mimicked by using silica-based nanostructures and macromolecular crowding agents were mimicked by high molecular mass dextrans. The data suggested that when tagged molecules like dextran-tetramethylrhodamine or eGFP-CaM are placed in heterogeneous environments as described above, there exist various mechanisms of diffusion. The first one being characterized as Fickian diffusion which is normal but slowed diffusion due to crowding. In some data it was also observed that molecules have a less hindered mobility due to relative size between tracer molecule and dimensions of crowding agents. In other data the molecule showed a subdiffusive like behavior and hindered mobility and it is explained by the idea that molecules can be trapped in either mobile or immobile cages (Sanabria et al., 2007).

There exists a pH gradient across many biological membranes. The pH gradient play important role in cellular functioning like modulating bilayer asymmetry, loading of vesicles with molecules bearing charge like amino acid, peptide, protein, controlling of fusion process and maintaining degradative functions in acidic organelles. It was observed that vesicles with a pH gradient across their membrane have different electrophoretic mobilities and is due to pH associated changes in surface density (Hope et al., 1989). Using capillary electrophoresis with laser induced fluorescence detection it was shown that pH gradient across liposomal membrane induce electrophoretic mobility shifts based on capacity theory. Moreover, it was also proved that mobilities of acidic organelles are in congruency with predictions based on liposomal models (Chen et al., 2007).

The major determinants of cytoplasmic rheology are fluid-phase viscosity and translational diffusion coefficient. For smaller solutes the collisions with intracellular components was determined to be the principal diffusive barrier which hindered translational diffusion (Kao et al., 1993). Seksek et al have reported that macromolecule size solutes (FITC-dextrans and Ficoll) when microinjected into fibroblasts and epithelial cells, the translational diffusion

slowed three- to four folds in cytoplasm and nucleus compared with water and the degree of slowing did not depend on molecular size up to at least 300 A° gyration radius (Seksek et al., 1997). The mobility of DNA in cytoplasm after it is released from a nanocarrier is a very important aspect to assess the efficacy of gene delivery using nanocarriers. Mobility of DNA in cytoplasm is also assessed by the same parameter described above which is translational diffusion. Fluorescein-labeled double stranded DNA fragments of increasing sizes (in base pairs (bp): 21, 100, 250, 500, 1000, 2000, 3000, 6000) were microinjected into cytoplasm and nucleus of HeLa cell and their diffusion was measured using photobleaching. Results indicated that the translational diffusion of smaller DNA fragments was not greatly impeded but the larger DNA fragments showed little or no diffusion especially for DNAs > 2000 bp. Such a slowing of DNA mobility is largely due to a combination of collisional interactions and macromolecular crowding effects. Interestingly, in nucleus DNA fragments of all sizes showed no mobility and this immobilization was attributed to extensive DNA binding to nuclear components like histones (Lukacs et al., 2000).

In an attempt to establish the exact mechanism involved in this reduced mobility with increase in DNA size, the diffusion studies were performed in presence of crowded solutions containing predominantly actin filaments. The results indicated that actin mesh rather than cytoplasmic crowding is the major barrier for cellular diffusion of large DNA. This result was consolidated by fact that when actin filaments were disrupted using cytochalasin D (5 μM) the size dependent reduction in mobility was not seen (Dauty et al., 2004). Thus, cytoskeletal barrier is an important limiting factor for non-viral gene delivery vectors. However, some viruses such as SV40 antigen overcome this barrier by activating tyrosine kinase-induced signaling cascades which dissociates the filamentous actin.

5. Effect of size and shape of nanoparticles in subcellular trafficking

Particle size of the nanocarriers have played a pivotal role in undermining many useful characteristics of the nanocarriers like enhanced permeation and retention (EPR) effect, cellular internalization and cellular trafficking. For example, to efficiently utilize the EPR effect the nanocarrier must fall in between a size range of 10 nm to 100 nm. If the nanocarrier is smaller than 10nm they will be rapidly cleared by kidneys and larger carriers are cleared by reticuloendothelial system. Particle sizes of nanocarrier also influence some of the very important characteristics such as degradation and clearance. It was reported that degradation of particles is size-dependent and degradation products formed within the particle can diffuse freely to the surface if they are from smaller particles (Dunne et al., 2000; Panyam et al., 2003). Particle size more importantly dictates the endocytic mechanism which will be engaged to internalize the nanocarrier. Large particles 2-3 μm are internalized by phagocytosis by macrophages. Internalization of considerably smaller particles >1 μm is facilitated by macropinocytosis, much smaller nanosacle range particles are internalized by caveolar-mediated (~60 nm), clathrin-mediated (~120 nm) and clathrin-independent and caveolin-independent endocytosis (~90 nm) processes (Petros et al., 2010). However, it is not just the particle size that governs the properties of the nanocarriers, particle shape also have a strong impact on the carrier performance.

The impact of particle shape was poorly understood earlier, perhaps due to lack of appropriate fabrication techniques. Recently, many fabrication techniques for synthesis of conventional non-spherical particles were reported in literature. The fabrication methods generally use techniques such as lithography, microfluidics, film-stretching, non-wetting

molding and photopolymerization. Many times, these techniques will be used in combination. By virtue of these fabrication methods particles of various morphologies like disks, toroids, ellipsoids form a variety of polymers such as poly(ethyleneglycol), poly styrene, poly vinyl alcohol, poly lactide-co-glycolide, poly(methylmethacrylate) etc, were synthesized and their properties were evaluated. Non-wetting molding and film-stretching methods produced a variety of two and three dimensional shapes in diameters of nanoscale (Champion et al., 2007).

Polystyrene particles of various shapes and sizes were prepared and their phagocytosis was studied in alveolar macrophages(Champion et al., 2006). The polystyrene particles were fabricated into six different shapes encompassing various shape characteristics such as aspect ratio, size, concavity and curvature. The geometric shapes of particles fabricated are spheres (radius 1.0-12.5 μm), oblate ellipsoids (major axis 4μm, aspect ratio 4), prolate ellipsoids (major axis 2-6 μm, aspect ratio 1.3-3), elliptical disks (EDs) (major axis 3-14 μm, aspect ratio 2-4, thickness 400-1,000 nm), rectangular disks (major axis 4-8 μm, aspect ratio 1.5-4.5), and UFO shaped particles (sphere radius 1.5μm, ring radius 4μm). These poly styrene particles were investigated in their nonopsonized and IgG-opsonized forms. It was reported that particle shape at the contact, not the size, dictates whether cells will proceed with phagocytosis or merely spread on the particle size. However, particle size was shown to affect the completion of phagocytosis especially when volume of particle is greater than macrophage volume. The interesting observation of this study was that except for spheres point of initial contact of particle with cell was major determinant in internalization. It was reported that internalization did not occur when cells initial point of contact was concave region whilst, attachment to dome or ring regions proceeded internalization process. The mechanism of internalization as elucidated by formation of actin cup was also influenced by point of initial contact and was in accordance with observations reported above (Champion et al., 2006). The following Figure 1 depicts the influence of point of initial contact on internalization of particles.

The influence of particle shape on *in vivo* circulation and its extravasation through microvasculature was also investigated in rodents using filamentous micelles (Geng et al., 2007). Cylindrically shaped filamentous micelles called filomicelles were fabricated using hydrophilic polyethyleneglycol (PEG) and hydrophobic polycaprolactone (biodegradable) or polyethylene (nonbiodgeradable). These stable filomicelles were fluorescently labeled and then compared with spherical micelles for transport and trafficking after intravenous injection into mice. Filomicelles have shown to exist in circulation for upto one week and filomicelles with longer initial lengths showed increased circulation times. It was also shown that these filomicelles enter cells under static conditions but under conditions of constant flow due to hydrodynamic shear cylindrical filomicelles are pulled off from phagocytes as they come into contact. It was also reported that when a chemotherapeutic agent Paclitaxel was loaded into these filomicelles, they shrunk tumors with longer filomicelles being more effective at a given dose (Geng et al., 2007).

A novel top-down lithographic fabrication method called PRINT (Particle Replication In Non-wetting Templates) was used to prepare micro and nanoparticles from cationic, cross linked poly (ethyleneglycol) hydrogels. These particles were then investigated for the interdependent effect of size, shape and surface charge (zeta potential) on cellular internalization by human cervical carcinoma epithelial (HeLa) cells. Using this technology three different types of particles were fabricated, a micrometer-sized series of cubic-shape

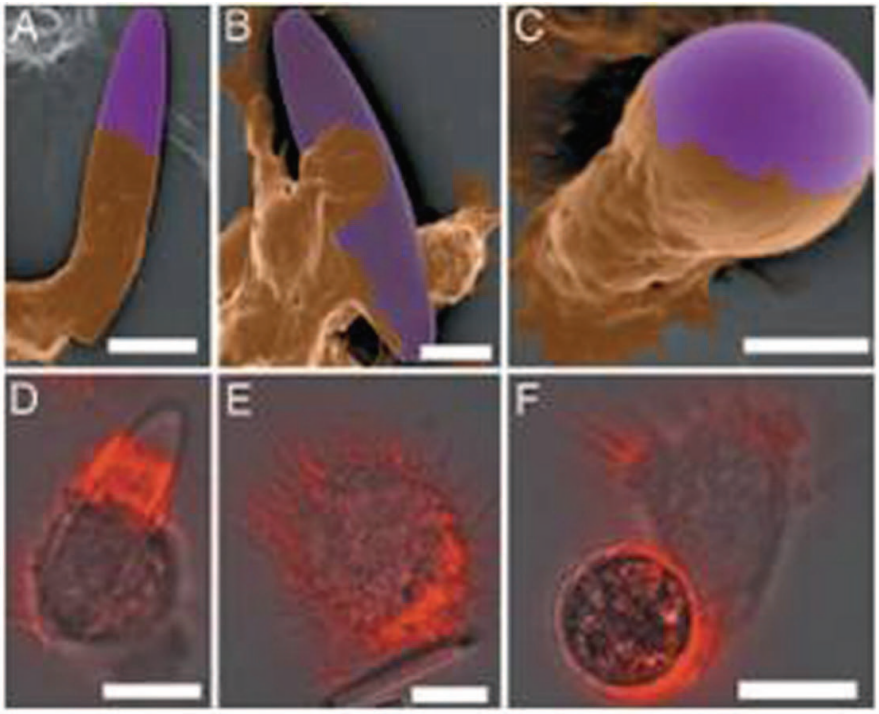

Fig. 1. Scanning electron micrographs (A-C) and actin staining (D-F) depicting the influence of initial point of contact on phagocytosis. In figures A-C cells and particles were colored brown and purple, respectively. (A) The elliptical disk (ED) which was opsonized can be seen to be engulfed by the cell. (Scale bar: 10μm). (B) The flat side of the particle (ED) attached by the cell. (C) A spherical particle with approximately half of its surface was covered by the cell membrane. Figures D-F depict the overlays of bright and fluorescent images after fixing and staining actin cells with rhodamine phalloidin. (D) At the leading edge of membrane new actin polymerization enables membrane to progress over an opsonized ED and formation of actin ring can be seen. (E) No actin cup or ring is visible when cell is attached to the flat side of the opsonized ED. (F) Formation of actin cup at the end of the sphere as internalization begins after attachment. (Scale bars in D-F: 10μm.) Numbers of cells observed for each orientation of each particle were not less than five. Figure was obtained with permission from reference by (Champion et al., 2006) © The National Academy of Sciences of the USA.

particles, micrometer-sized cylindrical particles with same heights but different diameters and finally a cylindrical-shape nanoparticle series. A very important observation of this work was that particles upto 3 μm sizes were internalized by the nonphagocytic HeLa cells. This contradicts the current dogma that upper limit of the size of any nanoparticle to be internalized into cells by means of nonspecific endocytosis is 150 nm. It was reported that of all shapes, rod like cylindrical nanoparticles with high aspect ratio (ratio of height to diameter) were internalized ~4 times faster than low-aspect ratio particles, and cylindrical particles with varying size and volume have shown varying degrees of internalization. It

was also shown that 84% of positively charged nanoparticles were internalized in comparison with <5% of identically shaped negatively charged indicating the importance of surface chemistry in cellular internalization (Gratton et al., 2008).
Similarly, Huang et al fabricated mesoporous silica nanoparticles with various shapes and aspect ratios and their cellular internalization was studied. The three shapes fabricated were spheres, short and long rods each with similar diameter but with different aspect ratios (ARs, 1, 2, and 4). The results indicated that particles with larger aspect ratios were internalized at faster rates and to a greater extent. It was also reported that long rod-shaped particles disrupted the cell cytoskeleton, where as spherical and short rod shaped particles did not. Finally, the long rod-shaped nanoparticles reduced the cell viability to a greater extent when compared to short-rods and spheres (Huang et al., 2010).

6. Intracellular fate of nanocarriers and factors affecting the intracellular fate

Nanocarriers are used profusely for many biomedical applications like drug delivery, gene delivery, imaging, targeted chemotherapy etc. Thus, it is of great importance to understand the intricate complex processes that regulate the intracellular fate of nanocarriers as they govern major properties of nanocarriers like biocompatibility, targeting efficiency etc.,. The traditional paradigm of intracellular fate of nanocarriers suggest that after cellular internalization, nanoparticles gets entrapped in endosomes which later fuse with acidic lysosomes and results in degradation of its contents (Watson et al., 2005). However, in recent years nanocarriers are designed in a way to protect its contents from lysosomal degradation. The strategies involve use of targeting ligands to directly target the organelle of interest, make nanocarriers pH sensitive thus making them endosmolytic or using alternative endocytic mechanisms which evade lysosomal degradation. Thus, intracellular fate of nanocarriers has become highly subjective and varies depending on physical and chemical characteristics of the nanocarrier.
The poly(lactide)-co-glycolide (PLGA) nanoparticles (NPs) were shown to follow a typical endocytosis-exocytosis route. The NPs initially encounter endosomes followed by retrieval or escape from the compartment and then interact with exocytic organelles of the cell like endoplasmic reticulum, golgi apparatus and secretory vesicles. PLGA NPs avoid lysosomes and are capable to bypass intracellular digestive compartment (Cartiera et al., 2009), Whereas, polystyrene NPs once internalized were not contained in endosomes or lysosomes but were found to localize within mitochondria of cell lines. It was also reported that polystyrene NPs were accumulated within bile canaliculi suggesting that NPs can be eliminated within bile. In another study where intracellular fate of a Tat-conjugated quantum dots were investigated, the results have shown that Tat peptide was digested in lysosomes by enzymes leaving the Tat-detached quantum dots in lysosomes for excretion (Xiong et al., 2010).
Liposomes are another important class of nanocarriers whose intracellular fate has been investigated. Tat-peptide conjugated liposomes were fluorescently labeled using Rhodamine-phosphatidylethanolamine (Rh-PE) and their intracellular fate was investigated using epi-fluorescence and differential interference contrast microscopy. The results have shown a typical time dependent pattern of distribution of liposomes inside the cell. The localization of intact liposomes within cytoplasm was observed after 1 hr. The liposomes after 2 and 4 hr were seen clustered in perinuclear region and at 9 hr the degradation of liposomes was observed. Finally, after 24 hr no liposome was seen inside cell (Torchilin, 2005).

7. Current approaches and strategies for optimal organelle targeting

7.A NUCLEUS

Nucleus is perhaps the most important cellular organelle which needs efficient targeting because it is the ultimate target for treatment for genetic diseases. Gene therapy which underscores the use of therapeutic DNA usually fails due to lack of transfection of DNA into nucleus. Thus it is very important to develop strategies that improve targeting of DNA molecules directly to nucleus. The prime barrier for a DNA or any molecule whose intended site of action is nucleus is the nuclear envelope. Both active and passive transport in and out of nucleus takes place via nuclear pore complexes (NPCs) embedded in nuclear envelope. The NPC structurally can be divided into three components, a central domain and a nuclear and cytoplasmic ring constructed from 50 different nucleoporin proteins. The central domain forms a aqueous channel through the nuclear envelope of ~9 nm in diameter. Passive diffusion is the mechanism underlying translocation of small molecules. Whereas, molecules >45 kDa must possess a nuclear localizing signal (NLS) which are recognized by importin family proteins which in turn mediate the nuclear transport (Poon et al., 2005).

In order to efficiently deliver the cargo into the nucleus we have to overcome two major impediments. One is the passage through cytoplasm and other is translocation via nuclear membrane. The common consideration in both these constraints is the size of the cargo. As described in previous sections, cytoplasm is a highly crowded environment containing organelles, macromolecules and cytoskeleton forming a `cytoplasmic sieve'. Large molecules thus diffuse slowly in cytoplasm, in comparison with their diffusion in water. To overcome this impediment a successful strategy was provided by taking example of trafficking of herpes simplex virus (HSV). HSV utilizes the microtubule cytoskeleton motor proteins dynein and kinesins for nuclear transport (Dohner et al., 2005). The transport of 125 nm HSV caspid to nucleus clearly demonstrates that by utilizing the cytoskeleton we can efficiently diffuse large molecules through cytoplasm to nucleus. In order to counteract the second barrier which is presented by NPC, the cargo should be packaged to a size/structure below ~40 -60 nm and a NLS has to be attached (Pante et al., 2002). Thus, it is imperative to consider these two barriers in designing strategies to deliver macromolecules like DNA to nucleus.

The most important strategy to target nucleus is by using DNA binding proteins. Most DNA binding proteins are inherently equipped with NLS to enable them to efficiently translocate into the nucleus to perform necessary functions. GAL4, a yeast transcription factor whose N-terminal 147 residues contain a DNA binding domain and the first 74 amino acids of this domain function as NLS. Thus, it was reported that enhanced GAL4-mediated gene delivery was achieved when a large SV40 T antigen was linked to the complex. This addition resulted in switching of nuclear import from an importin β-mediated to an importin α/β-mediated pathway(Chan et al., 2001). Nuclear factor κ B (NF κB) also has a NLS through which it internalizes into nucleus via a importin dependent fashion. The NFκB protein p50 which have NLS was shown not only to enhance the nuclear transport of DNA but also enhanced the migration of DNA towards nucleus from cytoplasm through microtubules (Mesika et al., 2005).

There has been an increase in considerable amount of interest in recent years in active targeting to nucleus. This stemmed from the rise of non-viral gene therapy to deliver large molecules of DNA to the nucleus. Initially, active targeting involved co-administration of NLS peptide after microinjection of DNA, but with this strategy, there remains a question

whether NLS and DNA remain bound in endosome or cytoplasm, or even after binding of NLS to importin. Thus coupling of NLS peptides to DNA emerged as a more attractive approach for nuclear targeting. But this strategy was not highly efficient as increase in gene expression was limited to 2 to 5 fold only (Pouton et al., 2007). An alternative to this strategy would be attaching a polypeptide or protein containing an NLS to the DNA by using covalent coupling, reversible interaction or streptavidin-biotin binding technology. This strategy of attaching a NLS as a part of protein or polypeptide would make NLS more likely to be presented with appropriate tertiary structure to impinge strong binding to the importin or relevant nuclear transport protein involved (Pouton et al., 2007). NLS-streptavidin was coupled to DNA molecules with a single biotinylated nucleotide end and microinjected into cells or administered to digitonin-permeabilised cells to investigate uptake of DNA by nucleus. This study reported that NLS-mediated transport delivered the DNA into nucleus, but there exists a size limitation of approximately 1 kb DNA.

Another strategy which is still in its infancy is the use of multifunctional fusion proteins which are equipped with a DNA binding domain and nuclear import moiety which allows efficient delivery and trafficking of DNA to the nucleus. A recombinant fusion protein was constructed based on multidomain structure of the bacterial pseudomonas exotoxin A. The protein consists of ErbB-2 specific antibody which imparts target cell specificity, the exotoxin A translocation domain executes endosomal escape and a DNA binding domain from yeast GAL4 enable sequence-specific high affinity binding to DNA. Transient expression of the luciferase gene was observed and correlates with the amount of carrier protein in the complex and when carrier protein was truncated lacking either cell recognition domain or translocation domain resulted in failure of DNA transfer (Fominaya et al., 1996).

7.B Mitochondria

Mitochondria are distinct cellular organelles which occupy a major volume of animal cell cytoplasm. Mitochondria are also called as power plants of the cell because they provide the bulk of cellular ATP. Mitochondria structurally are composed of two membranes and are primarily composed of phospholipid bilayers with proteins embedded in them. Because of presence of two membranes there exists two aqueous spaces, the inner one being called the matrix space and the one between the membranes is called the intermembrane space. The outer membrane consists of channel-forming protein called voltage-dependent anion channel (VDAC) and limits the passage of molecules to intermembrane space to a MW of 5000 Da or less. Proteins and other large molecules use a unique protein import apparatus to cross the outer membrane. The inner membrane has a composition distinct from outer membrane, it is more proteinaceous and contains an unusual phospholipid, cardiolipin. The major function of mitochondria, the ATP synthesis occurs in matrix thus inner membrane is then major barrier which governs the transport of molecules in and out of matrix. Inner membrane is thus embedded with many transporters which allow specific compounds with a specific ligand to reach matrix space. One of those transporters is the ATP/ADP carrier which transfers ATP out from matrix space while simultaneously allowing ADP to cross inner membrane (Mukhopadhyay et al., 2007).

Mitochondrial proteins synthesized in cytosolic ribosomes are translocated into mitochondria by receptor-translocator complexes present in inner and outer membranes, which are TIM (translocator inner membrane) and TOM (translocator outer membrane) respectively. An *N*-terminal leader sequence of protein is recognized by the TIM and TOM

complexes and thus enables protein to be translocated into mitochondria. Apart from their central role in energy metabolism and bioenergetics, mitochondria are also involved in regulation of apoptotic cell death, calcium metabolism, cardio protection and free radical formation (Biasutto et al., 2010). Thus, targeting mitochondria would benefit in alleviating many pathological conditions whose causes underlie in mitochondrial functioning.

The initial attempts to target mitochondria involved use of hydrophobic molecules which take the advantage of the hydrophobic nature of the membrane and diffuse across the membrane. The first compound showing that property was triphenyl phosphonium ion (Jauslin et al., 2003). We can either attach functional groups to the phosphorous atom or the phenyl rings can be modified to attach drugs and thus carried into the matrix space and released there. The important advantage of triphenyl phosphonium (TPP) or a methyl derivative of TPP is that without requiring a receptor they can penetrate into mitochondria due to their hydrophobicity and delocalized positive charge (Jauslin et al., 2003). TPP has been proved useful in targeting antioxidants to mitochondria which protect oxidative damage (Adlam et al., 2005; Sheu et al., 2006). Polymer based targeting of mitochondria was also investigated by attaching TPP covalently to HPMA copolymer. Results have indicated that TPP was able to transport electrically neutral and very low molecular weight conjugates (Callahan et al., 2006). A more recent study involved targeting of cyclosporin A to mitochondria using TPP. The results have shown that this conjugate amplified CsA activity, which is to abolish cell necrosis which in turn is due to deprivation of oxygen and glucose (Malouitre et al., 2009). TPP was also used to target peptide nucleic acid to mitochondria for treatment of mtDNA diseases (Muratovska et al., 2001). Dequalinium (DQA) is another dicationic amphiphilic compound which has the potential to localize exclusively in mitochondria. Another advantage of DQA is that it has shown to form liposome like aggregates in water called DQAsomes which can also bind plasmid DNA. Weissig et al have demonstrated that DQAsomes complexed with plasmid DNA can transport and release nucleic acid into mitochondria after interacting with mitochondrial membrane (D'Souza et al., 2003).

Mitochondrial proteins encoded in nucleus carry targeting signal that allow delivery into mitochondria through translocases, TIM and TOM. Peptides representing those signal sequences termed as mitochiondrial-targeted peptides are used to target mitochondria. Yamamoto and co-workers attached mitochondrial targeting peptide to *n*-trioctlyphosphine oxide (TOPO)-capped quantum dots and this conjugate exhibited a strong mitochondrial localization in contrast to quantum dots covered with a control peptide (Hoshino et al., 2004). In another attempt to internalize peptide nucleic acid into isolated mitochondria, a presequence of cytochrome *c* oxidase subunit VIII was used (Chinnery et al., 1999). These peptides thus provide a promising approach for targeting small molecules and nucleic acids to mitochondria.

Mitochondrial targeting also witnessed some reports where without any targeting sequence or lipophilic cation, molecules were localized in mitochondria. Block copolymer micelles that were made of poly(caprolactone)-b-poly(ethylene oxide) were unexpectedly localized into mitochondria suggesting these micelles have a important role in mitochondrial targeting (Savic et al., 2003).

There are many strategies used to target mitochondria in the pretext of cancer. Cardiolipin a mitochiondrial inner membrane phospholipid is important component for efficient functioning of various carriers, protein import apparatus and for respiratory chain. Cardiolipin also plays an important role in apoptosis as it is an essential collaborator for

caspase-8, t-Bid and Bax thus indicating an avenue for mitochondrial targeting which can promote or prevent apoptosis. Mitochondrial channels like VDAC (porin), Shaker-type K^+ have been shown to play a role in apoptosis. Thus, these channels can be attacked to promote apoptosis in cancer cells. Certain mitochondriotropic polyphenols having antioxidant activity were used to counteract the production of reactive oxygen species. Antioxidants quercetin and resveratrol were shown to preferentially accumulate in mitochondria when conjugated with TPP (Biasutto et al., 2010).

7.C Endoplasmic Reticulum

Endoplasmic reticulum (ER) is an important intracellular organelle involved in expression and control of functional proteins required for the cellular communication and activity. ER is characterized by extensive membrane surfaces in cell extending from the nuclear envelope to cell periphery. ER embedded with ribosomes is known as rough ER and it's continuity with smooth membranes that provide surfaces for vesicle formation at ER exit sites. ER is involved in many key activities of the cell including biosynthesis of lipids, assembly and folding of proteins, homeostasis and control of Ca^{2+} signaling. Protein assembly and folding by the ER is the most important function which affects many essential biological activities. Improper handling of protein in ER leads to development of a myriad of unrelated diseases affecting different organs like heart, thyroid, kidney etc. (Aridor et al., 1999). The protein assembly and folding are controlled by two execution pathways. In one pathway, proteins are directed towards proteasome degradation from a folding pathway and in other folded and assembled proteins are transported to golgi complex by virtue of activity of cytosolic protein complex, the COPII coat.

Dysfunctional processing of proteins by ER can lead to either loss or gain of essential cellular functions. Loss of function of protein is the basis of many ER derived diseases and arise due to mutations that hinder protein folding which eventually lead to retention and degradation in ER. One such example is the loss of cystic fibrosis transmembrane conductance regulator (CFTR) function due to mutation of protein leading to lung fibrosis (Rowe et al., 2005). Inhibition of degradation of mutant protein leading to accumulation and aggregation of protein is the underlying cause of gain of function related ER processing diseases. This leads to generation of signals by transmembrane receptors in ER and can lead to induction of inflammatory response and can culminate in cellular apoptotic response leading to degenerative disease. A mutated protein α 1-antitrypsin which regulates elastase activity when not efficiently degraded gets accumulated in ER and lead to propagation of inflammatory response. This inflammatory response results in liver injury along with normal manifestation of lung emphysema (Hidvegi et al., 2005).

Unfolded protein response (UPR) is an extensive adaptive response that initially upregulates cellular biosynthetic activities in response to signals generated from the ER. Chronic UPR activation leads to apoptotic cell death and UPR is observed in many neurodegenerative diseases.

Stabilization of protein folding is the prime target to alleviate a variety of ER derived diseases. When protein kinetics of ΔF508 mutant of CFTR were modulated at reduced temperatures (27°C) the protein efficiently folds and egresses from the ER (French et al., 1996). Chemical chaperones can also be used to support protein folding without direct binding to mutant proteins. One important class of chemical chaperones is presented by osmolytes. These osmolytes like glycerol, trimethylamine-*N*-oxide or deuterated water

increase the hydration layer of folding intermediates in the ER. The other classes of chemical chaperones include dimethyl sulfoxide, 4-phenyl butyrate (4-PBA). 4-PBA an FDA approved drug for urea cycle disorders was also found to be effective in reducing UPR in cells and animal models. Thus, these chemical chaperones can be utilized to target certain proteins to ER. Another approach for targeting protein in ER is using hydrophobic ligands capable to enter cells and stabilize protein function. Glibenclamide a sulfonylurea, binds to the sulfonylurea receptor protein SUR1 and rescues the ER-retained mutated SUR1 to support delivery and expression of Kir6.2 which otherwise lead to development of congenital hyperinsulinism (Yan et al., 2006).
The above strategies can be applied to efficiently target endoplasmic reticulum to alleviate ER derived disease due to improper processing of proteins.

7.D Endosomes / lysosomes

Endocytosis is the primary route of uptake of many small drugs and macromolecular therapeutics. Especially, the receptor mediated endocytosis is probably the most efficient one for specific uptake of therapeutics. The receptor mediated endocytosis usually begins with uptake of molecule at plasma membrane by binding to cell surface receptors at clathrin coated pits. This complex then in the form of clathrin coated vesicles called endosomes, enters the cell. These early endosomes then mature into late endosomes and then fuse with and release their contents into lysosomes, where the contents are degraded. So targeting of endosomes and lysosomes has to be studied together since the entry of molecules into these organelles in interconnected. Moreover, even though targeting of therapeutics to endosomal uptake pathway enhances the intracellular concentration of molecules up to 1000 fold it is not devoid of demerits (Breunig et al., 2008). Since the end point of the therapeutics like peptides, proteins, DNAzymes is either nucleus or any other organelles, these molecules have to be released in cytosol intact. But unfortunately acidic pH and degradative enzymes in lysosomes degrade those molecules and render them ineffective. Thus, targeting endosomes/lysosomes have to be studied in two contrasting parts. In one part we discuss strategies to enhance intracellular uptake of therapeutics mediated by receptor mediated endocytosis which explains direct targeting to endo-/lysosomes. The other part involves strategies applied for escape of molecules from endo-/lysosomes so that therapeutics are not degraded and delivered intact into cytosol for further action into other organelles.

7.D.1 Targeting endosomes / lysosomes

Apart from being the channel for degradation and recycling of molecules and receptors at cell surface, endosomal system is also an essential site of signal transduction. So, targeting endosomes or endosomal signaling pathway has very important therapeutic effects. For example, endosomal ECE-1 (endothelin-converting enzyme 1) is important target for diseases involving inflammation and pain, an ECE-1 inhibitor exhibited an anti-inflammatory effect thus proving the potential of targeting endosomal signaling pathway (Cattaruzza et al., 2009). The other important way of targeting endosomes involves use of cell surface receptors like folate, transferrin, and low density lipoprotein (LDL) receptors. Several anti cancer drugs like doxorubicin, cisplatin, chlorambucil, mitomycin, gemcitabine and DNAzymes were efficiently targeted to tumor cells through transferrin receptors (Breunig et al., 2008).

Lysosomes apart from serving as acidic organelles involved in degradation of extracellular molecules, are also responsible for turnover of intracellular cytosolic molecules and organelles by a process known as autophagy. In this process lysosomal enzymes are secreted by rough endoplasmic reticulum and reach lysosomes via mannose-6-phosphate receptors. The deficiency of lysosomal enzymes can lead to accumulation of missing enzyme's substrate and eventually leads to metabolic disorders called lysosomal storage diseases like pompe's disease, gaucher's disease, fabry's disease and hurler-Scheie syndrome (Pastores et al., 2005). Thus, targeting of degradative enzymes to lysosomes would result in reversing of those disease conditions. For example, enzyme replacement therapy with agalsidase alpha which is an exogenous source of α-galactosidase A is proved to be effective in treatment of Fabry's disease (Mehta et al., 2010).

7.D.2 Endo-/lysosomal escape strategies

The important strategy for endo-/lysosomal escape of protein and nucleotide therapeutics is to use pH responsive carriers. These pH responsive carriers utilize the low pH of endosomes to release the therapeutics into the cytoplasm. Several approaches have been proposed to achieve this task like use of fusogenic peptides, use of pH sensitive polymers use of cell penetrating peptides or photochemical internalization which involve rupture of endosomal membrane loaded with photosensitizing molecules.

The fusogenic peptides at pH 7 assume a random coil structure and when encounters acidic pH, a conformational transition takes place which enables their interaction with phospholipid membrane resulting in pore formation or lysis or membrane fusion. These peptides can be of natural origin like N-terminus of hemagglutinin subunit HA-2 of influenza virus or synthetic like WEAALAEALAEALAEHLAEALAEALEALAA(GALA), orWEAKLAKALAKALAKHLAKALAKALKACEA (KALA). Intracellular delivery of many therapeutics including oligonucleotides, peptides or plasmid DNA was enhanced when these peptides were incorporated into delivery systems. When a siRNA to silence suppressor of cytokine signaling 1 (SOCS1) gene was loaded in an octaarginine (R8) modified lipid envelope type nanoparticles (R8-MEND) with a fusogenic peptide GALA, successful endosomal escape was achieved. The results have shown that siRNA loaded R8/GALA- MEND nanoparticles efficiently suppress endogenous gene expression (Akita et al., 2010).

Many cationic polymers have an intrinsic endosmolytic activity which results in swelling and rupture of endosomes due to proton sponge effect. Cationic polymers such as polyethyleneimine (PEI) can result in endosomal escape and subsequent release of DNA into cytoplasm by two mechanisms. One involves proton sponge effect where PEI buffers endosomal environment resulting in osmotic swelling of vesicle and subsequent burst of endosome which leads to release of DNA into cytoplasm (Behr, 1994). Other involves direct interaction of PEI with endosomal membrane creating holes in the membrane (Bieber et al., 2002). In another attempt, hydrophobic groups were attached to dendrimers by an acid-sensitive acetal linkage and thus in acidic conditions the complex will lose its hydrophobic molecules making it hydrophilic and thus destabilizing the micelle and allow escape of drug doxorubicin (Gillies et al., 2005).

Inspite of the advantages offered by above strategies for delivery of therapeutics into cytosol a direct targeting strategy to cytosol, would be of prime importance. In order to achieve this objective, a novel approach using "cell penetrating peptides" (CPPs) or "protein

transduction domains" (PTDs) has been described. These peptides are tethered either directly to the therapeutic molecules or to the delivery system and then transported through cellular membrane. The cell penetrating peptides involve either chimeric CPPs like transportan and MPG or synthetic CPPs like oligoarginine and model amphipathic peptides (MAP). The protein transduction domains reported are penetratin and Tat peptide (Said Hassane et al., 2010). CPPs were successful in transporting many therapeutics and carriers such as proteins, DNA, antibodies and liposomes, nanoparticles into the mammalian cells (Gupta et al., 2005).

7.E GOLGI APPARATUS

Camillo Golgi more than 100 years ago first described the Golgi apparatus as 'internal reticular apparatus'. Golgi apparatus is central organelle of the cell secretory pathway and interacts with ER on both sides of the stack. It is comprised of a flattened stacks of cisternae arranged into a ribbon that are punctuated by openings of various sizes through which tubules project and vesicles move. Golgi apparatus mainly contains enzymes involved in processes like phosphorylation, acylation, glycosylation, methylation and sulphation which are post translational modification processes of newly synthesized proteins. The trans golgi network (TGN) is where cargo is sorted before it is sent to various organelles inside the cell and for secretion outside the cell (Marsh et al., 2002). Transport of cargo through golgi is bidirectional, one is anterograde (that is forward transport of cargo) and other is retrograde (transport of molecules backward within the golgi, and from the golgi to ER) and is mediated by vesicles, tubules or the process of cisternal progression/maturation. Both the anterograde and retrograde transport processes are mediated by coatomer protein complex I (COPI) dependent mechanism of vesicular transport (Marsh et al., 2002). The retrograde trafficking pathway gained importance since it involves delivery of drugs and macromolecules from endosomes to Golgi apparatus and to ER thus bypassing the acidic and hydrolytic environment of the lysosome.

The retrograde transport route was mainly reported to be exploited by toxins like shiga toxin and shiga like toxin to reach ER where they translocate into cytosol to exert their toxic effect. Movement of this toxin to ER is considered to follow a COPI independent process involving Rab6 which normally operates in cycling of enzymes from golgi apparatus to ER. Shiga toxin has two subunits A and B. Targeting property of the Shiga toxin resides in the B subunit of the toxin and subunit A involves in cell death. The B subunit binds to the cell surface glycosphingolipid, globotriaosylceramide or Gb_3 and internalizes the toxin in a receptor mediated endocytosis process. Gb_3 is also shown to be expressed in antigen presenting cells such as dendritic cells and some B cells. The unique retrograde trafficking pathway of shiga toxin combined with the expression of Gb_3 provides us with a strategy to deliver antigens to the MHC class I pathway of APC using shiga toxin as vector. It was also shown that expression of Gb_3 was enhanced in various cancers such as ovarian carcinoma, lymphoma, breast cancer cells, astrocytoma cells, malignant meningiomas, colon cancer and testicular cancer. Thus, shiga toxin can also be used to target the cancerous tissues for diagnostic as well as therapeutic purposes. Finally, since the B subunits of the shiga toxin can be produced as a polypeptide it can be used to target drugs, genes or proteins (antigens) for treatment and diagnosis of cancer and for delivery of antigens to MHC class I pathway (Tarrago-Trani et al., 2007).

7.F PEROXISOMES

Peroxisomes initially described as "microbodies" are single membrane bound organelles found in cytoplasm that encompass large variety of functions in all eukaryotic cells (Platta et al., 2007). Peroxisomes are multifunctional organelles responsible for a wide variety of biochemical and metabolic processes. Peroxisomes house biosynthetic pathways for bile acids, docosahexanenoic acids and ether phospholipids. By virtue of α- and β-oxidation reactions peroxisomes, degrade variety of fatty acids including 3-methyl-branched fatty acids, eicosanoids, prostaglandins, thromboxanes and leukotrienes. Detoxification of certain xenobiotics such as glyoxylate and hydrogen peroxide were also performed by peroxisomes (Wanders et al., 2006).

Peroxisomal enzymes are encoded by nuclear genes and then transported into lumen of peroxisomes from cytosol. The peroxisomal enzymes destined for matrix are first recognized by a peroxisomal targeting signal (PTS) type 1 or 2 (PTS1 or PTS2). A group of specific protein called peroxins play crucial role in proper formation and trafficking of matrix enzymes. Two peroxin receptors Pex5p and Pex7p recognize the enzyme's PTS1 and PTS2 respectively. These peroxin receptors bind to the cargo in cytosol and transport the complex to peroxisome membrane. The peculiar feature of protein import to peroxisomes which is different from mitochondria is its ability to accommodate fully folded, oligomeric, and co-factor bound proteins by shuttling receptors peroxins. Peroxins Pex 13p and Pex 14p which are membrane associated peroxins initiate the receptor/cargo docking. The translocation is then advanced by formation of RING membrane protein network which forms a larger complex the importomer from peroxins Pex2p, Pex 10p, ad Pex12p (Rosenkranz et al., 2006). Peroxisome membrane proteins are transported to membrane by chaperone peroxin Pex19p and other membrane-associated peroxins Pex3p and Pex16p. Subsequent to release of cargo Pex5p, Pex7p and Pex20p are exported back to cytosol for further transportation (Platta et al., 2007).

Peroxisomal related disorders include peroxisome biogenesis disorders, single enzyme deficiencies, or pathological situations associated with oxidative stress. The peroxisome assembly, protein import and consequent metabolic pathways are severely affected if there are any defects in genes encoding peroxins. Sometimes this might also result in complete loss of peroxisomal function. Thus, it would be of great clinical value if therapeutic proteins can be transported to peroxisomes of above discussed disease conditions.

As mentioned earlier peroxisomes have unique ability of internalizing fully folded and oligomeric proteins. Thus, in many peroxisome related conditions when therapeutic proteins or enzymes are targeted directly to peroxisomes they will alleviate the disease conditions. The most important criteria for a protein to be targeted to peroxisomes is that it should possess PTSs. PTS1 and PTS2 are the two PTSs. PTS1 is the best characterized as a short carboxy terminal sequence specifically recognized by the peroxin Pex5p and PTS2 is a N-terminal sequence recognized and transported by peroxin Pex7p (Miyata et al., 2009).

In order to target proteins which do not possess these targeting signal related sequences can be easily introduced through recombinant DNA molecular biology. For example an SKL sequence can be introduced to make protein accessible to PTS1 receptor Pex5p. One important consideration is that the attached sequence must be accessible and should not be buried inside the protein. To make it accessible spacers such as polyglycine can be introduced (Terlecky et al., 2007).

One classic example of peroxisomal targeting is administration of catalase enzyme in peroxisomal hypocatalasemia. In this condition, hydrogen peroxide and related ROS initiate

a 'peroxisomal deterioration spiral' which affect the peroxisomal import apparatus and hence catalase with weak KANL PTS1 is specifically affected. To overcome this, catalase was engineered such that it contains a high affinity SKL PTS1 and hence, its import into peroxisomes is enhanced. The results have shown that when catalase-SKL along with a cell penetrating peptide Pep-1 delivered into human hypocatalasemic fibroblasts, it reduced cellular hydrogen peroxide levels by 80% (Wood et al., 2006).
The following table summarizes various nuclear import machineries and their substrates present in cellular organelles.

Cellular Organelle	Import Apparatus	Substrates (Examples)	References
Nucleus	Importin α and β	Nuclear localization signals (SV40, GAL4)	(Chan et al., 2001)
Mitochondria	ATP/ADP Carrier	ATP, ADP	(Mukhopadhyay et al., 2007)
	TIM, TOM	Mitochondrial targeting peptide	(Hoshino et al., 2004)
	Mitochondrial membrane	Triphenyl phosphinium, Dequalinium	(Jauslin et al., 2003), (D'Souza et al., 2003)
Endosomes/lysosomes	Folate, transferrin cell surface receptors	Folic acid, Transferrin	(Breunig et al., 2008)
Golgi Apparatus	Coatomer protein complex 1 (COP I)	Shiga toxin	(Tarrago-Trani et al., 2007)
Peroxisomes	Peroxins	Peroxisomal targeting signal	(Miyata et al., 2009)

Table 2. Table of import machineries of various cellular organelles and their respective susbtrates.

8. Conclusions and future perspectives

The strategy of targeting therapeutics like small molecule drugs, proteins, enzymes, siRNA, DNA etc to specific cell population like cancer was proved to be successful. But that itself does not ensure the therapeutic efficiency of the molecule, since the molecule has to reach its intracellular target where it acts. Thus, it is very important to understand the concept of intracellular organelle targeting and intricacies involved therein. In order to establish a pattern for strategies it is first very important to understand how these intracellular organelles communicate with each other and how do they overcome the barriers due to intracellular environment. Next, it is very important to understand how do some natural viruses and other pathogens decode the intracellular processes and evade the organelle of interest and make it defective. When we combine both the strategies, we can definitely provide excellent strategies to target therapeutics of organelle of interest. Improvements in the field of polymer chemistry and molecular biology allowed us to design novel carriers

and signaling agents for targeting organelles with superior level of sophistication. Like other fields of biomedical research, targeting to organelles has to evolve from a laboratory experiments to clinical success.

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